STRUCTURAL and FUNCTIONAL STUDIES on the NON-MUSCLE ISOFORM of α-ACTININ.

by

DAVID BRIAN MILLAKE.

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<th>Description</th>
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<tr>
<td>Axbm</td>
<td>absorbance at xnm</td>
</tr>
<tr>
<td>ABD</td>
<td>actin-binding domain</td>
</tr>
<tr>
<td>ABP-120</td>
<td>Dictyostelium actin-binding protein -120kDa (also known as the Dictyostelium gelation factor)</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-indolylphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>c</td>
<td>centi</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CEF</td>
<td>chick embryo fibroblast</td>
</tr>
<tr>
<td>Ci</td>
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</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>CNBr</td>
<td>cyanogen bromide</td>
</tr>
<tr>
<td>CPM</td>
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<tr>
<td>CTP</td>
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DRP  dystrophin related protein
DTT  dithiothreitol
dTTP  deoxythymidine triphosphate
E  extinction coefficient
E. Coli  Escherichia coli
ECM  extracellular matrix
EDC  1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide
EDTA  ethylenediaminetetraacetic acid
EF-Brain  the plasmid pGEX-1 with a cDNA fragment encoding both EF-hands of chick brain α-actinin cloned into the Bam HI site
EF-Smooth  the plasmid pGEX-1 with a cDNA fragment encoding both EF-hands of chick smooth α-actinin cloned into the Bam HI site
EGF  epidermal growth factor
EGTA  ethyleneglycol-bis-(β-aminoethyl ether) N, N', N'-tetraacetic acid
ELISA  enzyme-linked-immunosorbant assay
FPLC  fast protein liquid chromatography
GTP  guanosine triphosphate
HBS  HEPES buffered saline
HCC  hepatocellular carcinoma
HEPES  N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid
HPLC  high performance liquid chromatography
IPTG  isopropyl β-D-thiogalactopyranoside
k  kilo
kb  kilobase
Kd  dissociation constant
LMP  low melting point
m  milli
mm  millimetre
μ  micro
M  molar
MCS  multiple cloning site
MES  2-[N-morpholino]-ethanesulfonic acid
MRLC  myosin regulatory light chains
mRNA  messenger RNA
MTJ  myotendinous junction
nano
7-chloro-4-nitro-benzeno-2-oxa-1,3-diazole-
nitro-blue-tetrazolium
newborn calf serum
nuclear magnetic resonance
optical density
polyacrylamide gel electrophoresis
polymerase chain reaction
platelet-derived growth factor
polyethylene glycol 6000 and 8000
isoelectric point
protein kinase C
phenylmethyl-sulphonyl fluoride
the chick brain α-actinin cDNA 7a/9a cloned
into the eukaryotic expression vector pECE
polyvinylidene difluoride
polyvinylpyrrolidine
ribonucleic acid
ribonuclease
rous sarcoma virus
sodium dodecyl sulphate
one-, two-, or three-dimensional
N, N, N', N', -tetramethyl-ethylene-diamine
1-O-tetradecanoylphorbal-13-acetate
thymidine triphosphate
(hydroxymethyl) aminomethane
uridine triphosphate
ultraviolet light
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
## AMINO ACIDS

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<tr>
<td>Valine</td>
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Preface.

The work described within this thesis was performed between October 1987 and September 1990 in the Department of Biochemistry, University of Leicester. No part of this work has been submitted to any other university, and unless otherwise stated all work was carried out unaided.

I am most grateful to my supervisor Dr. D. R. Critchley for advice and encouragement throughout the course of this work. I am also grateful to Professor B. Brammer in whose department this work was undertaken. In addition, I would like to thank Dr. J. Sampson and Dr. C. Bagshaw for their advice and discussion. I would like to thank Dr. A. Weeds (LMB, Cambridge) for allowing me to spend a day in his laboratory, and to Mr. B. Pope (LMB, Cambridge) for advice concerning the 'dot-blot' $^{45}$Ca$^{2+}$ overlay technique. All my love to my family. I am extremely grateful to the following: Mr. B. Patel for technical assistance; Mrs. B. Colley for cleaning my glassware; Mr. J. Kyte for making synthetic oligonucleotides; Mr. D. Gamble for stores; and I. Riddel for photographic services. Many thanks to the following, for stimulating discussions etc: A. D. Blanchard; M. D. Davison; P. Jackson; A. Walmsley; L. Hemmings; G. Waite; I. Graham; T. Harrisson; I. Eperon; R. Cooper; T. Maxwell; P. Jones; M. Way; G. Price; V. Ohanion; A. Gilmore; P. Senior; A. McGregor; E. Corben; P. Moore; M. Moore; S. Morris; A. Gargaro; A. Britton; G. Banting; W. Mawby; L. Bruce; P. Martin; K. Ridgwell; G. Bloomberg; and M. J. A. Tanner. I acknowledge the MRC for funding this study.

David Brian Millake.
Publications.


Abstract.

α-Actinin was purified from adult chicken brains, cleaved with thermolysin and analysed by SDS-PAGE. Chicken brain α-actinin gave a similar (but distinct) pattern of thermolytic peptides to the chicken smooth and skeletal muscle α-actinins, suggesting that all three isoforms have similar domain structures. Several polypeptides derived from the actin-binding and repeat domains of chick brain α-actinin were found to be identical in sequence with the corresponding regions of the chick smooth muscle isoform. Indeed, the complete deduced sequence of chick brain α-actinin (893 amino acids) was only divergent with that of the smooth muscle isoform in the region of the N-terminal EF-hand, where 27 residues in brain α-actinin are replaced by 22 residues in smooth muscle α-actinin. These isoforms therefore arise by the alternative splicing of a transcript produced by a single gene. Chick brain α-actinin is predicted to bind two calcium ions per dimer, whereas chick smooth muscle α-actinin is not predicted to bind any. This may explain why chick brain (but not chick smooth muscle) α-actinin binds actin in a calcium-sensitive manner (Duhanian and Bamburg, 1984). However, in this analysis chick brain α-actinin was not found to bind calcium using two different in-vitro assay procedures. Both chick brain and smooth muscle isoforms became incorporated into stress-fibres and cell-matrix junctions when complete cDNAs were expressed in monkey COS cells, suggesting that these isoforms are not differentially localised in non-muscle cells. The complete cDNA sequence of a human placental α-actinin was found to display a high level of identity with the chick brain sequence across the transcribed regions (85 and 97% identity at the DNA and protein levels respectively).
Chapter 1. Introduction.
The protein α-actinin was first identified in chicken skeletal muscle by Ebashi et al. (1964). It was shown to increase the Mg^{2+}-dependent ATPase activity of reconstituted acto-myosin leading early investigators to suggest that it might be involved in the regulation of the actin-myosin interaction (Maruyama, 1966; Temple and Goll, 1970). α-Actinin has since been isolated from smooth and cardiac muscles (Singh et al., 1977; Feramisco and Burrige, 1980), as well as from a wide variety of non-muscle tissues (Kuo et al., 1982; Bennett et al., 1984; Duhaiman and Bamburg, 1984; Landon et al., 1985). The absolute function of α-actinin is still not known, but the location of the protein within the cell suggests that it may bind and organize F-actin filaments within the cytoplasm. For example, in skeletal muscle α-actinin is found at the Z-disc (Endo and Masaki, 1982) and at membranous sites (Endo and Masaki, 1984). Similarly, it is located in the Z-disc and intercalated discs of cardiac muscle cells (Tokuyasu et al., 1981), and in the cytoplasmic dense plaques and dense bodies of smooth muscle cells (Geiger et al., 1981). In fibroblasts and other non-muscle cells α-actinin is found distributed at periodic intervals (1-2μm) along actin thin filaments and stress fibres, and at the junction of these cytoskeletal structures with the plasma membrane, including the adherens-type cell-cell contacts and the cell-substratum attachment sites (adhesion plaques or focal contacts) (Lazarides and Burrige, 1975).

The native molecule is a homodimer composed of non-covalently linked polypeptide chains (~100-kDa), which are antiparallel in orientation (Wallraff et al., 1986). Electron micrographs have shown that α-actinin is a long rod-shaped molecule (2-4 x 30-40nm), and that actin-binding is confined to the ends of the rod (Wallraff et al., 1986). The 3 main isoforms of the protein, skeletal, smooth and non-muscle α-actinin, show clear differences in primary sequence as revealed by peptide mapping and immunological properties (Bretscher et al., 1979; Endo and Masaki, 1982; Endo and Masaki, 1984; Duhaiman and Bamburg, 1984). Unlike α-actins derived from muscle tissues, those derived from non-muscle sources bind to F-actin in a calcium-sensitive manner (Burrige and Feramisco, 1981). Presumably, this is a reflection of the different roles played by α-actinin in muscle and non-muscle cells.

1.1 Structure of α-Actinin.

Several complete cDNAs encoding α-actinin have been isolated and sequenced. These include cDNAs encoding chicken smooth muscle α-actinin (Baron et al., 1987b), chicken skeletal muscle α-actinin (Arimura et al., 1988), human skeletal muscle α-actinin (Beggs et al., 1992), and Dictyostelium α-actinin (Noegal et al., 1987). These sequences have shown that each isoform is composed of three major
The Domain Structure of Alpha-actinin.

Figure 1.1 Schematic Diagram Illustrating the Structure of the α-Actinin Homodimer.

The two subunits of the α-actinin homodimer are arranged in an antiparallel manner with the N-terminal actin-binding domain (ABD) displayed at opposite ends of the molecule. The central domain of the protein contains 4 repeats homologous with those found in spectrin. The C-terminal domain contains two EF-hand motifs which may or may not bind calcium. In this diagram the subunits are shown to be co-extensive, although this has never been demonstrated experimentally.
domains (Figure 1.1). Firstly, there is a N-terminal actin-binding domain (ABD), which is highly conserved across species. Secondly, there is a central domain containing 4 repeats of approximately 120 residues in length, which are homologous with the 106-residue repeats found in the erythrocyte cytoskeletal protein, spectrin. Thirdly, there are two calcium-binding loops at the C-terminal end of the subunit, which are homologous with the EF-hand structures found in calcium-modulated proteins such as troponin-C.

1.1.i Actin-Binding Domain.

When α-actinin is cleaved with thermolysin, one of the major fragments liberated is a 27-kDa polypeptide, which has been shown to bind F-actin in sedimentation experiments (Mimura and Asano, 1986). The 27-kDa fragment of chicken smooth muscle α-actinin has been sequenced, and starts at Leu 25 within the complete deduced sequence of the protein (Davison et al., 1989). This data indicates that the ABD of α-actinin is contained within a 27-kDa polypeptide derived from the N-terminal region of the molecule. Electron microscopy of rotary shadowed free α-actinin reveals that most molecules form extended rods, with no detectable thickening at the ends (Wallraff et al., 1986). However, some of the molecules form shorter rods with globular N-termini (Wallraff et al., 1986). This latter result has been confirmed by gel filtration studies which have shown that the 27-kDa thermolytic fragment of α-actinin is globular (Mimura and Asano, 1987). The ABD of α-actinin may therefore take on both globular and non-globular confirmations. Interestingly, a monoclonal antibody that binds the ABD, was observed to induce a globular confirmation at the binding site (Wallraff et al., 1986). This antibody can bind to both ends of the α-actinin rod, confirming the antiparallel configuration of the α-actinin homodimer. The simultaneous binding of two such antibody molecules to α-actinin often caused the dimer to split into two subunits joined at opposite ends, suggesting that both subunits extend throughout the whole length of the α-actinin molecule (Wallraff et al., 1986).

The amino acid sequence of animal and fungal actins is highly conserved, whereas plant actins appear to be more divergent (Hightower and Meagher, 1986; Mclean et al., 1990). In turn one might also expect the sequence of the ABD of α-actinin to be highly conserved in animals and fungi. This theory is borne out when one compares the sequence of α-actinins from chicken (Baron et al., 1987b), Dictyostelium (Noegal et al., 1987), Drosophila (Fyrberg et al., 1990), and Nematode (Barstead et al., 1991) (Figure 1.2). The ABDs of Dictyostelium, Nematode, Drosophila α-actinin display 58, 77, and 83% identity respectively with residues 1-255 of chicken smooth muscle α-actinin. This data suggests that the gene encoding the ABD appeared at a very early stage during evolution, and has since been highly conserved. The level of identity
Figure 1.2 Sequence Alignment of Several α-Actinin-Type Actin-Binding Domains.

The alignment contains amino acid sequence deduced from cDNA clones. The single letter amino acid code is used throughout. Pad characters (-) are introduced to maximize alignments. Residues which are identical across all sequences are indicated with an asterisk (*) at the bottom of the alignment. Substitutions which conserve amino acid property are indicated with a dot (•).

- **DGF** 120-kDa *Dictyostelium* actin-gelation factor (Noegal et al., 1989);
- **CSM** chick smooth muscle α-actinin (Baron et al., 1987b);
- **CSK** chick skeletal muscle α-actinin (Arimura et al., 1988);
- **DAA** *Dictyostelium* α-actinin (Noegal et al., 1987);
- **FAA** *Drosophila* α-actinin (Fyrberg et al., 1990);
- **HDY** human dystrophin (Koenig et al., 1988);
- **CDY** chick dystrophin (Lemaire et al., 1988);
- **FSP** *Drosophila* β-spectrin (Byers et al., 1989);
- **FM1** and **FM2** the N- and C-terminal ABDs respectively of chick intestinal fimbrin (de Arruda et al., 1990); and
- **ABP** human endothelial filamin (Gorlin et al., 1990).

The numbering refers to the *Dictyostelium* 120-kDa actin-gelation factor (Noegal et al., 1989). A putative actin binding site within the sequence ABP-120 is underlined (Bresnick et al., 1990). Two further actin-binding sequences have been detected in human dystrophin by proton NMR spectroscopy, and these sequences have been double-underlined (Levine et al., 1990; Levine et al., 1992).
between these α-actinins extends C-terminal to the ABD, but is markedly reduced (see Chapter 4).

ABDs homologous with α-actinin have been detected in a number of other actin-binding proteins, including: β-spectrin (Winkelmann et al. 1988; Byers et al., 1989); dystrophin (Koenig et al., 1988; Lemaire et al., 1988); filamin (Gorlin et al., 1990); Dictyostelium gelation factor, ABP-120 (Noegal et al., 1989); α- and β-adducin (Joshi et al., 1991; Tripodi et al., 1991; Gilligan and Bennett, 1991); and fimbrin (de Arruda et al., 1990) (Figure 1.2). Values of 19-49% identity are scored between residues 1-255 of chicken smooth muscle α-actinin and the ABDs of these proteins. The level of amino acid identity across the ABD is far higher between α-actinins derived from different species than between α-actinin and α-actinin-related proteins. During evolution the ancestral gene encoding the first protein with an α-actinin-type ABD must have duplicated to give similar ABDs in different actin-binding proteins. These proteins then evolved independently, producing ABDs with distinct amino acid sequences and function(s) pertinent to the specific protein. For example, the 27-kDa thermolytic fragment of α-actinin contains, in addition to its F-actin binding site, a site for binding to the adhesion plaque protein zyxin (Crawford et al., 1991; Crawford et al., 1992). The ABDs of the α-actinin-related proteins are likely to contain sites for different ligands, the nature of which will vary according to the function of the protein and its localisation within the cell.

Fimbrin contains two α-actinin-like ABDs which are arranged tandemly in the C-terminal core region of the protein (de Arruda et al., 1990). Residues 1-255 of chicken smooth muscle α-actinin are 19 and 23% identical with the first and second ABDs respectively of chick intestinal fimbrin (Figure 1.2). de Arruda et al. (1990) have observed that there is a weak repeating structure within each ABD of Fimbrin, and also in the ABD of α-actinin and the other ABD containing proteins (Figure 1.3). This repeat divides the ABD into two segments of 125 residues. The majority of the repeated residues are hydrophobic in nature, and they may have a role in maintaining the structure or conformation of the domains (de Arruda et al., 1990).

When a cDNA (C17) encoding the complete sequence of chicken smooth muscle α-actinin was transfected into monkey (non-muscle) COS cells the expressed protein was found to be incorporated into both the stress fibres and adhesion plaque structures (Jackson et al., 1988). However, when the the region encoding the ABD was deleted from the cDNA, the mutant protein failed to localise at these sites (Hemmings et al., 1992). The ABD is therefore essential for normal incorporation, and the regions outside of the ABD are not capable of targeting α-actinin to the actin-containing structures within the cell. A chimaeric protein containing the ABD of human dystrophin
Figure 1.3  Repeated Hydrophobic Consensus Pattern Within the α-Actinin-Type ABD.

The ABD can be divided into a repeated hydrophobic consensus sequence (boxed residues). The repeats are referred to as the A and B domains. Lines 1 and 3 - the N- and C-terminal ABDs of chick intestinal fimbrin respectively (de Arruda et al., 1990); lines 2 and 4 - the N- and C-terminal ABDs of human T-plasminogen respectively (Lin et al., 1988, 1990); line 5 - human dystrophin (Koenig et al., 1988); line 6 - chick dystrophin (Lemaire et al., 1988); line 7 - chick smooth muscle α-actinin (Baron et al., 1987b); line 8 - Drosophila B-spectrin (Byers et al., 1989); and line 9 - 120-kDa Dictyostelium actin gelation factor (Noegel et al., 1989). Numbering refers to the sequence positions at the N- and C-terminal end of each ABD. The amino acids shown at the bottom of the alignment indicate those residues conserved within the A and B domains. [Taken from de Arruda et al. (1990)].
(residues 1-233) and the repeat and EF-hand regions of chicken smooth muscle α-actinin (residues 244-888) was also shown to localize to the F-actin stress fibres and adhesion plaques when expressed in COS cells (Hemmings et al., 1992). This suggests that dystrophin can also bind to F-actin, and that the ABD may function in an analogous manner in each of the α-actinin-related proteins. Several recent studies have attempted to identify sequences within the ABD which are essential for binding actin. For example, Hemmings et al. (1992) introduced a number of N- and C-terminal deletions into the ABD of chick smooth muscle α-actinin and looked at the incorporation of the expressed mutant proteins in monkey non-muscle COS cells. The residues important for actin-binding were recognised by virtue of their ability to target α-actinin molecules to the actin-containing structures within these cells. Using this strategy a functional actin-binding site was localised to amino acid residues 20-195 within the chick smooth muscle α-actinin ABD. This has been supported by the work of several other groups. By co-sedimenting small tryptic fragments of human β-spectrin with F-actin Karinch et al. (1990) identified a small actin-binding peptide of 16.5-kDa. This fragment was found to extend from Ala 47 to Lys 186 within the complete human β-spectrin sequence, aligning with residues 25 to 158 of chick smooth muscle α-actinin (Baron et al., 1987b). Using a similar strategy, Bresnick et al. (1990) identified a small actin-binding sequence between residues Leu 89 to Argus of Dictyostelium ABP-120. A synthetic 27mer containing this sequence was found to co-sediment with F-actin, and to inhibit the F-actin cross-linking ability of native Dictyostelium ABP-120 (Bresnick et al., 1991). Antibodies to the 27mer were found to react with the native protein, indicating that at least part of this sequence is accessible to solvent and perhaps available for binding F-actin. The 27mer is fairly central within the ABD, and is highly conserved in the alignment (Figure 1.2). Values of 63-70% identity are scored between residues 89 and 115 of Dictyostelium ABP-120 and the corresponding regions of Drosophila β-spectrin, chicken smooth muscle α-actinin, human dystrophin, chick dystrophin, Dictyostelium α-actinin, and human non-muscle filamin. Several residues in the alignment are totally conserved, whilst several others maintain physico-chemical property. Experiments involving deletion mutagenesis have shown that the actin-binding site is located within the C-terminal half of this 27mer in chick smooth muscle α-actinin (residues 120-134) (Kuhlman et al., 1992). This sequence (NVKMTLGMIWTIILR) contains several hydrophobic residues, and their mutation to alanine was found to cause a substantial reduction in actin-binding activity (Kuhlman et al., 1992). The interaction between α-actinin and F-actin may therefore be mediated by hydrophobic bonds. This is consistent with the observation that binding is relatively independent of salt concentration (Kuhlman et al., 1992). Using proton NMR spectroscopy, Levine et al. (1990) have identified another potential actin-binding site within a synthetic peptide containing residues 10-32 of human dystrophin (Levine et al.,
This peptide was found to interact with residues 83-117 on the actin sequence (Levine et al., 1992). This is in partial agreement with the EDC-mediated cross-linking experiments of Mimura and Asano, 1987. In these experiments the 27-kDa thermolytic fragment of α-actinin was shown to interact with residues 1-12 and 86-119 on the actin subunit. Values of 50-90% identity scored between residues 19 to 27 (KTFTKWVNA) of human dystrophin and the corresponding regions of chick dystrophin, Drosophila β-spectrin, human non-muscle filamin, Dictyostelium ABP-120, and chicken smooth muscle α-actinin. Within this site the sequence KTFT is particularly well conserved. However, in transfection experiments chick smooth muscle α-actinins with mutated KTFT sites were found to undergo normal incorporation into F-actin structures when expressed in COS cells (Hemmings et al., 1992). Indeed, when the ABDs derived from these mutants were expressed as fusion proteins in E. coli, each was found to retain the ability to bind F-actin (albeit at a reduced level: 22-57% of the wild-type binding activity) (Kuhlman et al., 1992). Therefore, although the KTFT motif does not appear to be essential for binding F-actin it does seem to involved in the interaction. Using proton NMR spectroscopy, Levine et al. (1992) identified a further actin-binding site, within a synthetic peptide corresponding to residues 128-156 of human dystrophin. A 29-mer containing this sequence (MAGLQQTNSKILLSWVRQSTRNYPQNV) was shown to bind a peptide containing the C-terminal region of the actin molecule, residues 350-375 (Levine et al., 1992). Indeed, a similar peptide was found to inhibit the interaction between G-actin and chick smooth muscle α-actinin (see below) (Lebart et al., 1990). The α-actinin-binding sites on the actin subunit (residues 83-117 and 350-375) lie fairly close (1-1.5nm) on the outer domain of the 3D-model presented by Kabsch et al. (1990). However, at this stage it is not known if the small actin-binding sequences within the ABD of α-actinin associate with one another to form a larger contact site.

1.1.ii Spectrin-Like Repeats.

The central domain of α-actinin contains 4 repeats of 120 residues, which show homology with the 106-residue repeat found in spectrin (Baron et al., 1987a) and the 110-residue repeat found in dystrophin (Davison and Critchley, 1988). The function of the α-actinin repeat domain is still not known absolutely, though it may be involved in the formation of the antiparallel dimer. When chicken smooth muscle α-actinin is cleaved with the protease thermolysin, a stable fragment of 53-kDa is liberated, which migrates as a dimer when analysed by gel filtration (Mimura and Asano, 1986). The N-terminus of this fragment is at Leu 267 within the complete chicken smooth muscle sequence which is close to the start of the repeats, Phe 246 (Blanchard et al., 1989).
Given that each α-actinin repeat is predicted to have a molecular weight of 12-kDa, the 53-kDa thermolytic fragment is large enough to contain all 4 repeats (Davison and Critchley, 1988). Taken together, these data suggest that the α-actinin repeat domain is on its own capable of forming a dimer. Further evidence has come from the analysis of chymotryptic fragments of α-actinin (Imamura et al., 1988; Kahana and Gratzer, 1991). Under non-denaturing conditions chymotrypsin releases two major fragments from α-actinin with molecular weights of 55- and 36-kDa, which are analogous with the 53- and 27-kDa thermolytic polypeptides respectively (Imamura et al., 1988). The 55-kDa chymotryptic fragment contains all 4 repeats and starts at Ala267 within the complete sequence of chicken smooth muscle α-actinin (Imamura et al., 1988). When reacted with the zero-length cross-linking reagent EDC, the 55-kDa fragment migrates with an apparent molecular weight of 110-kDa, suggesting that the native fragment is dimeric (Imamura et al., 1988). In contrast, the 36-kDa chymotryptic fragment containing the ABD of the molecule is not cross-linked by EDC, suggesting that it is monomeric (Imamura et al., 1988). To define interactive sites more precisely within the 55-kDa dimeric fragment, Imamura et al. (1988) cleaved the polypeptide with chymotrypsin under denaturing conditions, with or without EDC-mediated cross-linking. In the absence of cross-linking the 55-kDa fragment liberated polypeptides of 37-, 34-, 30-, 26-, 19- and 16-kDa. The 37-, 34- and 30-kDa polypeptides were found to label with DACM, a reagent that binds to cysteines which are localised within the N-terminal half of the intact 55-kDa fragment (Imamura et al., 1988). In contrast, the 26-, 19- and 16-kDa fragments reacted with a monoclonal antibody (CP2-1) specific for the C-terminal end of the intact 55-kDa (Imamura et al., 1988). Cleavage of the cross-linked product (110-kDa) released 3 unique fragments of 52-, 48- and 44-kDa. Each of these polypeptides reacted with both CP2-1 and DACM, suggesting that they form the interactive sites at the ends of the antiparallel 55-kDa dimeric fragment. Imamura et al. (1988) propose that the 52- and 48-kDa fragments are derived from a combination of the C-terminal 19-kDa polypeptide derived from one subunit with the N-terminal 30- or 34-kDa fragment derived from the other subunit. The 44-kDa polypeptide may contain the N-terminal 30-kDa chymotryptic fragment and the C-terminal 16-kDa fragment. This narrows down the interactive sites to within the first two repeats on one subunit and the fourth repeat on the opposite subunit.

The 55-kDa chymotryptic fragment forms an extremely stable dimer which can only be dissociated under denaturing conditions, and is much more resistant to dissociation by urea than is the spectrin heterodimer (Kahana and Gratzer, 1991). The cross-linking data has shown that this strong association can only be due interactions taking place between the repeat units of α-actinin. Under the electron microscope, the chymotryptic 55-kDa fragment of chicken smooth muscle α-actinin is rod-shaped, with
dimensions of 4 x 25nm (Imamura et al., 1988). Consequently, the repeat domain must account for 72% of the molecular length of the α-actinin molecule. Circular dichroism measurements (Imamura et al., 1988; Kahana and Gratzer, 1991) have shown that the 55-kDa chymotryptic fragment of chicken smooth muscle α-actinin contains a higher α-helical content (74-85%) than the intact α-actinin molecule (62%). The values obtained for the 55-kDa fragment fall within a range measured for intact erythroid spectrin (Gratzer, 1983). The α-helical composition of the repeats may therefore induce the rod-shape of both α-actinin and spectrin.

A number of models predicting the secondary structure of the repeat unit have been presented (Figure 1.4), based on multiple sequence alignments of repeats from spectrin (Speicher and Marchesi, 1984), α-actinin and spectrin (Davison et al., 1989) and dystrophin (Cross et al., 1990). Speicher and Marchesi derived their model using the Chou-Fasman method on 8 repeats from α-spectrin. Each repeat segment was predicted to fold into a triple helical structure, in which the helices are joined by two short regions of turn/coil (Speicher and Marchesi, 1984). The repeat units are joined by a connecting peptide where the helix is broken, but the sequence is not turn/coil. The model presented by Davison et al. (1989) is derived from 8 different predictive methods and is based on the analysis of a greater number of repeats (13 from spectrin and 8 from α-actinin). This model is distinct in that each repeat is folded into a four helical arrangement, in which the helices are separated by 3 strong regions of turn/coil (Figure 1.4). The first helix is the longest (36 residues) and is interrupted by 10 helix breaking residues, which correspond to the 'connecting peptide' predicted by Speicher and Marchesi. This region occurs two thirds of the way through the long helix, and is immediately preceded by a protease sensitive region that can be cleaved with thermolysin, trypsin and chymotrypsin (Davison et al., 1989). The long helix may lie at an angle to the filament axis, placing the protease sensitive region in a relatively exposed region of the repeat units (Figure 1.4). Tryptic digests of α-actinin liberate fragments of 55-, 40- and 12-kDa (Davison et al., 1989). The N-terminal amino acid sequence of the 55- and 40-kDa fragments places these polypeptides within the protease sensitive region of the first and second repeats respectively. The 55- and 40-kDa fragments are fairly resistant to further proteolytic digestion, suggesting that they form compact structures. However, extensive digestion with trypsin liberates the individual 12-kDa repeat units. Helices 2, 3 and 4 of the Davison model are shorter (6 to 18 residues) and more buried within the repeat unit than the first helix, which is consistent with the relative resistance of these helices to proteolysis. Both secondary models described above agree in the approximate position of the turn/coil regions connecting helices 2 and 3 (numbers refer to the Davison model) and helices 4 and 1. However, the Davison model is distinct from the first in predicting a region of turn/coil
**Figure 1.4 Structural Models for the Spectrin-Like Repeats of α-Actinin.**

A. The model proposed by Speicher and Marchesi, (1984). Each repeat unit is folded into a triple helical structure in which the helices are separated by two regions of turn/coil (a and b). A connecting region (c) is shown where the helices break but are not in turn/coil configuration. B. The model proposed by Davison et al. (1989). The size constraints of the individual repeat units (6.25 x 2nm) are indicated by the background rectangle. Each repeat is divided into 4 α-helices, which are separated by regions of turn/coil. Helix 1 is shown as a long interrupted helix which overlaps adjacent domains. The interruption in helix 1 is not represented as turn/coil because the discontinuity at this site is weak. The arrows pointing to helix 1 mark the approximate position of the protease-susceptible region. The internal structure is not specified in this diagram, but may resemble other proteins with 4 helical bundles, such as influenza haemagglutinin (Wilson et al., 1981) or myohemerythrin (Weber and Salemme, 1980). C. The model proposed by Cross et al. (1990). This model predicts a repeat unit with two types of α-helices, H1 (unshaded) and H2 (shaded), separated by regions of turn/coil. I. The H1 helices wrap around one another to form a coiled-coil structure, which is probably reinforced by H2 which folds back to form a triple-coil. II. Successive repeat units nest together to form a continuous rod, with H2 helices occupying alternate grooves.
between helices 3 and 4, in contrast to the continuous helix predicted by Speicher and Machesi (1984).

Davison et al. (1989) have shown that there are no positions in the spectrin and \( \alpha \)-actinin repeats where absolute amino acid identity is retained (Figure 1.5). However, physico-chemical property is conserved at 25 positions, and these are clustered into discrete regions of their consensus model. The first site occurs at the start of the repeat unit (positions 1 to 8), the second at the end of helix 1/loop (positions 35-47), and the third across the whole of helix 3 (positions 62-77). These regions may play an important role in defining the structure of the repeat unit (Davison et al., 1989).

A third model has also been presented (Cross et al., 1990), based on the central domain of dystrophin (Figure 1.4). All of the dystrophin repeats were found to carry a heptad repeat, that had not previously been recognised in \( \alpha \)-actinin or spectrin. If the heptad repeat is represented by \( a-b-c-d-e-f-g \), then residues \( a \) and \( d \) are usually hydrophobic, whilst the other residues are frequently hydrophilic. The heptad repeats were used to align all 25 repeats of human dystrophin (Koenig et al., 1988). The alignment indicated that each repeat consisted of two \( \alpha \)-helical regions, \( H_1 \) and \( H_2 \), joined by two turn/coil regions, \( L_1 \) and \( L_2 \), rich in proline and glycine (Cross et al., 1990). \( L_1 \) and \( L_2 \) agree with two of the turn/coil regions predicted in the models presented by Speicher and Machesi (1984) and Davison et al. (1989). \( H_1 \) is constant in length (54 residues) and is the most highly conserved heptad-rich core of the repeat. \( H_2 \) is variable in length, and exhibits a weaker heptad pattern than \( H_1 \) (Cross et al., 1990). Cross et al. (1990) suggest that \( H_1 \) \( \alpha \)-helices wrap around one another to form a coiled-coil structure. Alternate \( H_2 \) helices might then occupy the alternate grooves formed by the \( H_1 \) helices to produce a triple coil. In this way successive repeats along the subunit may nest together to form a continuous rod with elastic properties. \( H_1 \) helices may slide relative to one another during stretching, dissociating helix \( H_2 \). The opposing return force would come from the \( H_1 \) helices regaining full overlap. This model may also be applicable to the repeats of spectrin and \( \alpha \)-actinin. If so the rod domain of each protein may confer longitudinal elasticity on the molecule.

Despite the low level of amino acid identity observed between the repeats of spectrin and \( \alpha \)-actinin, antibodies to a synthetic peptide corresponding to residues 48-70 in the sequence of chick \( \alpha \)-spectrin were found to cross-react with \( \alpha \)-actinin (Narvanen et al., 1987). Furthermore, antibodies to the repeats of dystrophin cross-react with a specific variant of skeletal muscle \( \alpha \)-actinin (Hoffman et al., 1989). This suggests that these 3 proteins belong to a family containing distinct but structurally homologous repeat units (Davison et al., 1989).

A highly conserved feature of the \( \alpha \)-actinin, spectrin and dystrophin repeats are the two aromatic residues, usually tryptophan, at positions 5 and 46 within predicted
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Figure 1.5 Optimal Alignment of α-Actinin and Spectrin Repeats.

C=A-CT. m 1-4 refers to chick smooth muscle α-actinin (Baron et al., 1987b), Ds-ACT. m 1-4 to Dictyostelium α-actinin (Noegal et al., 1987), H-SPEC.p 1-5 to erythroid α-spectrin (Speicher and Marchesi, 1983), H-SPEC.m 1-2 to human erythroid α-spectrin (Curtis et al., 1985). M-SPEC. m 1-2 to mouse erythroid α-spectrin (Curtis et al., 1985). C-SPEC. m 1-4 to chicken non-erythroid α-spectrin (Birkenmeier et al., 1985).

m= deduced, p= determined by protein sequencing. Below the abbreviation identifying each repeat are the inclusive amino acid residue numbers. Pad characters are indicated by (-). Proline residues are boxed. Positions in the alignment where conservative substitutions are always, or generally, maintained are marked by vertical lines. The conserved Trp46 is marked by a thick horizontal line. [Taken from Davison et al. (1989)].
loop segments of the model presented by Davison et al. (1989), or at positions 12 and 46 within the core-helix of the model presented by Cross et al. (1990). The fluorescence of these residues is not perturbed during subunit dissociation suggesting that they do not form inter-chain binding sites (Kahana and Gratzer, 1991). However, the long chain fatty acid 2-bromostearate has been shown to quench nearly 60% of the tryptophan fluorescence of the chymotryptic 55-kDa fragment of chicken smooth muscle α-actinin (Kahana and Gratzer, 1991). Spectrin also interacts with 2-bromostearate, but more strongly (Isenberg et al., 1981). This data suggests that the repeat units contain lipophilic binding sites in close proximity to tryptophan side-chains (Kahana and Gratzer, 1991). This is in accordance with the observed interaction between α-actinin and model membranes (Meyer et al., 1982) or hydrophobic ligands such as phosphatidylinositol 4, 5-bisphosphate, palmitic acid and diacylglycerol (see Section 1.4.ii) (Burn et al., 1985; Fukami et al., 1992). 2-Bromostearate is effective at low concentrations, suggesting that the interaction is site-specific (Kahana and Gratzer, 1991). The heavy atom of the fatty acid may interact with the fluorophore via van der Waals forces (Kahana and Gratzer, 1991). Kahana and Gratzer (1991) suggest that the 2-bromostearate binding sites in α-actinin could also accept the head group of a membrane lipid in a similar manner. The interaction between α-actinin and lipid may form one mode of attachment between the cell membrane and the actin-cytoskeleton. Another mode of attachment may involve the observed interaction between α-actinin and the transmembrane receptor integrin (Otey et al., 1990; Otey et al., 1991). Interestingly, the binding site for integrin has also been found to lie within the repeat units of α-actinin (see Section 1.4.ii).

1.1.iii EF-Hand Calcium-Binding Domain.

The C-terminal domain of α-actinin contains two structural motifs, termed EF-hands, which are homologous with the calcium-binding structures present in such proteins as calmodulin and troponin-C (Strynadka and James, 1989). When these proteins bind calcium they change their conformation, and this enables them to interact with their respective target molecules (reviewed by Strynadka and James, 1991). In this way [Ca^{2+}] can modulate the activity of secondary target proteins involved in such processes as skeletal muscle contraction (troponin-C) and cellular metabolism (calmodulin).

The EF-hand motif consists of a calcium-binding loop (12 residues in length) flanked on either side by two α-helices (also 12 residues in length) (Strynadka and James, 1989). This structural principle was first identified in the crystal structure of the calcium-binding protein, carp parvalbumin (Figure 1.6, Moews and Kretsinger, 1975).
The 'EF-1' of EF-hand refers to the E- and F-helices of this protein (Moews and Kretzinger, 1975). The helix-loop-helix motif has been likened to an index finger (E-helix), a curled second finger (the loop), and thumb (F-helix) of the right hand. Five residues of the loop contain a carboxyl (or a hydroxyl group) in their side chain, and the oxygens provide co-ordination points for the calcium ion (Strynadka and James, 1989). By nomenclature, the five essential oxygen containing residues occupy positions: X (residue 1); Y (residue 3); Z (residue 5); -X (residue 9); and -Z (residue 12) of the loop (Figure 1.6). In addition, a main chain carbonyl at -Y (residue 6) provides an oxygen for the co-ordination of the Ca\(^{2+}\). This residue is the most variable in the loop in terms of the nature of the amino acid, since the side-chain extends into the solvent (Strynadka and James, 1989). The residue at the -X vertex (residue 9) is sometimes too short (e.g. aspartate, serine, glycine etc) to co-ordinate the Ca\(^{2+}\) ion directly, and in such cases it does so by way of a water-bridge (Strynadka and James, 1989). Aspartate at the X vertex (residue 1) is essential, since the carboxylate side chain participates in the co-ordination of the calcium ion, and it also forms stabilizing hydrogen bonds with residues 4, 5 and 6 of the loop (Putkey et al., 1989; Geiser et al., 1991; Strynadka and James, 1989). Mutation of this aspartate to alanine, asparagine, or glutamate in the second loop of troponin C causes a significant reduction in calcium-binding affinity, and totally removes the ability of the mutant protein to induce muscle contraction (Putkey et al., 1989; Babu et al., 1992). In functional EF-hands the amino acid at -Z (residue 12) is invariably a glutamate, and both oxygens of its carboxylate moiety participate in co-ordinating the Ca\(^{2+}\) ion. This residue also participates in stabilizing long range hydrogen bonds with the main chain NH groups of residues 2, 3 and 9 of the loop. Mutation of this glutamate to valine in each of the EF-hands of yeast calmodulin (see Figure 1.6) reduced the calcium affinity by 100-fold (Geiser et al., 1991). The calcium liganding residues of most EF-hands are arranged in a pentagonal bi-pyramidal arrangement (Strynadka and James, 1989). The bi-dendate co-ordination at -Z, along with oxygens from positions Y, Z and -Y form an approximate planar pentagonal arrangement around the Ca\(^{2+}\). Residues at X and -X provide the bi-pyramidal aspect of this arrangement.

The remaining residues of the loop stabilize the geometry of the loop (necessary for calcium-binding) by providing hydrogen bonds from main chain NH-groups (Strynadka and James, 1989). Most calcium-binding proteins contain multiple (2 to 8) copies of the EF-hand motif. Troponin-C and calmodulin each contain four EF-hands with dissociation constants ranging from 10\(^{-7}\) to 10\(^{-5}\) M (Strynadka and James, 1991). Pairs of EF-hands associate intimately, via the loops and the helices, to enhance their affinity for calcium (Strynadka and James, 1989; Heizmann and Hunziker, 1991). The loops of adjacent EF-hands associate via two anti-parallel \(\beta\)-sheet hydrogen bonds. The helices
Figure 1.6 Ca\(^{2+}\) Binding Motifs of α-Actinin and Calmodulin.

A. The crystal structure of the EF-hands of carp muscle parvalbumin, resolved to 1.9Å. The calcium ion is represented with a * . Taken from Kretsinger and Barry, 1975.

B. Depicted are the EF-hands of: yeast calmodulin (CAL, Davis et al., 1986); chick smooth muscle α-actinin (CSM, Baron et al., 1987b); chick skeletal muscle α-actinin (CSK, Arimura et al., 1988); two variants of human skeletal muscle α-actinin, HuActSk1 (HSK1, Beggs et al., 1992) and HuActSk2 (HSK2, Beggs et al., 1992); Dictyostelium α-actinin (DAA, Noegal et al., 1987); Nematode α-actinin (NAA, Barstead et al., 1991); Drosophila α-actinin (DFA, Fyrberg et al., 1990) and the partial N-terminal EF-hand of a putative chick non-muscle α-actinin (CNM, Baron et al., 1987a). The positions of the calcium chelating residues of the EF-hand loop are denoted by the letters X, Y, Z, -Y, -X and -Z. The hydrophobic or inner aspects of the helices are located under columns headed n.
A.

Calcium binding loop

- Ca²⁺

B.

N-terminal EF-hand

C-terminal EF-hand

| CAL | EFKEAFALFKDNNNSSISSSELATVMRSLGLSP...-SEAEVNDLMEIDVDGYNHQIEFSEFLALMSRQ |
| CSM | EFRASFHVFRKHKTGMDIENBCLAISGMGYNM...-GEAEFARIMSIDPNRPMGVTFRQAPIDFMSRE |
| CSH | DFRAFNAVDRKVDKMGDHFRAVCISGMGYNM...-GEAEFARIMSLVDPNQGGTFTQIFDIFMTRE |
| HSK1 | EFRAFSNFHDKRGDKMDHEDFRAVCSGMGYNM...-GEAEFARIMTVLPDQGGTFTQIFDIFMTRE |
| HSK2 | EFRASFHVFRKNGMEFDDFRAVCISGMGYNM...-GEAEFARIMTVLPNAGGTVFTQIFDIFMTRE |
| DAA | EFKACOHDFKDNKLNKLFPESSPEIGDEL...-TEBQNVQTVKIDTDGKTSGPEPADFMRE |
| NAA | EFRASFHDFKERAG.LDPEQRSLISGTVTIPREGDAAELHKVLAHVDPNMPGRVPEAPLDFTKA |
| DFA | EFRASFHVFRKNGMVFDDFRAVCISGMGYNM...-GEAEFARIMTVLPNAGGTVFTQIFDIFMTRE |
| CNM | EFRASFHVDKNSGTLP |

Linker region
are amphipathic and associate with each other by forming intra- and inter-chain hydrogen bonds. The hydrophilic faces are exposed to solvent.

Using the above information, one can attempt to predict whether an EF-hand is likely to bind calcium from its sequence. The EF-hands of several α-actinins are aligned in Figure 1.6. Both EF-hands of Dictyostelium α-actinin meet all the criteria of fully functional EF-hands, and this might be expected of a calcium-sensitive non-muscle isoform (Noegal et al., 1987). In contrast, the smooth and skeletal muscle isoforms of chicken α-actinin are insensitive to calcium, and their EF-hands are predicted to be non-functional (Baron et al., 1987b; Arimura et al., 1988; Beggs et al., 1992). For example, the N-terminal EF-hand of chicken smooth muscle α-actinin contains a lysine at the Y vertex and an aspartate at the -Z vertex (Figure 1.6). The lysine at Y would not be able to contribute an oxygen towards liganding a calcium ion, and its positive charge would be expected to repel it. Aspartate contains a carboxylate side chain but it is too small to carry out the same functions as the most preferred residue at this site, glutamate, and this has been demonstrated by mutagenesis experiments involving troponin-C (Babu et al., 1992). Consequently, the N-terminal EF-hand of chicken smooth muscle α-actinin would not be expected to bind calcium (Baron et al., 1987b). The C-terminal EF-hand of chicken smooth muscle α-actinin contains a methionine and an alanine at the Z and -Z vertices respectively. Neither of these residues contain an oxygen in their side chains, and so this EF-hand would also not be expected to function (Baron et al., 1987b). Similar arguments may be applied to the N-terminal EF-hand of the chicken skeletal muscle α-actinin isolated by Arimura et al. (1988), which contains the positively charged amino acid arginine at the Y vertex and an aspartate at the -Z vertex. There is an oxygen-containing residue at each of the vertices of the C-terminal EF-hand of chicken skeletal muscle α-actinin (Arimura et al., 1988). However, this EF-hand contains a serine at the -Z vertex, and so would not be expected to be functional. The EF-hands of human skeletal muscle α-actinin are also predicted to be non-functional (Figure 1.6) (Beggs et al., 1992). Thus calcium-sensitivity may follow a simple rule, whereby non-muscle α-actinins with functional EF-hands bind actin in a calcium-sensitive fashion, whereas muscle α-actinins with non-functional EF-hands are insensitive towards calcium (Baron et al., 1987b). However, caution should be taken when making predictions from the primary sequence of an EF-hand, because residues far removed from the immediate environment of the metal ion co-ordination site can still have profound effects on the calcium-binding affinity. For example, the replacement of a surface carboxylate residue outside of the calcium-binding site of calbindin 9K with the isologous neutral amide reduced the calcium-binding affinity (Martin et al., 1990). Furthermore, when a disulfide bridge is introduced into troponin-C at sites far removed from the calcium-binding sites, the calcium-binding affinity of the oxidised state of the protein is decreased and there is a
loss of in-vivo activity (Grabarek et al., 1990). When the disulfide bonds are reduced normal calcium affinity and regulatory activity are reinstated. The calcium-binding affinity of a given EF-hand is therefore reliant on a complex interplay of structural features within the protein.

Drosophila and Nematode α-actinins contain five extra residues between the EF-hands, which are not present in chicken smooth muscle, chicken skeletal muscle, human skeletal muscle or Dictyostelium α-actinins. This linker region does not appear to be conserved in sequence, and so the length of this insertion must be important to its function and not the absolute structure (Figure 1.6). The N-terminal EF-hand of Nematode α-actinin (Barstead et al., 1991) contains an alanine at the Z vertex and a glutamine at the -Z vertex and is unlikely to bind calcium (Figure 1.6). The C-terminal EF-hand of this protein is also unlikely to be functional because it contains a methionine at the Z vertex, a proline at the -X vertex, and an alanine at the -Z vertex (Barstead et al., 1991). Consequently, this isoform of Nematode α-actinin is likely to be a calcium-insensitive, muscle-type isoform of α-actinin (Barstead et al., 1991). The N-terminal EF-hand of Drosophila α-actinin (Fyrberg et al., 1990) appears to fit all the criteria of a functional EF-hand (Figure 1.6). The C-terminal EF-hand of this isoform contains a histidine at the -X vertex, and an alanine at the -Z vertex. Neither of these residues contains oxygen-containing side chains, and so the C-terminal EF-hand is likely to be non-functional. Drosophila α-actinin is therefore predicted to contain 1 functional EF-hand per subunit. Drosophila α-actinin is encoded by a single alternatively spliced gene, and the EF-hands of both muscle and non-muscle isoforms are identical in sequence (Roulier et al., 1992). Consequently, these isoforms may not differ in calcium-binding affinity, and in Drosophila (at least ?) the non-muscle and muscle isoforms of α-actinin may not differ in calcium-sensitivity.

There is evidence that the function of the N-terminal EF-hand of α-actinin may be impaired when coupled with a non-functional C-terminal EF-hand. Witke et al. (1991) introduced a short sequence into the C-terminal EF-hand of Dictyostelium α-actinin by substituting residues DTDGN with DGSLGDIEPYDSS (see Figure 1.6), and tested the calcium-binding activity of the mutant protein. The altered confirmation of the C-terminal EF-hand was found to cause a significant reduction in calcium-binding affinity, and similar results were observed when point mutations were introduced into either one or both of the EF-hands of Dictyostelium α-actinin (Schleicher et al., 1991). Mutation of the N-terminal EF-hand alone was found to completely abolish calcium-sensitivity. Mutation of the C-terminal EF-hand alone did not abolish calcium-sensitivity, but 500 times more calcium was required to bring about inhibition. The calcium-binding affinity of the Dictyostelium α-actinin N-terminal EF-hand must therefore be reduced significantly upon mutation of the C-terminal EF-hand (Schleicher
et al., 1991). Furthermore, the C-terminal EF-hand is likely to have a co-operative affect on calcium-binding in the whole molecule (Schleicher et al., 1991). The binding of calcium to the C-terminal EF-hand in native Dictyostelium α-actinin may alter the confirmation of the molecule and this may enable the N-terminal EF-hand to bind calcium with high affinity.

1.2 Functional Properties of α-Actinin In-Vitro.

1.2.i Actin-Binding.

Actin occurs in two major states, monomeric (or G-actin) and filamentous (or F-actin) (reviewed by Bremer and Aebi, 1992). When α-actinin is added to F-actin solution there is a corresponding increase in viscosity, and this has frequently been demonstrated by falling-ball viscometry (Fechheimer et al., 1982; Duhaiman and Bamburg, 1984; Bennett et al., 1984; Burridge and Feramisco, 1981; Landon et al., 1985; Ohtaki et al., 1985; and Pollard, 1981). This technique measures the time taken for a steel ball to fall a fixed distance through a tube containing a solution of F-actin. The increase in viscosity is maximal at a ratio of 1 dimer of α-actinin per 9-13 actin monomers. This value approximates to 1 α-actinin dimer per cross-over repeat (i.e. 36nm) of the F-actin helix (Meyer and Aebi, 1990).

The binding affinity of α-actinin for F-actin is in the micromolar range and is temperature dependent (Meyer and Aebi, 1990; Bennett et al., 1984). Meyer and Aebi (1990) report that the binding affinities of chicken gizzard, Dictyostelium and Acanthamoeba α-actinin decrease with increasing temperature. For example, the $K_d$ of chicken gizzard α-actinin is $0.4\mu M$ at 22°C, and $1.2\mu M$ at 37°C. However, Bennett et al. (1984) have shown the exact opposite with rabbit macrophage α-actinin, i.e. increasing the temperature resulted in tighter binding. This discrepancy may result from the different sources or different states of purity of the proteins used by these workers. The affect of temperature on the cross-linking ability of α-actinin has also been investigated (Bennett et al., 1984; Duhaiman and Bamburg, 1984; Goll et al., 1972; Ohtaki et al., 1985; and Landon et al., 1985). These studies have shown that increasing the temperature diminishes the actin viscosity induced by α-actinin, and a much higher α-actinin to actin ratio is required to yield the same amount of bound α-actinin at 37°C than at 0°C. Using a large excess of α-actinin, Bennett et al. (1984) have shown that the maximum capacity of F-actin for α--actinin at 37°C is high corresponding to one α-actinin molecule per 2-3 actin monomers. However, α-actinin did not induce F-actin gel formation at this temperature. Despite these in-vitro observations, there is still speculation that α-actinin can cross-link F-actin under physiological conditions. For
example, Grazi et al., (1991) have found that α-actinin is an efficient F-actin gelling protein, even at 37°C, providing the concentration of actin is low (1.2-2.4 μM). This is because, as the F-actin concentration increases the rigidity of the gel is also increased, and more work is required to bring two F-actin filaments into the reaction distance of α-actinin (Grazi et al., 1991). Furthermore, it has been shown that PEG 6000 (at 6% w/v) can greatly assist the gelation of F-actin filaments by α-actinin at 37°C (Grazi et al., 1990). PEG 6000 exerts its effect by sequestering water molecules, and thereby inducing an osmotic effect. Since all macromolecules have some ability to induce osmotic effects, it is likely that the proteins of the cell sap may also enhance the F-actin cross-linking ability of α-actinin (Grazi et al., 1990). Recent evidence suggests that phosphatidylinositol 4, 5-bisphosphate (PtdInsP₂) may actually control the F-actin-gelating activity of α-actinin in-vivo (Fukami et al., 1992). PtdInsP₂ is a component of the phospholipid membrane and Z-disk, and its breakdown is enhanced during mitogenic stimulation of cells by growth factors (see Section 1.6.ii). Chicken skeletal muscle α-actinin contains large amounts of endogenous PtdInsP₂ (20-30 mol/mol α-actinin), whereas chicken smooth muscle α-actinin only contains approximately 2% of this level of endogenous PtdInsP₂ (Fukami et al., 1992). However, the smooth muscle isoform was found to bind exogenously added PtdInsP₂ (Fukami et al., 1992). Using falling-ball viscometry, the skeletal muscle isoform was found to induce F-actin gelation at 25°C, but smooth muscle α-actinin showed only weak gelating activity. However, PtdInsP₂ had a marked stimulating affect on the smooth muscle isoform, increasing its gelating activity to a level comparable with skeletal muscle α-actinin. These results strongly suggests that α-actinin requires the presence of PtdInsP₂ for maximal gelation activity, and this might explain why the gelating activity of skeletal muscle α-actinin is apparently higher than the smooth muscle isoform (Fukami et al., 1992).

The two subunits of the α-actinin molecule are arranged in an antiparallel orientation with the NH₂-terminal ABD located on the "knob-like" protrusion at the end of the rod (Mimura and Asano, 1987; Imamura et al., 1988). As a consequence α-actinin cross-links F-actin by binding a filament at each end (Podlubnaya et al., 1975). The cross-linked actin filaments are not randomly arranged, but are specifically orientated dependent upon the α-actinin under investigation (Meyer and Aebi, 1990). For example, chicken gizzard and Dictyostelium α-actinin cross-link F-actin filaments in an antiparallel fashion, whereas Acanthamoeba α-actinin links filaments preferentially in a parallel fashion. The in-vivo significance of this observation is not known.

Using electron microscopy, Meyer and Aebi (1990) have shown that the mean molecular lengths of Acanthamoeba and Dictyostelium α-actinin shorten significantly upon binding to F-actin. This effect is more prominent with Acanthamoeba (44.1 to 35.0nm) than Dictyostelium (31.4 to 28.1nm) α-actinin. However, shortening was not
observed with chicken gizzard (34.8 to 36.1nm) α-actinin. The mechanism underlying molecular shortening is not known, but it may occur as a result of the individual α-actinin subunits sliding past one another, or by a change in conformation within regions of the molecule such as the globular ABD.

The ability of α-actinin to bundle F-actin filaments has been reported by several workers, including Podlubnaya et al. (1975), Condeelis and Vahey (1982), Endo and Masaki (1982), Burn et al. (1985) and Meyer and Aebi (1990). Bundles consist of parallel arrays of F-actin filaments, which are linked to one another at regular intervals by α-actinin molecules arranged like the rungs of a ladder. Meyer and Aebi (1990) have shown that a critical molar ratio of α-actinin to actin (approximately 1 : 20) must be exceeded for bundle formation to occur. Bundles were found to disintegrate within seconds of removing or diluting out the free α-actinin. It is not known whether this phenomenon occurs in-vivo or not, but it is tempting to speculate that the intracellular free concentration of α-actinin may provide a means of controlling F-actin bundle formation within the cell. Meyer and Aebi propose that F-actin bundles may switch configuration, from "tight" to "loose" bundles, by lowering the surrounding pool of free α-actinin. In the tight configuration α-actinin molecules may be arranged end-to-end along the F-actin filaments, with their long axes running parallel to the filament axes. In contrast, α-actinin molecules within the loose configuration may be orientated with their long axes perpendicular with the filament axes. These configurations may interchange via "obliquely" orientated α-actinin intermediates.

Meyer and Aebi (1990) have shown that Dictyostelium α-actinin, unlike Acanthamoeba and chicken gizzard α-actinin, will not form F-actin filament bundles even at the critical bundling concentration. Instead, this protein was found to organise F-actin into network structures, with filaments radiating from foci containing α-actinin. This type of filament organisation is very similar to that reported for chicken liver α-actinin (Ohtaki et al., 1985). Meyer and Aebi propose that the length of the bound α-actinin molecule may determine its spacing along the F-actin filament, and this in-turn may decide whether or not the protein can induce F-actin bundle formation. When bound to F-actin, chicken gizzard and Acanthamoeba α-actinin molecules space themselves at intervals equivalent to the size of the bound α-actinin molecule, a distance which also approximates with the cross-over repeat of the double stranded F-actin helix (i.e. 36nm). This may be a pre-requisite for bundle formation. In contrast, the length of the bound Dictyostelium α-actinin molecule is only 28nm, and as a consequence its spacing interval along the F-actin filament is far shorter than the cross-over repeat. The length of the Dictyostelium α-actinin molecule may perturb F-actin bundle formation. This property of Dictyostelium α-actinin may be shared by all α-actinins that form networks of F-actin filaments in preference to bundles. The actin-severing protein actophorin has
been shown to promote the formation of F-actin bundles by α-actinin in-vitro (Maciver et al., 1991). Such proteins may cut F-actin into shorter filaments which are more free to diffuse, rotate and interact laterally with other short filaments via α-actinin to form bundles of filaments (Maciver et al., 1991). Maciver and co-workers suggest that this may be one of the major functions of actin severing proteins in the cell.

The interaction between α-actinin and G-actin has also been studied (Ohtaki et al., 1985, Lebart et al., 1990). Using gel filtration chromatography, Ohtaki et al., (1985) failed to detect any interaction. However, these experiments were performed in 20μM MgCl₂, which is lower than the critical 2mM MgCl₂ required for the polymerisation of G-actin into F-actin. Lebart et al., (1990) suggest that 2mM MgCl₂ might also induce conformation changes in actin monomers which affect their ability to interact with α-actinin. To study these salt affects Lebart and co-workers investigated the interaction between G-actin and chicken gizzard α-actinin in the presence of 2mM MgCl₂ using an ELISA technique. In these experiments G-actin was immobilised onto plastic to prevent it polymerizing. Under these conditions α-actinin was found to bind G-actin with an affinity comparable with F-actin (Kd = 2x10⁻⁶M). Using synthetic peptides and polyclonal antibodies against known actin sequences, these workers found that α-actinin interacts with 2 segments on the actin sequence, the first located near residue 103 and the second in the last twenty amino acids of the molecule (residues 355-375). These results are in agreement with the EDC-catalysed cross-linking experiments of Mimura and Asano (1987), and the proton NMR experiments of Levine et al., (1992) (see above).

The structure of F-actin and G-actin have been reviewed by Bremer and Aeber (1992). The actin monomer consists of a large and a small subunit, between which is a cleft containing a bound molecule of adenine nucleotide (ATP/ADP) and a calcium ion. The two subunits can be further subdivided to give 4 subdomains, which lie roughly at the corners of a square. The small subunit (comprising sub-domains 1 and 2) is at a larger radius from the F-actin helix axis than the large subunit (comprising subdomains 3 and 4). α-Actinin binds to actin residues 83-117 and 350-375, which lie fairly close (1-1.5nm) on subdomain 1 of the 3D-model presented by Kabsch et al., (1990). This domain is readily accessible in filamentous actin and known to interact with several actin-binding proteins such as tropomyosin and profilin (Kabsch et al., 1990). Profilin is a small actin-binding protein expressed in all eukaryotic cells (Vandekerchove et al., 1989; Cao et al., 1992). Actin forms a 1:1 complex with profilin, forming profilactin. α-Actinin can promote the polymerisation of actin from profilactin complex, and this might be explained by a direct competition between these proteins for actin-binding sites (Blikstad et al., 1990). Indeed, profilin interacts with the C-terminal region of actin (near residue 364) which also binds to α-actinin (Blikstad et al., 1990; Vanderckhove et al.,
1989). Tropomyosin binds along the groove of actin filaments, and interacts with Arg95 (Johnson and Blazyk, 1978) and Cys374 within the C-terminal domain of actin (Moir and Levine, 1986). α-Actinin and filamin can bind simultaneously to F-actin, whilst tropomyosin exhibits marked competition with α-actinin for sites on actin filaments (Zeece et al., 1979). The competitive affect of tropomyosin is temperature dependent, and whilst α-actinin can displace tropomyosin from F-actin at 0°C, the reverse occurs at 37°C. At this temperature tropomyosin almost completely abolishes the effect of α-actinin on F-actin viscosity. Goll et al. (1972) have shown that α-actinin binds preferentially to the filament ends of tropomyosin-decorated F-actin at 37°C, with a binding ratio of approximately two α-actinin molecules per F-actin strand of length 1μm (Goll et al., 1972). Recently, Grazi et al. (1991) have confirmed that α-actinin does bind the F-actin/tropomyosin complex less tightly than F-actin alone at 37°C, but they found no evidence that α-actinin binds to the filament ends at this temperature.

1.2.ii Calcium-Sensitivity of Non-Muscle α-Actinin.

Unlike α-actinins derived from muscle sources, those derived from non-muscle tissues bind to F-actin in a calcium-sensitive manner (Burridge and Feramisco, 1981; Blanchard et al., 1989). This property has been demonstrated with non-muscle α-actinin derived from Ehrlich tumour cells (Mimura and Asano, 1979), Acanthamoeba (Pollard, 1981), Dictyostelium (Fechheimer et al., 1982), Hela cells (Burridge and Feramisco, 1981), human platelets (Rosenberg et al., 1981; Landon et al., 1985), rat liver (Kuo et al., 1982; Ohtaki et al., 1985), porcine kidney (Kobayashi and Tashima, 1983), rabbit alveolar macrophages (Bennett et al., 1984) and chicken brain (Duhammad and Bamburg, 1984). In most of these studies calcium-sensitivity was observed using falling-ball viscometry (Fechheimer et al., 1982; Duhammad and Bamburg, 1984; Bennett et al., 1984; Burridge and Feramisco, 1981; Landon et al., 1985; Ohtaki et al., 1985; and Pollard, 1981). For example, in the absence of free calcium (<10^-7M) the addition of Hela cell α-actinin to a pre-formed solution of F-actin caused the onset of filament gelation (marked by a sharp increase in viscosity) (Burridge and Feramisco, 1981). However, when the concentration of free calcium was made to exceed 10^-7M, there was a sharp fall in viscosity, and at 10^-5M Ca^{2+} no actin gelation activity could be detected. The viscosity was found to fall to a level comparable with that of pre-formed F-actin alone, indicating that the cross-linking activity of α-actinin had been totally removed. When the same experiment was performed with smooth muscle α-actinin instead of Hela α-actinin, the viscosity of the F-actin solution was not affected over the range of free calcium studied (Burridge and Feramisco, 1981). The effect of calcium on the F-actin
cross-linking activity of non-muscle α-actinin is apparently reversible (Fechheimer et al., 1982; Landon et al., 1985).

The calcium-sensitivity of non-muscle α-actinin has also been demonstrated by F-actin sedimentation (Fechheimer et al., 1982; Kuo et al., 1982; Duhaiman and Bamburg, 1984; Bennet et al., 1984; Kobayashi and Tashima, 1983; Rosenberg et al., 1981; Mimura and Asano, 1979; Landon et al., 1985; Ohtaki et al., 1985). For example, Duhaiman and Bamburg (1984) incubated radioactively labelled chicken brain α-actinin with F-actin for 3 hours at 4°C in the presence or absence of 2mM Ca^{2+}. Following ultra-centrifugation, the pellets (containing F-actin and bound α-actinin) were removed for scintillation counting and for SDS-PAGE/densitometry analysis. In the absence of calcium (<10^{-7}M), chicken brain α-actinin was found to bind F-actin at a level comparable with chicken smooth muscle or chicken skeletal muscle α-actinin (~1 α-actinin dimer per 9-11 actin monomers). The addition of 2mM Ca^{2+} to the assay buffer did not affect the binding of either of the muscle α-actinins to F-actin, but the binding of chicken brain α-actinin was reduced by approximately 50%. Similar F-actin co-sedimentation results were obtained using non-muscle α-actinins derived from rabbit macrophage (Bennet et al., 1984), human platelet (Landon et al., 1985), rat liver (Ohtaki et al., 1985), and porcine kidney (Kobayashi and Tashima, 1983). However, when one considers that chicken brain α-actinin is completely incapable of increasing the viscosity of F-actin at concentrations of free Ca^{2+} greater than 10^{-5}M (see above), it is surprising that the level of F-actin-binding was only reduced by 50% (Duhaiman and Bamburg, 1984). This suggests that both actin-binding sites of the α-actinin dimer are required for cross-linking F-actin, and that both are fully functional in the absence of free calcium (i.e. below 10^{-7}M). However, in the presence of calcium, one of the actin-binding sites must become inoperative, leaving the α-actinin dimer incapable of cross-linking F-actin, but capable of binding F-actin at a reduced level. Similar, conclusions were drawn by Ohtaki et al. (1985), when looked at the interaction between F-actin and α-actinin in the presence and in the absence of Ca^{2+} by electron microscopy of rotary shadowed samples. Under conditions of low [Ca^{2+}] rat liver α-actinin was found to cross-link F-actin into network structures, with filaments radiating from "spot-like" structures containing α-actinin. In the presence of calcium (0.4mM) no spot-like structures were observed but numerous α-actinin molecules were detected binding along the side of actin filaments, using only one of their actin-binding sites.

So far, the stoichiometry by which Ca^{2+} binds to non-muscle α-actinin has only been determined for rabbit macrophage α-actinin (Bennett et al., 1984). In this study, the binding of \textsuperscript{45}Ca^{2+} to rabbit macrophage α-actinin at 4°C was determined by equilibrium dialysis. The protein was found to bind four calcium ions per dimer with an affinity of 4 \times 10^{-6}M^{-1}. If this data is accurate, then macrophage α-actinin must contain
two functional EF-hands per polypeptide. However, using $^{45}\text{Ca}^{2+}$-gel overlay Pacaud and Molla (1987) failed to detect any binding of $^{45}\text{Ca}^{2+}$ to macrophage $\alpha$-actinin present in low-calcium cytosolic extracts of rabbit macrophage cells. These extracts contain a macromolecular complex of actin, filamin, $\alpha$-actinin, and two proteins of 70-kDa and 50-kDa. $^{45}\text{Ca}^{2+}$ was found to bind strongly to the 70-kDa protein and to calmodulin (positive control) transferred onto the same blot. Pacaud and Molla (1987) suggest that $\alpha$-actinin may not have bound $^{45}\text{Ca}^{2+}$ in this experiment due to insufficient renaturation of the protein following SDS-PAGE and immobilisation to nitrocellulose.

The molecular mechanism underlying the calcium-sensitivity of the non-muscle isoform is not known. However, since $\alpha$-actinin forms an antiparallel homodimer, it is possible that the interaction between calcium and the EF-hands of one subunit may directly inhibit the binding of F-actin at the ABD of the apposing subunit (Noegal et al., 1987). In this model, the conformation of the EF-hands becomes altered on binding $\text{Ca}^{2+}$, and this sterically inhibits the binding of F-actin. However, these changes are too small to be detected in rotary shadowed images of non-muscle $\alpha$-actinin prepared in the presence or absence of $\text{Ca}^{2+}$ (Fechheimer et al., 1982; Bennett et al., 1984). The molecular re-arrangements that accompany the binding of calcium to non-muscle $\alpha$-actinin will only become fully known when the 3-D structure of the protein (in the calcium-free and calcium-bound states) has been determined.

1.3 Isoforms of $\alpha$-Actinin.

$\alpha$-Actinin has been classified into 3 major isoforms, namely skeletal muscle, smooth muscle and non-muscle $\alpha$-actinin (Burridge and Feramisco, 1981; Blanchard et al., 1989). A major functional difference between these isoforms is the calcium-sensitivity of non-muscle $\alpha$-actinin (see Section 1.2.ii). However, a number of lines of data suggest that these proteins are both chemically and immunologically distinct (Bretscher et al., 1979; Endo and Masaki, 1982; and Duhaicaim and Bamburg, 1984). For example, all 3 isotypes have similar but distinct total amino acid compositions and one-dimensional V8-protease cleavage profiles (Bretscher et al., 1979; Endo and Masaki, 1982; and Duhaicaim and Bamburg, 1984). Furthermore, antisera raised against chicken skeletal muscle or chicken smooth muscle $\alpha$-actinin were found to react strongly with their own antigens in double-immunodiffusion experiments, but antigen cross-reactivity was not detected using this technique (Bretscher et al., 1979; Endo and Masaki, 1982). However, both antisera were found to stain $\alpha$-actinin in skeletal and smooth muscle cryostat sections using the more sensitive technique of immunofluorescence microscopy (Endo and Masaki, 1982). This data suggests that although chicken skeletal muscle and smooth muscle $\alpha$-actinins contain many distinct
epitopes, they do appear to share some common antigenic determinants (Endo and Masaki, 1982). Chicken smooth muscle α-actinin has been shown to bind far less endogenous phosphatidylinositol 4,5-bisphosphate (PtdInsP$_2$) than the chick skeletal muscle isoform (see Section 1.2.ii) (Fukami et al., 1992). PtdInsP$_2$ is required for the maximal F-actin gelation activity of α-actinin, and this may explain why smooth muscle isoform was found to induce gelation at a far lower level than the skeletal muscle isoform in the absence of exogenously added PtdInsP$_2$ (see Section 1.2.ii) (Fukami et al., 1992).

The chick smooth muscle and chick skeletal muscle isoforms of α-actinin are 74% identical with one another at the cDNA level (Baron et al., 1987b; Arimura et al., 1988), and their deduced amino acid sequences are particularly divergent at the N-terminal end of the molecule. Chick skeletal muscle α-actinin is 9 amino acids longer at its N-terminal end, and has a novel sequence over the next 12 amino acids (Figure 1.2). Outside of this region these proteins differ at numerous locations which are evenly distributed throughout the molecule. It is therefore clear that these isoforms are the products of separate genes. This conclusion is supported by the failure of the chick smooth muscle and skeletal muscle cDNAs to cross-hybridise with their respective mRNAs in northern blots (Baron et al., 1987b; Arimura et al., 1988). Chromosome mapping experiments involving fluorescent in-situ hybridisation and somatic cell hybrid panels have shown that the smooth muscle and skeletal muscle α-actinin genes are located on different genes (Youssoufian et al., 1990; Beggs et al., 1992). The gene encoding the smooth muscle/non-muscle isoform of human α-actinin is located chromosome 14q22-24 (Youssoufian et al., 1990). There are least two genes encoding human skeletal muscle α-actinin, HuActSk1 on chromosome 1q42-q43 and HuActSk2 on chromosome 11q13-14 (Beggs et al., 1992). The α-actinin encoded by HuActSk1 (104-kDa) is the human homologue of the chicken skeletal α-actinin variant identified by Arimura et al. (1988), but the HuActSk2 gene product (103-kDa) is a distinct variant of skeletal muscle α-actinin. HuActSk1 and HuActSk2 differ in amino acid sequence at sporadic intervals along the molecule, but are particularly divergent with one another at the extreme N-terminus (Figure 4.11, Chapter 4). Northern blot analysis has shown that HuActSk1 is expressed in both skeletal and cardiac muscle, whereas HuActSk2 is expressed in skeletal muscle only (Beggs et al., 1992). At this stage, it is not known if these variants display distinct functional properties or function at distinct sites within the cell.

The exact number of variants of each major isoform of α-actinin is not known, but approximately 11 variants of α-actinin have been identified in chicken smooth, skeletal and cardiac muscles by isoelectric focusing (Endo and Masaki, 1982). The isoelectric variants were found to range between pH 5.46 to pH 6.60, and the
Relative amounts of each variant were found to vary from muscle to muscle (Endo and Masaki, 1982). Suzuki et al. (1973) have detected distinct tissue-specific forms of porcine $\alpha$-actinin in fast (also known as white) and red (also known as slow) skeletal muscle. Slow skeletal muscle $\alpha$-actinin contained more aspartic acid residues than fast skeletal muscle $\alpha$-actinin, but both variants contained similar amounts of positively charged amino acids. Consequently, slow skeletal muscle $\alpha$-actinin could be separated from fast skeletal muscle $\alpha$-actinin by DEAE-cellulose chromatography or polyacrylamide gel electrophoresis in the absence of SDS (Suzuki et al., 1973). Slow skeletal muscle fibers have significantly thicker Z-lines than fast fibers, and this may be a consequence of the distinct types of $\alpha$-actinin expressed by these muscle types. Distinct variants of $\alpha$-actinin have also been identified in rabbit fast twitch skeletal muscle (Kobayashi et al., 1984; Schachat et al., 1985). Schachat et al., (1985) resolved two variants of rabbit fast skeletal muscle $\alpha$-actinin by SDS-PAGE, termed $\alpha$-A1F (104-kDa) and $\alpha$-A2F (100-kDa). $\alpha$-A1F was most abundant in tongue muscle, whereas $\alpha$-A2F was most predominant in psoas, adductor magnus and rectus femoris muscle. The two variants were found to produce totally distinct CNBr peptide cleavage profiles indicating that there are extensive differences in amino acid sequences. However, $\alpha$-A1F was found to exhibit the same molecular weight as slow skeletal muscle $\alpha$-actinin by SDS-PAGE, and also produced an identical CNBr cleavage profile as this protein. $\alpha$-A1F is also similar to slow skeletal muscle $\alpha$-actinin in that it is expressed in fast skeletal muscle fibers which exhibit significantly thicker Z-lines than those expressing $\alpha$-A2F (Schachat et al., 1985; Suzuki et al., 1973). Schachat et al., (1985) suggest that $\alpha$-A1F and $\alpha$-A2F may be equivalent to the red and white skeletal muscle $\alpha$-actins respectively recognised by Suzuki et al. (1973) in pig, and that both variants may be expressed simultaneously in the same muscle fibres albeit at different levels. Two variants of fast skeletal muscle $\alpha$-actinin were also identified by Kobayashi et al. (1984). Type I $\alpha$-actinin is enriched in rabbit psoas muscle, whereas type II $\alpha$-actinin is abundant in longissimus dorsi muscle. Type II $\alpha$-actinin contains more aspartic acid and isoleucine than type I $\alpha$-actinin, but fewer glycine and valine residues (Kobayashi et al., 1984). The two $\alpha$-actins also display distinct two-dimensional tryptic peptide maps, and intact type II $\alpha$-actinin was found to migrate more slowly (112-kDa) than type I $\alpha$-actinin (100-kDa) on 5% SDS-PAGE (Kobayashi et al., 1984). The relationship between the two rabbit fast skeletal variants identified by Schachat et al. (1985) and the two identified by Kobayashi et al. (1984) is not clear. Schachat et al. (1985) did not identify a species as large as 112-kDa in their fast skeletal muscle preparations, indeed they found that $\alpha$-A2F made up more than 85% of the total $\alpha$-actinin in both psoas and longissimus dorsi muscles. There are two types of fast twitch fibres, termed fast twitch glycolytic and fast twitch oxidative-glycolytic, which have
distinct histochemical, physiological, metabolic and ultrastructural properties. Interestingly, an antibody raised against the rod domain of dystrophin was found to cross-react with a specific variant of α-actinin (90-kDa) found only in fast-twitch glycolytic skeletal muscle (Hoffman et al., 1989). This variant is severely depleted in patients with symptomatic Duchene muscular dystrophy (see later) (Minetti et al., 1991). This data clearly shows that there are distinct tissue-specific variants of α-actinin expressed in discrete skeletal muscle fibres. However, at this stage it is not known how many of these variants arise via post-translational modification of a common protein core. The physiological significance of the tissue-specific variation of skeletal muscle α-actinin also remains to be established. Interestingly, not one variant of skeletal muscle α-actinin has yet been identified with unique biological properties (such as F-actin-binding and stimulation of the acto-myosin Mg²⁺ATPase).

Distinct variants of skeletal muscle α-actinin have also been identified in insect muscles. In Drosophila, there are two variants of skeletal muscle α-actinin which differ in subunit molecular weight by immunoblotting analyses (Vigoreaux et al., 1991). Preparations of flight muscles, jump muscles and leg muscles have significantly more of the smaller variant of Drosophila α-actinin, whilst larval intersegmental muscles have more of the larger variant. The larval intersegmental muscles are supercontractile, and their Z-bands contain perforations which allow filaments from one sarcomere to pass into adjacent ones. The extent of sarcomeric shortening is therefore not limited by the length of the thin and thick filaments. The larger supercontractile' variant of Drosophila α-actinin may allow for the Z-band distortions which occur during supercontraction, such as the observed widening of the perforations (Vigoreaux et al., 1991). The single gene encoding Drosophila α-actinin contains one variable exon which is variably spliced to give one non-muscle and the 2 muscle isoforms, i.e. adult muscle and supercontractile muscle α-actinins (Roulier et al., 1992). In all 3 isoforms the ABD, repeats 2-4, and the EF-hands are identical in sequence (Figure 1.7). The single variable exon encodes a peptide located at the junction of the ABD and the first central repeat (Roulier et al., 1992). The specific roles of each isoform of Drosophila α-actinin must therefore be conferred by this peptide. The non-muscle and adult muscle isoforms are 72% identical in this region (Figure 1.7). The Drosophila supercontractile muscle α-actinin is identical with adult muscle α-actinin over this stretch of 29 amino acids, but contains an additional 22 residues (see Figure 1.7). An isoform of Nematode α-actinin has also be shown to contain an insertion of sequence (27 residues), in exactly the same position as Drosophila supercontractile muscle α-actinin (Barstead et al., 1991). The sequence of the Nematode insertion is not similar to the that of Drosophila, but both regions contain
Figure 1.7 Comparison of the Amino Acid Sequences of 3 Isoforms of *Drosophila* α-Actinin with the Sequence of *Nematode* α-Actinin.

Three alternative isoforms of α-actinin are encoded by a single *Drosophila* α-actinin gene (Roulier et al., 1992). These are the non-muscle (DNM), the adult muscle (DAM), and the supercontractile muscle (DSM) isoforms of *Drosophila* α-actinin. The amino acid sequences encoded by codons 229-257 of non-muscle and adult muscle mRNAs are 72% identical. The supercontractile isoform mRNA encodes an extra 22 amino acids in the region between the ABD and the repeats. A similar spacer stretch has been identified in *Nematode* α-actinin (NAA), but not any other previously identified α-actinins (Barstead et al., 1991). The spacer peptides of DSM and NAA are not related in sequence, except for being rich in proline residues (underlined). Vertical lines (1) indicate regions of total amino acid sequence identity. The numbering refers to the amino acid sequence of the supercontractile isoform of *Drosophila* α-actinin. [Modified from Roulier et al., (1992)].

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<td>DNM</td>
<td>DLQNTALPDERA VMTYVSSYYHCFSGAQK</td>
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<td>DAM</td>
<td>DLI NT PQDERA IMTYVSCY YHAFQGAQQ</td>
<td>I I I I I I I I I I I I</td>
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<td>I I I I I I I I I I</td>
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</tr>
<tr>
<td>DSM</td>
<td>DLI NT PQDERA IMTYVSCY YHAFQGAQQVGNVTHVEP TRQYTVPNYN</td>
<td>I I I I I I I I I I I I</td>
<td>I I I I I I I I I I I</td>
<td>I I I I I I I I I I</td>
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<tr>
<td>NAA</td>
<td>HPDEKSTMTYT VSCFYHAFRNMRDPPPPVI RQPPPQ RVVVAAPPPPERDWRKD</td>
<td>I I I I I I I I I I I I</td>
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several proline residues (see Figure 1.7). This stretch of sequence may therefore form a non-helical spacer or hinge region between the ABD and repeat domains of the protein (Roulier et al., 1992).

A developmental sequence of α-actinin expression has been observed in chick skeletal muscle (Kobayashi et al., 1989). In extracts of total skeletal muscle from 8-10-day-old embryos, a so called 'embryonic' isoform of α-actinin was detected. In extracts from back, pectoral, forelimb or hindlimb skeletal muscles derived from 15 day old embryos a second isoform of α-actinin, the 'adult' isoform, was also present. However, by 21 days only the adult isoform was evident in these tissues. The embryonic isoform had a molecular mass of 112-kDa and an isoelectric point of 5.8 whereas the values for the adult isoform were 100-kDa and 5.85 respectively. The embryonic isoform also had a distinct tryptic peptide map when compared with the adult isoform in 15-day-old-embryo skeletal muscle (and α-actins purified from adult chicken pectoral and gizzard muscles). The adult isoform detected in 15-day-old embryo muscle gave an identical peptide map to the adult chicken pectoral muscle α-actinin, but a map distinct from that of adult chicken gizzard α-actinin. This suggests that there are distinct embryonic and adult variants of chick skeletal muscle α-actinin, and that the adult variant replaces the embryonic variant during embryogenesis (Kobayashi et al., 1989). A similar developmental analysis has been performed on extracts derived from rabbit fast skeletal muscles (Briggs et al., 1990). In fetal muscle only the larger variant of fast skeletal muscle α-actinin (α-A₁F, 104-kDa) was detected. However, within a week following birth the smaller variant of fast skeletal muscle α-actinin (α-A₂F, 100-kDa) was also expressed, and this variant then predominated into adult tissues (Briggs et al., 1990).

A complete cDNA (C17) encoding what appears to be the smooth muscle isoform of α-actinin has been isolated from a CEF cDNA library (Baron et al., 1987b). The amino acid sequence deduced from this cDNA was found to identical with the sequence of several proteolytic fragments derived from several regions along the chicken smooth muscle α-actinin molecule, including the EF-hands (Baron et al., 1987b). As mentioned above, both EF-hands of smooth muscle α-actinin contain unsuitable residues at vital positions within the loop, and would not be expected to bind calcium (see Figure 1.6). A partial cDNA (C18) isolated from the same CEF library was found to be identical with C17 in the region encoding the ABD and repeats (Baron et al., 1987a). However, the last eight amino acids encoded by C18 were found to divergent with C17 in the region of the N-terminal EF-hand (see Figure 1.6). As a result, the Y vertex of the α-actinin encoded by C18 was found to contain an aspartate residue, which is the most preferred residue at this site. Despite lacking some N-terminal and C-terminal coding sequence, the partial cDNA C18 appears to encode the chick non-muscle isoform
of α-actinin. If so, the smooth muscle and non-muscle isoforms are likely to arise via alternative splicing. The ABD and repeats may be totally conserved between these isoforms, and may confer similar functions in both proteins. However, the smooth muscle and non-muscle isoforms differ with respect to their sensitivity towards calcium, and their EF-hand domains may therefore be distinct and encoded by separate exons. These exons may be transcribed onto the same transcript, and then differentially spliced to give two distinct mRNAs. This situation will only become clarified when cDNAs encoding the complete coding sequence of chick non-muscle α-actinin and chick genomic clones derived from the EF-hand region of the smooth muscle/non-muscle α-actinin gene have been isolated and sequenced.

There is little information on the number of isoelectric variants of non-muscle α-actinin, but two distinct types of platelet α-actinin have been observed (Landon et al., 1985), which differ in molecular weight (type aa, 97-kDa and type cc, 94-kDa). These variants have markedly different V8-protease cleavage profiles, and distinct immunological characteristics. Both platelet α-actinins stimulate the acto-myosin Mg^2+ATPase, bind F-actin and cross-link F-actin in a calcium-sensitive manner (Landon et al., 1985). The F-actin cross-linking activity of both aa and cc was inhibited to the same extent by calcium, but the F-actin-binding ability of aa was inhibited far more strongly by calcium than cc. This result suggests that non-muscle cells may contain structurally and functionally distinct variants of calcium-sensitive α-actinin.

So far, the only clearly demonstrated differential localisation of α-actinin isoforms within a cell has been observed in cultured chick skeletal muscle cells (Endo and Masaki, 1984). In this study, antibodies rendered monospecific for either chick skeletal muscle or chick smooth muscle α-actinin by cross-adsorption were used to stain both mononucleated and fused chick skeletal muscle myotubes. The smooth muscle α-actinin specific antiserum was found to stain the cytoplasm and membranous structures of mononucleated cells, but only the membranous structures of fused myotubes (Endo and Masaki, 1984). In contrast, the antiserum specific for skeletal muscle α-actinin did not stain myogenic cells before fusion, but after fusion labelling was restricted to the Z-discs. These results suggest that the skeletal muscle and smooth muscle α-actinins are present in distinct subcellular structures in the cell and do not co-localise (Endo and Masaki, 1984). The isolation of a smooth muscle α-actinin cDNA from a non-muscle cDNA library has given rise to the suggestion that smooth muscle and non-muscle isoforms of α-actinin may be expressed simultaneously in non-muscle cells, where they function in unique areas (Baron et al., 1987b). For example, the smooth muscle-type calcium-insensitive isoform may be targeted to the non-muscle stress fibres which are sarcomere-like in both structure and function (see Section 1.4.iii), so that these structures can maintain tension even during periods of increased intracellular [Ca^{2+}]
(Baron et al., 1987b). In contrast, non-muscle-type calcium-sensitive α-actinin may be targeted to the actin-filament networks and filament/membrane junctions of non-muscle cells. Elevated intracellular [Ca$^{2+}$] may cause the release of actin from α-actinin bound at these sites. This may occur under the influence of external factors such as hormones and growth factors, providing another mechanism by which control can be exerted over cell adhesion. However, a differential localisation of α-actinin isoforms in vertebrate non-muscle cells was not observed when α-actinins purified from calf thymus (Meigs and Wang, 1986), chick smooth muscle (Feramisco, 1979; McKenna et al., 1985) and chick skeletal muscle (Sanger et al., 1986) were microinjected into cells and observed by fluorescence microscopy. Each protein was incorporated into both the stress fibres and adhesion plaques of injected non-muscle cells. Since α-actinin extracted from tissues is likely to consist of a mixture of isoforms and isoform variants, conclusions drawn from such experiments are obviously open to uncertainty.

1.4 Structure and Function of the Major Cellular Components Containing α-Actinin.

The precise function of α-actinin in-vivo is not known. However, the location of this protein within the cell suggests that it may bundle F-actin filaments and anchor them to various intracellular structures. The dense bodies of smooth muscle are analogous in function with the Z-discs of skeletal muscle. In these structures α-actinin may bind actin filaments securely, and hold them in register within the cytoplasm of the cell for even contraction (Endo and Masaki, 1982). Similarly, the membrane associated dense plaques of smooth muscle and the intercalated disks of cardiac muscle may function in an analogous manner to the adhesion plaques of non-muscle cells. At these loci α-actinin may participate in attachment of cytoplasmic bundles of actin filaments to the membrane thus transmitting their tension across the membrane to the ECM (Endo and Masaki, 1982; Burridge et al., 1988). The precise reason why calcium-sensitivity is restricted to the non-muscle isoform of α-actinin is not known, but this property may reflect the different requirements of the non-muscle and muscle contractile systems (Burridge and Feramisco, 1981). A stable calcium-insensitive actin contractile apparatus may be required in muscle for efficient repeated contraction. In this situation, an α-actinin isoform that stopped binding actin in the presence of calcium would be a distinct disadvantage. In contrast, the cytoskeleton of non-muscle cells may require almost constant re-organisation to support the shape changes accompanying cell movement. This process may be controlled, in part, by the intracellular concentration of free calcium. Disassembly of the actin network may follow a calcium-sensitive loss of cross-linking activity. If so, non-muscle α-actinin may be one of the chief calcium sensors
involved in this system in vertebrate cells. The calcium-sensitivity of non-muscle \( \alpha \)-actinin might also be involved in the release of vesicles attached to the actin microfilaments of secretory cells (Duhaiman and Bamburg, 1984). \( \alpha \)-Actinin has been detected in the secretory vesicles of both platelets and adrenal chromaffin cells (Jockusch et al., 1977), and has been partially purified from the coated vesicle fraction of bovine brain (Schook et al., 1978). Synaptic vesicles have been shown to bind to F-actin microfilaments (LeBeaux and Willemot, 1975), and their release is dependent on elevated \([Ca^{2+}]\) near the synapse of nerve endings (McGraw et al., 1980). \( \alpha \)-Actinin may therefore be involved in the binding of these vesicles to microfilaments, and their release in response to elevated calcium (Duhaiman and Bamburg, 1984).

The following sections describe 3 of the major intracellular structures with which \( \alpha \)-actinin is associated, and outline the putative functions of \( \alpha \)-actinin within them.

1.4.i The Z-disc.

The basic repeat unit of skeletal muscle is the sarcomere (reviewed by Amos et al., 1985). Adjacent sarcomeres within the myofibril are connected at the Z-disc. The thin (F-actin) filaments from adjacent sarcomeres bind tightly to the Z-disc, and are aligned laterally to ensure even contraction (Figure 1.8). \( \alpha \)-Actinin is the major component of the Z-disc, forming 2\% of the total myofibrillar protein (Ebashi and Ebashi, 1965). The major role of \( \alpha \)-actinin within this structure may be to bind the actin filaments and hold them in register at this site during the contraction-relaxation cycle (Hurridge and Feramisco, 1981).

In addition to \( \alpha \)-actinin, the Z-disc contains a number of proteins including: desmin (Lazarides, 1978); vimentin (Granger and Lazarides, 1979); eu-actinin (Kuroda et al., 1981); synemin (Granger and Lazarides, 1980); filamin (Gomer and Lazarides, 1981); Z-protein (Ohashi and Maruyama, 1979); Z-nin (Suzuki and Nonami, 1982); and glycogen phosphorylase (Maruyama et al., 1985). Desmin, vimentin and synemin are known as the intermediate filaments because their filamentous diameters are intermediate between those of the actin "thin" filaments (9nm) and the myosin "thick" filaments (15nm). Using immunofluorescence, the intermediate filaments (and also filamin) can be detected in a network of interconnected "collar-like rings" on the periphery of the Z-disc (Lazarides and Hubbard, 1976; Gomer and Lazarides, 1981; Granger and Lazarides, 1980). The intermediate filaments are thought to co-aggregate to form the cytoplasmic bundles which link the individual Z-discs to each other and to the plasma membrane. However, these proteins are not essential for myofibrillogenesis (Schultheiss et al., 1991).
The interior of the Z-disc is composed of at least two phases: Z-filaments and an amorphous matrix (Kelly and Cahill, 1972). The Z-filaments provide the structural backbone of the Z-disc (Figure 1.8). The amorphous matrix fills the interspace of the Z-disc and cements neighbouring Z-filaments. The major components of the amorphous phase are 75-kDa and 55-kDa proteins, which are assumed to be phosphorylase and Z-protein respectively (Takahashi and Hattori, 1988). Ohashi and Maruyama (1979) have shown that Z-protein will form lattice structures (of periodicity 8nm) when left for a few days in 1mM NaHCO₃. Z-protein is therefore a major candidate for the tetragonal lattice structure of the Z-disc.

Takahashi and Hattori (1988) have shown that the amorphous matrix can be removed non-enzymatically, by treating glycerinated vertebrate skeletal muscle fibres with a solution containing 0.1mM Ca²⁺. The amount of α-actinin in the Z-disc remains unchanged during this process, suggesting that α-actinin is a major component of the Z-filaments. The removal of the amorphous matrix by Ca²⁺ pre-treatment allows an uninterrupted view into the centre of the Z-disc (Takahashi and Hattori, 1988). The actin filaments are connected to the Z-disc via rod-like projections, 11 x 40nm in dimension (Figure 1.8). These structures are rigid, and are connected laterally at the N₁-line. At the Z-disc, the rod-like projections branch off into two single Z-filaments (5nm in diameter). These filaments are more flexible than the rods. Z-filaments emanating from adjacent rod-like projections within the myofibril unite at an angle of 90°, to form thicker Z-filaments (7nm in diameter). Thick Z-filaments from adjacent sarcomeres interdigitate at the centre of the Z-disc and are separated by a gap of 6.5nm. Two molecules of α-actinin can form a side-to-side aggregate 4 to 5nm in diameter (Podlubnaya et al., 1975), which is equivalent to the diameter of a single Z-filament. Antibodies raised against α-actinin react strongly with Z-filaments, further suggesting that these structures may be formed from α-actinin. Figure 1.8 shows a possible arrangement of α-actinin molecules within the Z-filaments (Takahashi and Hattori, 1988). Antibodies to α-actinin also bind the N₁-line, suggesting that α-actinin may link the actin thin filaments to the rods at this site (Takahashi and Hattori, 1988). The precise role of eu-actinin (42-kDa) in the Z-disc is not known, but this protein has been shown to bind α-actinin (Kuroda et al., 1981). Like α-actinin, eu-actinin is located in the interior of the Z-line and is not released by Ca²⁺-treatment (Takahashi and Hattori, 1988). Eu-actinin also interacts with F-actin to form bundles, but unlike α-actinin eu-actinin inhibits the onset of superprecipitation of acto-myosin. Takahashi and Hattori (1988) suggest that eu-actinin may be a component of the rod-like projections, and may support α-actinin in the role of attaching actin filaments to the rods. Goll et al., (1991) have shown that both μ- and m-calpain (i.e. the micro- and millimolar Ca²⁺ requiring Ca²⁺-dependent proteinases) release both actin and α-actinin from the Z-discs intact. This result implies that one or
Figure 1.8

A. Schematic Diagram Illustrating a Longitudinal Section Through a Skeletal Muscle Cell.

At high magnification a series of light (I) and dark (A) bands can be seen in each sarcomere. A dense line in the centre of each light band separates one sarcomere from the next and is known as the Z-line or Z-disc. The plus end of each actin thin filament is embedded in the Z-disc, whilst the minus end points towards the myosin thick filaments. The myosin thick filaments reverse polarity at the mid-line of the sarcomere (the M-line). During contraction the thick filaments slide past the thin filaments, thus reducing the length of the sarcomere.

B. The Internal Structure of the Z-disc.

Treatment of glycerinated skeletal muscle fibres with a solution containing 0.1mM Ca\(^{2+}\) removes the amorphous matrix surrounding the Z-disc, permitting an uninterrupted view of the Z-filaments (5-7nm). The thick black bars show a putative arrangement of four \(\alpha\)-actinin molecules in the Z-filament structure. The unit length is in nm. [Modified from Takahashi and Hattori, (1989)].
A

- M line
- Z Line
- Actin thin filament
- Myosin thick filament
- H zone
- I band
- A band
- 2.5μM

B

- Z-line
- 40
- 122
- F-actin filaments
- Rod-like projections
- N1-line
- Intrinsic Z-disc
more proteins of the Z-disc may strengthen the interaction between actin and α-actinin or act as 'linkers' between them. These proteins may be degraded by calpain, so releasing α-actinin from the Z-disc.

α-Actinin may also be involved in attaching some components of the 3rd filament system to the Z-disc, because in-vitro binding studies have shown that nebulin and titin can bind to α-actinin (Nave et al., 1990; Wang and Jeng, 1990). These proteins form separate parallel filaments extending from the A-band (in the case of titin) or the I-band (in the case of nebulin) to the Z-disc (Wang and Wright, 1988; Furst et al., 1988; Komiyama et al., 1990). It has been postulated that nebulin and titin may act as organizing templates and length determining factors for the actin and myosin filaments respectively (Wang and Wright, 1988). Nebulin (600-900-kDa) contains a large number of highly conserved repeats of 31 to 38 amino acids (Jin and Wang, 1991). Nebulin may regulate the length of sarcomeric thin filaments by aligning its repeat domains with an equal number of helical repeats of the actin filaments (Kruger et al., 1991; Jin and Wang, 1991).

A number of three-dimensional models have been proposed for the Z-disc based on electron microscope studies (Cheng and Deatherage, 1989; Deatherage et al., 1989; Morris et al., 1990). One of these reconstructions has been derived from the nemaline rod Z-band (Morris et al., 1990). Nemaline myopathy is an autosomal dominant neuromuscular disorder, characterized by an enlargement of the muscle Z-band (Jockusch et al., 1980; Goldstein et al., 1980). The nemaline Z-bands often extend to several microns in the direction of the muscle fibre long axis, whereas normal Z-bands are only 40-120nm in width (Morris et al., 1990). They are in fact natural crystals, composed of lateral polymers of the normal Z-disc (Jockusch et al., 1980; Goldstein et al., 1980). Immuno-fluorescence studies have shown that the nemaline Z-bands contain α-actinin (Jockusch et al., 1980), but recent linkage studies have ruled out the 3 known α-actinin genes as candidate genes for the disease (Laing et al., 1992). The nemaline Z-band displays an axial periodicity of 35nm (Yamaguchi et al., 1978; Goldstein et al., 1980). When glycerinated nemaline rods are treated with calcium activated protease (which releases α-actinin from the Z-band) this axial periodicity is removed leaving the longitudinal filaments intact (Yamaguchi et al., 1978). The longitudinal filaments running through the Z-disc can be decorated with heavy meromyosin, indicating that they consist of actin. This suggests that α-actinin may serve as a connecting filament linking the longitudinal F-actin filaments, and giving rise to transverse striations observed in the nemaline rods. Three-dimensional reconstructions derived from thin sections through the nemaline Z-band, have shown that each F-actin filament from one sarcomere is surrounded by four F-actin filaments of opposite polarity from the adjacent sarcomere (Morris et al., 1990). The F-actin filaments are linked by a structure (of about 36nm)
which in transverse section resembles the letter X with an extended central region. The arms of the X cross at an angle of 45°. This structure was found to link 4 actin filaments. The two uppermost points of the X bind two adjacent actin filaments of the same polarity at the same axial level. The extended central segment of the X runs parallel with the thin filaments for approximately 20nm. The lower two actin-binding points of the X-shaped structure bind to two actin filaments with opposite polarity to those bound by the uppermost points. Morris et al. (1990) suggest that the linking structure is composed of two α-actinin dimers. In this model, the ends of the dimers containing the ABDs are splayed outwards from the central repeat domain to form the X-shape. As mentioned above two molecules of α-actinin can form a side-to-side aggregate 4 to 5nm in diameter (Podlubnaya et al., 1975). This fits in well with the dimensions of the X-shaped structure i.e. 5nm by 36nm (Morris et al., 1990).

There is evidence that α-actinin may participate in the development of the Z-bands and in myofibrillogenesis. Mckenna et al. (1986) studied the formation and alignment of Z-bands in living cultured chick myotubes microinjected with rhodamine labelled α-actinin. Small aggregates of α-actinin (Z-bodies) were found to coalesce to form mature Z-bands during myotube development. Similar results were obtained when fluorescently labelled α-actinin was microinjected into cultured embryonic and cardiac myotubes (Sanger et al., 1986). During early myofibrillogenesis (i.e. within 2-3 days of culture), α-actinin was detected in closely spaced beads, distributed 0.3-1.5μM apart along cytoplasmic fibrils. These nascent Z-bands were found to grow apart and associate laterally with neighbouring arrays containing α-actinin to form sarcomeres of adult length (1.9-2.4μM). The myotendinous junction (MTJ) of stretched muscle fibres is another good site for studying myofibrillogenesis (Dix and Isenberg, 1990), because sarcomeres are added in series at this site during stretch induced growth. The entire sarcolemmal membrane at the MTJ of stretched fibres is particularly electron dense. These membrane complexes contain talin, vinculin and α-actinin (Tidball et al., 1986; Dix and Isenberg, 1990; and Trotter et al., 1983). Actin thin filaments attach to these membrane complexes thereby anchoring the elongating myofibril in place. Myosin thick filaments then attach to the thin filaments in a non-registered manner. α-Actinin containing Z-bodies were next found to assemble with the thin and thick filaments, before coalescing to form Z-bands (Dix and Isenberg, 1990). In summary, the incorporation of α-actinin into Z-bodies and subsequently into Z-bands is not required for the assembly of the thin or thick filaments during myofibrillogenesis. However, this process is required to set the assembled filaments into sarcomeric registry (Dix and Isenberg, 1990).
1.4.2 The Adhesion Plaques of Non-Muscle Cells.

Cultured non-muscle cells, such as fibroblasts, epithelial cells, endothelial cells, and platelets, form adhesion plaques when plated onto a suitable substrate (Burridge et al., 1988). At the adhesion plaque the cell makes its closest apposition (10-15nm) to the underlying substratum. Antibodies to α-actinin stain the adhesion plaques of stationary cells, and also their associated actin stress fibres at periodic intervals (Burridge, 1986; Burridge et al., 1988). The location of α-actinin at these sites strongly suggests that it may be involved in the organisation of microfilament bundles within the cytoplasm of cultured non-muscle cells, and in their attachment to the plasma membrane (Burridge et al., 1988).

Adhesion plaques consist of 3 distinct molecular domains (Figure 1.9). The outermost domain contains the extracellular matrix proteins to which the cell is attached. These include the proteins: fibronectin (Hynes, 1987a; Yamada, 1989); vitronectin (Hayman et al., 1983; Hayman et al., 1985) and laminin (Martin, 1987; Kleinman and Weeks, 1989). These proteins contain several common structural motifs (reviewed by Engel, 1991). The next domain contains the transmembrane receptors which link the extracellular matrix with the internal cytoskeleton (Akiyama et al., 1990). These receptors comprise a large family of glycoproteins, termed the integrins (Hynes, 1987b; Buck and Horwitz, 1987; Hynes, 1992). They are heterodimeric consisting of α- and β-subunits. Several α- and β-chains have now been cloned and sequenced (reviewed by Hynes, 1992). Each chain contains a putative transmembrane domain, a short C-terminal cytoplasmic domain, and a large extracellular domain. The β-chain contains a characteristic cysteine-rich repeat motif in its extracellular domain, with extensive intrachain disulfide bonding. So far 8 classes of β-polypeptide and 14 classes of α-integrin polypeptides have been identified. The β-chains are related and are probably derived from a common evolutionary precursor, as are the α-polypeptides. However, the αs and βs are not related to each other. ECM-binding diversity is generated through the association of different α-subunits with a common β-subunit (Cheresh et al., 1989). For example, the β1-integrin subclass contains receptors for fibronectin (α5β1); laminin (α6β1); and collagen (α2β1). The α-subunits must therefore be important in conferring substrate specificity. At the cell to cell contact, the receptors include members of the integrin, cadherin, selectin, epidermal growth factor, and immunoglobulin superfamilies (reviewed by Bock et al., 1991).

The next domain of the adhesion plaque consists of cytoplasmic components on the inner face of the membrane which are thought to link actin stress fibres to the transmembrane receptors (Burridge et al., 1988). The major cytoplasmic components include: talin; vinculin and α-actinin. Talin (230-kDa) was first isolated from chicken
Cultured cells adhere to their underlying substrate through discrete regions of the plasma membrane termed adhesion plaques. At these regions of close apposition between the plasma membrane (PM) and the glass (or plastic) substratum there is a continuum between the extracellular matrix and the intracellular cytoskeleton. Extracellular fibronectin (F) binds to one of a family of transmembrane receptors, the integrins (I), which are dimers of α and β subunits. There is evidence that talin (T, 215-kDa) can bind to the cytoplasmic domain of integrin and to another plaque protein, vinculin (V, 130-kDa). In turn vinculin is thought to bind to the actin-binding protein α-actinin (α-A, 100-kDa), completing the link between the adhesion plaque and the actin stress-fibres (S). This arrangement may be stabilised by other less well characterised plaque proteins, such as the vinculin-binding protein paxillin (P) or the α-actinin-binding protein zyxin (Z). Alternative modes of linkage may also operate at this locus. For instance, α-actinin can bind to integrin directly, and talin and vinculin may also interact with actin (see text).
gizzard (Burridge and Connell, 1983) and has been shown to bind vinculin with relatively high affinity (Kd of 10^{-8}M) (Burridge and Mangeat, 1984). It also binds the fibronectin receptor (integrin α5β1), but with low affinity (Horwitz et al., 1986). Talin is thought to consist of two functional domains, an N-terminal domain of 47-kDa and a C-terminal domain of 190-kDa, joined by a protease-sensitive linker (O'Halloran et al., 1985; Rees et al., 1990). The larger fragment retains the ability to bind vinculin (O'Halloran and Burridge, 1986; Lee et al., 1992) and integrin (Horwitz et al., 1986; Simon and Burridge, 1991). Recently, talin has been shown to interact with actin with a maximal ratio of 3 actin monomers per talin molecule (Muguruma et al., 1990; Kaufmann et al., 1991; Muguruma et al., 1992). Talin exhibits actin nucleation activity, but this activity is inhibited by vinculin (Goldmann et al., 1991). It has been shown to enhance the α-actinin-induced gelation of F-actin filaments, and lowers the amount of α-actinin necessary for gel formation (Maguruma et al., 1992). This property may reflect the potential F-actin cross-linking ability of oligomeric complexes of talin (Maguruma et al., 1992). Talin has also been shown to interact selectively with lipid bilayers (Heise et al., 1991), suggesting that it may have a direct role in linking actin filaments to the membrane. Cell to cell contacts between adjacent epithelial cells contain vinculin but not talin or paxillin (Geiger et al., 1985; Turner et al., 1990). Epithelial cells do contain a small amount of talin, but it is confined to the basal surface of the cell in contact with the underlying basement membrane (Burridge et al., 1988). When fluorescently labelled 190-kDa fragment of talin is microinjected into Madin Darby Canine Kidney cells it is localised to both the cell/cell and cell/matrix contacts, presumably because of its interaction with vinculin (Nuckolls et al., 1990). However, in similar experiments whole talin was found to target to cell/matrix contacts only. On this basis, Nuckolls et al. (1990) propose that the N-terminal domain of talin is responsible for the specific localisation of talin to cell/matrix contacts. The N-terminal 47-kDa domain displays homology with the erythroid cytoskeletal protein band 4.1 (80kDa), the microvillar brush border protein ezrin (77-81kDa), the cell to cell contact protein radixin (82kDa), and moesin (77kDa) (Rees et al., 1990; Funayama et al., 1991; Bretscher, 1983; Lankes and Furthmayr, 1991). This domain may be involved in the attachment of these cytoskeletal proteins to the membrane (Rees et al., 1990).

Vinculin (130-kDa) contains two structural domains, a large globular N-terminal head domain and a short linear C-terminal domain, both of which can be distinguished in electron micrographs of the protein (reviewed by Otto, 1990). V-8 protease digests of vinculin liberate fragments of 90-kDa, containing the globular head region, and 32- or 27-kDa fragments depending on which of the two possible protease sites are cleaved (Price et al., 1989). The V-8 protease cleavage sites are located within a proline-rich region separating the two domains. Talin binding activity is retained in the
90-kDa fragment, but not the 60-, 32-, or 27-kDa polypeptides (Price et al., 1989). Several cDNAs coding for chick and human vinculin have been isolated and sequenced in this laboratory (Price et al., 1989; Weller et al., 1990). The chick and human sequences both encode proteins of 1066 amino acids, with deduced molecular weights of around 117-kDa. The globular head region of vinculin was found to contain a triple internal repeat of 112 amino acids, of unknown function. The V-8 protease cleavage sites that liberate the 90-kDa, 32-kDa and 27-kDa fragments are located within the proline rich region. Chick and human vinculins are very highly conserved, with more than 95% amino acid similarity. One of the chick vinculin cDNAs, called cVin5, was found to lack some internal sequence encoding residues 167-207 in the globular head region of the protein (Price et al., 1989). Protein fragments expressed from this cDNA failed to bind to talin in-vitro and to localise to focal contacts in-vivo (Bendori et al., 1989; Jones et al., 1989). This strongly suggests that residues 167-207 of chick vinculin contain the talin-binding site (Bendori et al., 1989; Jones et al., 1989). Interestingly, protein fragments expressed from the C-terminal tail of vinculin were found to be incorporated into adhesion plaques of non-muscle cells (Bendori et al., 1989). This may be because this domain contains sites for binding to the adhesion plaque protein paxillin (Turner et al., 1990), and sites for vinculin self-association (Milam, 1985). The results documenting the interaction between vinculin and actin are controversial. While studies from several groups suggest that vinculin binds to F-actin and reduces its apparent viscosity (Burridge and Feramisco, 1982; Isenberg et al., 1982; Wilkins and Lin, 1982), recent experiments suggest that this effect is largely due to the presence of minor contaminants in the vinculin preparations used (Evans et al., 1984; Wilkins and Lin, 1985). Nevertheless, Westmeyer et al. (1990) have shown that NBD-actin can bind to nitrocellulose-bound vinculin, and this interaction is specifically inhibited by a monoclonal antibody (AS3) that interacts with the central region of the vinculin molecule (residues 587-851). Interestingly, this region of the protein also contains a putative phosphorylation site, tyrosine 822 (Price et al., 1987). If residues 587-851 contain an authentic actin-binding site then it is tempting to speculate that tyrosine kinase activity may control the interaction between these proteins within the cell (see Section 1.6.iii).

A weak interaction between vinculin and α-actinin has been detected using a number of techniques including gel overlay, equilibrium gel filtration, and fluorescence energy transfer (Otto, 1983; Wilkins et al., 1983; Burridge and Mangeat, 1984; Craig, 1985; Belkin and Koteliantsky, 1987; Wachsstock et al., 1987; Pavalko and Burridge, 1991). For example, Wachsstock et al. (1987) used fluorescence energy transfer and obtained a dissociation constant of approximately $10^{-6}$M, suggesting a low affinity for the interaction. Using gel-blot assays Pavalko and Burridge (1991) have shown that 125-I vinculin binds the 27-kDa thermolytic fragment of α-actinin, containing the ABD
of the protein, but not the 53-kDa thermolytic fragment, containing the spectrin-like repeats. If the interaction between vinculin and α-actinin observed in vitro also occurs in-vivo, then additional factors must regulate this interaction and confine vinculin to adhesion plaques, and prevent it from binding to α-actinin along stress fibres.

The interaction between the proteins: actin and α-actinin, α-actinin and vinculin, vinculin and talin, and talin and integrin suggests that there is a chain of specific protein interactions linking actin filaments to the plasma membrane (Figure 1.9). Indeed, α-actinin is located further from the membrane in cultured fibroblasts than vinculin or talin (Chen and Singer, 1982). The tension generated in stress fibres is transmitted across the membrane at the adhesion plaque, and one might expect the interactions taking place within this locus to be of reasonably high affinity. However, almost all of these interactions, with the exception of the interaction between talin and vinculin, are of low affinity. It is conceivable that there might be other proteins present in the adhesion plaque (currently unknown?) which are involved in stabilising this complex in-vivo. Furthermore, there might be several modes of linkage between the cytoskeleton and the membrane, and these may operate in union at the adhesion plaque (Burridge et al., 1988). Several recent studies involving α-actinin have provided evidence in support of this hypotheses.

Firstly, there is evidence of a direct interaction between α-actinin and integrin (Otey et al., 1990; Otey et al., 1991). In the first of these experiments, a peptide corresponding to residues 752-798 of the cytoplasmic domain of β1-integrin was immobilised onto a column of Sepharose, and this column was used to purify integrin-binding proteins from extracts of chick fibroblasts. A protein of approximately 100-kDa was eluted, which reacted with antibodies to α-actinin. In subsequent experiments, ^125^I α-actinin was shown to bind integrin adsorbed onto microtitre plates and was displaced by unlabelled α-actinin. The Kd of the interaction was found to be 2.5 x 10^-6 M and 2.1 x 10^-6 M for smooth muscle and platelet integrins respectively, suggesting a low affinity. This interaction was inhibited by the 53-kDa thermolytic fragment of α-actinin but not the 27-kDa thermolytic fragment (Otey et al., 1990). This strongly suggests that the integrin-binding site of α-actinin is located within the repeat units, and not the ABD. To map the integrin-binding site more precisely within this domain, a series of deletion mutants containing three out of four α-actinin repeats were expressed in E. Coli, and used in binding assays with the β1-integrin peptide (Otey et al., 1991). In these experiments the second repeat of α-actinin was found to interact with integrin. In similar experiments, using peptides corresponding to one quarter of the cytoplasmic domain of β1-integrin, residues 758-770 within β1-integrin were found to be important for binding to α-actinin (Otey et al., 1991).

Secondly, there is evidence that α-actinin may link actin filaments directly to
the membrane. For instance, Meyer et al. (1982) have observed a specific interaction between α-actinin and model membranes containing glycerides and fatty acids. Furthermore, Burn and co-workers (1985) have shown that in the presence of diacylglycerol and palmitic acid, a complex involving lipid, F-actin and α-actinin is formed in-vitro, with substructures reminiscent of the actin bundles observed in-vivo. The long-chain fatty acid, 2-bromostearate, has been shown to quench nearly 60% of the tryptophan fluorescence of the 55-kDa chymotryptic fragment of chicken smooth muscle α-actinin (Kahana and Gratzer, 1991), suggesting that the α-actinin repeats contain lipophilic binding sites in close proximity to the highly conserved tryptophan residues (see above). Finally and most importantly, phosphatidylinositol 4,5-bisphosphate has been shown to bind to α-actinin and to regulate its F-actin gelating activity (see Sections 1.2.i and 1.6.ii) (Fukami et al., 1992).

Thirdly, the interaction between α–actinin and lesser known components of the adhesion plaque may increase the overall stability of the adhesion plaque complex. One such component may be zyxin (Crawford et al., 1991; Crawford et al., 1992; Sadler-Riggleman and Beckerle, 1991). Zyxin (82-kDa) is a protein found at the adhesion plaques and stress fibres of non-muscle cells, that is phosphorylated on multiple sites in-vivo (Sadler-Riggleman and Beckerle, 1991). This protein is extremely rich in proline residues (15%) and in one stretch of 250 amino acids it reaches 27%. This region has a series of polyproline tracts (4 to 7 prolines) that may form polyproline helices. This would explain the long extended structure of the zyxin molecule (Sadler-Riggleman and Beckerle, 1991). The C-terminal region of zyxin is rich in histidines and cysteines, which are organised into repeated motifs reminiscent of metal co-ordinating sequences. Preliminary experiments have shown that zyxin can bind zinc (Sadler-Riggleman and Beckerle, 1991). It has also been shown to interact with α-actinin (Crawford et al., 1991). Zyxin co-sediments with F-actin in an α-actinin dependent manner, suggesting that it does not interact with F-actin directly but does so by way of α-actinin (Crawford et al., 1991). A direct interaction between α-actinin and zyxin has been demonstrated using analytical gel filtration and solid phase microtiter well binding assays (Crawford et al., 1991; Crawford et al., 1992). In the latter analysis, unlabelled α-actinin was found to competitively inhibit the binding of 125-I α-actinin to immobilized zyxin (Crawford et al., 1991). The Kd of the interaction was found to be 0.41μM, which indicates that the binding affinity is relatively low. By gel overlay analysis, 125-I zyxin has been shown to interact with the thermolytic 27-kDa fragment of α-actinin as well with the intact protein (Crawford et al., 1992). Double-labelling immunofluorescence experiments have shown that the localisation of zyxin and α-actinin in-vivo is co-extensive, suggesting that the in-vitro interaction may also occur in-vivo (Crawford et al., 1992). Zyxin may communicate tension between α-actinin and another
submembranous component(s). This pathway may be regulated by the phosphorylation of the zyxin molecule.

1.4.iii The Stress Fibres of Non-Muscle Cells.

In cultured cells, such as cultured chick embryo fibroblasts, adhesion plaques are found at the termini of large F-actin bundles that stretch across the cytoplasm of the cell, termed stress fibres (Burridge et al., 1988). The opposite end of a stress fibre can insert into a meshwork of intermediate filaments that surrounds the nucleus, or else into another adhesion plaque. Stress fibres are thought to communicate tensions, acquired during cell adhesion, from the cytoplasm to the membrane (Burridge et al., 1988). In addition to cells in culture, stress fibres have been found in various tissues in situ, including: endothelial cells (White et al., 1983); epithelial cells (Gordan et al., 1982); and fish scale fibroblasts (Byers and Fujiwara, 1982). The size and prominence of stress fibres in cultured cells is apparently due to the strong adhesion made with the rigid substrate (the culture dish or coverslip) and to the consequential isometric tensions induced by the associated microfilaments (Byers et al., 1984; Burridge et al., 1988). If the cells are grown in a less rigid substrate, such as in collagen gels, the cells lack discernible stress fibres and adhesion plaques (Tomasek et al., 1982).

Under the electron microscope stress fibres contain a sub-structure of alternating electron-dense and electron-lucid regions (Goldman et al., 1979). This pattern is thought to reflect a sarcomere-like arrangement of actin, α–actinin, tropomyosin, myosin, and myosin light chain kinase (Langanger et al., 1986; Sanger et al., 1986). This arrangement is depicted schematically in Figure 1.10. Immunocytochemical studies have shown that α–actinin is concentrated in the electron dense regions, spaced 0.9-1.5 μm apart (Langanger et al., 1984; Langanger et al., 1986; Sanger et al., 1986). These structures may function in an analogous manner to the skeletal muscle Z-discs. Thus α–actinin would serve to bind the actin filaments and hold them in register during contraction and relaxation. Filamin is localised in both the electron-dense and electron-lucid regions of the stress fibre (Langanger et al., 1984). Myosin and tropomyosin are co-localised in the electron-lucid regions (Langanger et al., 1986; Sanger et al., 1986). The electron-lucid regions can be subdivided into two areas with slightly increased electron density that contain cross-bridges, and a central band that is more electron-translucent (Langanger et al., 1986). Antibodies specific for the head region of myosin stain the two slightly more electron dense regions of the electron-lucid band (Langanger et al., 1986). In contrast antibodies specific for the myosin tail region
Stress fibres contain a sub-structure of alternating electron-dense and electron-lucid regions (Goldman et al., 1979). This pattern is thought to reflect a sarcomere-like arrangement of actin, α-actinin, tropomyosin, myosin, and myosin light chain kinase (Langanger et al., 1986; Sanger et al., 1986). α-Actinin is concentrated in the electron dense regions, spaced 0.9-1.5 μm apart (Langanger et al., 1984; Langanger et al., 1986; Sanger et al., 1986). Filamin is localised in both the electron-dense and electron-lucid regions of the stress fibre (Langanger et al., 1984). Myosin and tropomyosin are co-localised in the electron-lucid regions (Langanger et al., 1986; Sanger et al., 1986). The Myosin filaments may form dimers arranged in a bi-polar manner. (Modified from Langanger et al., 1986).
react with the more translucent region of the electron-lucid band. This suggests that myosin filaments are arranged in a bipolar arrangement in the stress-fibers, and may even be arranged into dimers or oligomers (Figure 1.10).

There is strong evidence that stress fibres may be contractile structures (Giuliano and Taylor, 1990; Kolega et al., 1991). When stress fibres are cut free from their attachment points with a laser microbeam in a permeabilised cell, they can be made to contract when supplied with Mg\(^{2+}\) and ATP, as observed by the closing up of the inter-myosin gaps (Amos and Bradshaw, 1991). When fibroblasts are stimulated by growth factors there is extensive stress fibre contraction, which is concomitant with increased motility, such as membrane ruffling and the formation of cellular protrusions (Giuliano and Taylor, 1990). Stress fibre contraction is also induced by cytochalasin, a potent inhibitor of actin polymerisation both in-vivo and in-vitro (Kolega et al., 1991). The arrangement of stress fibre myosin suggests that it may participate in a sliding acto-myosin movement similar to its role in smooth and skeletal muscle. In smooth muscle, contraction can be stimulated by the phosphorylation of the myosin regulatory light chains (MRLC), and inhibited by agonists of this process (Itoh et al., 1989). Similarly, there is increased phosphorylation of MRLC when stress fibres are reorganised following the stimulation of fibroblasts with growth factors (Bockus and Stiles, 1984). This data suggests that the phosphorylation of MRLC may be important in the contraction of non-muscle stress fibres in-vivo.

In addition to the ability of \(\alpha\)-actinin to exchange between the bound and soluble pools in living non-muscle cells (see later), an apparent translocation of \(\alpha\)-actinin along stress fibres has also been observed (McKenna and Wang, 1986). In this experiment, rhodamine-labelled \(\alpha\)-actinin was microinjected into embryonic chick cardiac fibroblasts, and a laser beam pulse was then used to photobleach small spots of \(\alpha\)-actinin incorporated into the stress fibres. The distance between the bleached spot and the terminus of the stress fibre was then measured at 2-3min intervals. Analysis of the bleached spots showed that the spots moved away from the terminus of the stress fibre towards the nucleus, even though the stress fibre and its tip remained stationary. Similar results were obtained when rhodamine labelled actin was microinjected into the cell, suggesting that the translocation process involves the movement of the whole macromolecular assembly along the stress fibre (McKenna and Wang, 1986). Translocation of proteins along stress fibres was found to occur predominantly at the trailing end of the cell, suggesting that this process may be responsible for the retraction of the trailing end during cell movement. The mechanism underlying translocation may involve the 'treadmilling' of actin subunits at the membrane associated tips of the stress fibre (McKenna and Wang, 1986). F-actin filaments are polar structures, and have two structurally different ends, referred to as the minus end (or pointed end) and the plus end.
(or barbed end). In-vivo the growth of F-actin filaments almost always occurs at the plus end. The actin filaments along the length of the stress fibre are of mixed polarity, and so are unlikely to support treadmilling (Sanger and Sanger, 1980). However, at the adhesion plaque the actin filaments are specifically arranged with their growing tips (plus end) facing the membrane (Sanger and Sanger, 1980). The preferential addition of new actin monomers at the adhesion plaque-associated end of the stress fibre would have the apparent effect of displacing the older actin subunits and their associated proteins away from the membrane (McKenna and Wang, 1986).

1.5 Physiological Significance of α-Actinin in Muscle and Non-Muscle Cells.

The high level of amino acid sequence identity exhibited between α-actinins from human, chicken, Drosophila, Dictyostelium, and Nematode suggests that this protein might perform an important function in-vivo (see Chapter 4) (Noegal et al., 1987; Baron et al., 1987b; Fyrberg et al., 1990; Barstead et al., 1991; Beggs et al., 1992). However, mutants of Dictyostelium lacking α-actinin were found to display almost normal growth, with no detectable alteration in cell motility, chemotaxis or morphogenesis (Wallraff et al., 1986; Witke et al., 1987; Schleicher et al., 1988). Like normal Dictyostelium amoeba, the mutants were found to be highly motile cells capable of reacting quickly to a chemotactic stimulus. They formed a normal multicellular slug composed of two cell types, which was motile and capable of forming a fruiting body consisting of a stalk and spore head. Similar results were obtained with Dictyostelium mutants lacking the F-actin severing protein severin (André et al., 1989) or the F-actin cross-linking protein ABP-120 (Brink et al., 1990; Cox et al., 1992). In contrast to each of the single mutations described above, Dictyostelium double-mutants lacking both α-actinin and ABP-120 were found to be severely disturbed in development (Witke et al., 1992). The double-mutant cells were able to aggregate but were rarely able to form fruiting bodies (Witke et al., 1992). However, this ability was rescued when the double-mutant cells were mixed with wild-type cells, or when a functional α-actinin gene was inserted into the mutants and expressed (Witke et al., 1992). This suggests that in Dictyostelium (at least) there is extensive overlapping redundancy among actin-binding proteins, and they can complement one another other to such an extent that the elimination of one protein does not produce significant effects (Bray and Vasiliev, 1989). Way and Weeds (1990) suggest that the effects produced on eliminating one or more actin-binding proteins is likely to vary according to the cell type, because of "differences in the construction of the cytoskeletal apparatus, how it is used and the extent of redundancy in its regulation". For example, in some cells the loss of α-actinin
may have dire consequences if there is no redundancy, i.e. if there are other available protein capable of attaching actin filaments to vinculin, or to integrin, or to the lipid bilayer etc (also see Section 1.6.iii).

Roulier et al. (1992) have characterized the gene encoding Drosophila α-actinin and analysed a number of distinct mutations at this locus (subdivision 2C of the X chromosome). The single gene encoding Drosophila α-actinin is variably spliced to give one non-muscle and two muscle variants, the adult muscle and the supercontractile muscle isoforms (see Section 1.3). Drosophila fliA mutants contain point mutations in the α-actinin gene, and are flightless due to abnormalities within their indirect flight muscles (Roulier et al., 1992). In fliA^4 there is an A to T mutation within the acceptor site of the last intron, which drastically cuts the level of expression of each isoform of α-actinin. This mutation produces negligible effects in adult non-muscle cells, and embryogenesis occurs as normal. However, all the adult muscles of fliA^4 mutants were found to be weakened and paralysed. This suggests that α-actinin may have an essential role in anchoring and stabilizing actin thin filaments in muscle cells. In contrast, the role of α-actinin in Drosophila non-muscle cells may be redundant, as observed in Dictyostelium (see above). In fliA^3, a G to A mutation occurs adjacent to an internal splice donor site in the variable muscle specific exon of the gene (see Section 1.3). As a consequence of this mutation the adult muscle isoform is not expressed, and the supercontractile muscle isoform is expressed at low levels in the wrong tissues, such as the flight and leg muscles. The non-muscle isoform is unaffected in this mutant. Paralysis and atrophy were found to occur in the flight muscles of fliA^3 (Roulier et al., 1992). The thorax and leg muscles of these flies were less severely affected, but were evidently weakened as demonstrated by the inability of the mutant flies to jump. These results confirm that α-actinin must play an important role in the Z-discs of skeletal muscle. Furthermore, in Drosophila normal skeletal muscle development may be absolutely dependent on the expression of the correct muscle isoform of α-actinin.

1.6 Dynamics of α-Actinin in Non-Muscle Cells.

The dynamic properties of α-actinin have been measured by fluorescence recovery after photobleaching, following the microinjection of fluorescent derivatives into live non-muscle cells (Kreis et al., 1982; Geiger et al., 1984). Sixty to eighty percent of the injected protein is free to diffuse in the cytoplasm. The α-actinin associated with the adhesion plaque is far less diffuseable. The half life of recovery (2-4 mins) indicates a slow exchange between the adhesion plaque and the surrounding soluble pool of proteins (Geiger et al., 1984). A similar approach has been used to examine the exchangeability of α-actinin in living cardiac fibroblasts and muscle cells.
(McKenna et al., 1985). α-Actinin in the Z-disc was found to be far less diffuseable than in the adhesion plaques or stress fibres of fibroblasts. The difference in exchangeability between the bound and soluble pools of α-actinin in muscle and non-muscle cells may reflect the difference in the dynamic properties of the cytoskeletal apparatus of these tissues. α-Actinin may be anchored more firmly (by protein-protein interactions) within the lattice structure of the Z-disc than within the stress fibres and adhesion plaques of non-muscle cells.

The following sections describe a number of agents which either directly or indirectly effect a re-organisation of α-actinin in non-muscle cells.

1.6 Rous Sarcoma Virus (RSV)-Transformation of Chick Embryo Fibroblasts.

RSV-transformed chick embryo fibroblasts are less adherent and more rounded than normal cells (Burridge, 1986). They contain fewer adhesion plaques, referred to as podosomes, which are smaller and more diffuse in structure than their normal counterparts (Burridge, 1986). Using immunofluorescence microscopy podosomes have been shown to contain a core of actin filaments and α-actinin, surrounded by a ring of talin and vinculin (Marchisio, 1987). In RSV-transformed cells, cytoplasmic actin cables are less abundant and less extensive than normal stress fibres (Kellie et al., 1986). Brightly staining aggregates of actin are present in the cytoplasm which are partly coincident with podosomes (Kellie et al., 1986). Stickel and Wang (1987) have looked at the dynamics of α-actinin within podosomes by fluorescence recovery after photobleaching. The half life of recovery of α-actinin fluorescence in podosomes was short (several seconds, as compared with 5 minutes for normal adhesions) indicating a rapid exchange between the podosome and the surrounding soluble pool of proteins (Stickel and Wang, 1987).

RSV contains a single oncogene encoding the tyrosine kinase pp60V-SRC (Hunter and Sefton, 1980). Large amounts of pp60V-SRC are found in the podosomes (Rohrschneider, 1980), and a number of proteins including talin, vinculin, integrin and paxillin are phosphorylated on tyrosine residues (Pasquale et al., 1986; DeClue and Martin, 1987; Sefton et al., 1981; Hirst et al., 1986; Glenney and Zokas, 1986). α-Actinin is not phosphorylated during transformation, it may be less stable in podosomes due to the perturbation of its interaction with integrin or vinculin etc. The increased mobilisation of α-actinin in podosomes may in turn contribute to the instability of microfilaments in RSV-transformed cells. In turn this may contribute to the uncoupling of the link between the extracellular matrix and the cytoskeleton. Protein phosphorylation at this locus might represent a pathway by which normal cell adhesion
is maintained. The aberration of this pathway during RSV-infection may cause the cells to become detached from their substrate and undergo transformation.

1.6.ii Tumour Promoters.

When added to cultured cells tumour promoters produce effects very similar to those induced upon RSV-transformation (Burridge et al., 1988). Tumour promoters activate the Ca\(^{2+}\) and phospholipid dependent kinase, protein kinase C (PKC, see Nishizuka, 1986). Several growth factors, such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF), also regulate PKC (Nishizuka, 1986). This is because they are able to stimulate phospholipase C, which catalyses the degradation of phosphatidylinositol 4,5-bisphosphate (PtdInsP\(_2\)). This reaction produces two second messengers: inositol triphosphate, which mobilises intracellular Ca\(^{2+}\) stores; and diacylglycerol, which stimulates PKC (Heldin and Westermark, 1990). Tumour promoters, such as the phorbol ester 1-O-tetradecanoylphorbal-13-acetate (TPA) mimic diacylglycerol, and bind and activate PKC (Castagna, 1982). The effects of TPA on BSC-1 cells has been investigated by Meigs and Wang (1986) who microinjected live cells with fluorescently labelled \(\alpha\)-actinin or vinculin. They found that adhesion plaques (as judged by vinculin fluorescence) remained intact for longer than stress fibres (as judged by \(\alpha\)-actinin fluorescence), following TPA treatment. However, the \(\alpha\)-actinin in adhesion plaques was found to dissociate away before the \(\alpha\)-actinin in stress fibers (Meigs and Wang, 1986). One of the earliest effects of TPA treatment may be to cause the stress fibres to disassemble from their points of attachment, with the result that \(\alpha\)-actinin closest to the adhesion plaque is the first to be lost (Meigs and Wang, 1986).

In normal rat embryo fibroblasts Type 3 PKC is found in adhesion plaques (Jaken et al., 1989), and it may regulate the stability of these structures by phosphorylating key proteins (such as vinculin and talin) on serine and threonine residues (Werth et al 1983; Litchfield and Ball, 1986). In support of this the level of vinculin phosphorylation has been shown to be elevated in cells treated with phorbol esters (Werth and Pastan, 1984). PtdInsP\(_2\) has been shown to bind \(\alpha\)-actinin, and to regulate its F-actin gelating activity (see Section 1.2.i) (Fukami et al., 1992). However, it is not known if these effects are influenced by the turnover of PtdInsP\(_2\) in response to extracellular stimuli, such as epidermal- and platelet-derived growth factors or treatment with phorbol esters.
1.6.iii Proteolytic Fragments derived From the \(\alpha\)-Actinin Molecule Perturb the Function of Endogenous \(\alpha\)-Actinin when Introduced into Non-Muscle Cells.

This was investigated by microinjecting fluorescently labelled fragments of chick smooth muscle \(\alpha\)-actinin into live rat embryo fibroblasts (Pavalko and Burridge, 1991). The 27-kDa thermolytic fragment of \(\alpha\)-actinin immediately became incorporated into the stress fibres and adhesion plaques of these cells. This is consistent with it containing the actin-binding site of the molecule, and also binding sites for the adhesion plaque proteins vinculin and zyxin (Mimura and Asano, 1986; Pavalko and Burridge, 1991; Crawford et al., 1992). The 53-kDa thermolytic only became incorporated into the adhesion plaques of rat embryo fibroblasts (Pavalko and Burridge, 1991), presumably due its binding sites for \(\beta\)1-integrin and lipid (Otey et al., 1990; Kahana and Gratzer, 1991). However, cells microinjected with either fragment were found to disassemble most of their adhesion plaques and stress fibres within 2 hours of injection. Pavalko and Burridge (1991) suggest that each fragment may produce its effects by interfering with the function of endogenous, intact \(\alpha\)-actinin. Interestingly, each fragment was found to induce stress fibre disassembly before adhesion plaque disassembly, and cells lacking stress fibres were often found to contain residual adhesion plaques which stain for talin, vinculin and integrin but not \(\alpha\)-actinin (Pavalko and Burridge, 1991). This suggests that the mobilisation of \(\alpha\)-actinin is an early event in adhesion plaque disassembly, leading to stress fibre detachment and disassembly. These observations are consistent with the studies of Meigs and Wang (1986) in which the loss of \(\alpha\)-actinin from adhesion plaques was found to precede loss of vinculin in cells treated with phorbol ester (see above), but are in contrast with the observations of Stickel et al., (1988), who cultured 3T3 cells in the presence of the synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS). This peptide mimics the cellular binding site of many adhesive proteins of the extracellular matrix, including fibronectin and vitronectin (Burridge et al., 1988). In cells treated with this peptide, vinculin and \(\alpha\)-actinin were found to dissociate from the adhesion plaque together as a complex, resulting in a gradual disappearance of plaque structure, and the rounding and detachment of spread cells (Stickel et al., 1988).

1.7 \(\alpha\)-Actinin Protein Superfamily.

A number of proteins have been shown to contain ABDs homologous with \(\alpha\)-actinin. These proteins include, \(\beta\)-spectrin (Prchal et al., 1987; Winkelmann et al., 1988; Byers et al., 1989), dystrophin (Koenig et al., 1988; Lemaire et al., 1988); filamin (Gorlin et al., 1990), Dictyostelium gelation factor, ABP-120 (Noegal et al.,...
1989), and fimbrin (de Arruda et al., 1990). These proteins can be divided into 3 classes (see Figure 1.11).

1.7.i Proteins with a Tandem Arrangement of ABDs.

These proteins include fimbrin (de Arruda et al., 1990) and its homologues, human L- and T-plastin (Lin et al., 1988; Lin et al., 1990) and yeast SAC6 (Adams et al., 1991). Fimbrin contains two major domains, an N-terminal headpiece and a C-terminal core region (de Arruda et al., 1990). The headpiece (115 residues) contains two EF-hand motifs (de Arruda et al., 1990; Matsudaira, 1991). The core domain is 500 residues in length and contains two α-actinin-like ABDs arranged in a tandem fashion. Each ABD within the core binds a single F-actin filament, and the bundles so formed are very tightly packed (de Arruda et al., 1990). This type of bundle provides structural support for finger-like projections of the membrane, such as microvilli and filopodia (Matsudaira, 1991). The stress fibres of non-muscle cells differ from the microvillar-type F-actin bundles in that they contain myosin and tropomyosin, and are able to contract in-situ on addition of ATP (Matsudaira, 1991). The F-actin filaments in stress fibre bundles are cross-linked by α-actinin and are spaced further apart (~40nm) than those cross-linked by fimbrin or villin (14nm). The large space offered by the α-actinin cross-link may allow space for myosin thick filaments to interact with F-actin filaments in the bundle (Figure 1.10) (Matsudaira, 1991).

1.7.ii α-Helical Proteins.

This class includes the proteins, α-actinin, spectrin, and dystrophin. Conserved features of this class include, an N-terminal ABD, a central repetitive segment of α-helical motif, C-terminal EF-hands, and oligomerization into an anti-parallel homodimer (Davison et al., 1989; Koenig et al., 1988; Bennett, 1990).

Spectrin is one of the major components of the erythroid cytoskeleton and consists of a heterodimer of α- (250-kDa) and β- (225-kDa) subunits (Bennett, 1990). These heterodimers associate to form elongated tetramers and higher order structures. In the erythrocyte cytoskeleton short filaments of actin (30-50nm) associate with five or six spectrin tetramers and a number of accessory proteins to form a sheet of five and six sided polygons underlying the membrane (Bennett, 1990). The elastic properties of this dome-shaped structure enable the erythrocyte to withstand repeated deformations as it passes through narrow capillaries (Bennett, 1990). Both α- and β-type spectrins have been cloned and sequenced (Prchal et al., 1987; Winkelmann et al., 1988; Byers et al., 1989; Wasenius et al., 1989; Hong and Doyle, 1989; Winkelmann et al., 1990). Both
These proteins can be divided into 3 separate classes. The first class are monomeric proteins, with two (or more?) tandemly arranged α-actinin-like actin binding domains (ABD). The best example found in this group is fimbrin. This protein contains two N-terminal EF-hands (⊕), and actin-binding may be modulated by calcium. This region of the protein also contains sites which are phosphorylated (P). The second class of proteins form dimers and higher order structures. They contain a single ABD per subunit, which is usually situated at the N-terminal end of the molecule. The N-terminal and C-terminal domains are separated by several repeats (¶¶) which are α-helical in nature. The repeats may be responsible for dimerization, and the folding of the molecule into a rod-shape. The C-terminal domain of these proteins contains: EF-hands (α-actinin, α-spectrin, and dystrophin); putative membrane association sites (dystrophin, ¶¶); or sites for self-association (β-spectrin, ¶). The third class are also dimeric proteins, but in this group the N-terminal and C-terminal domains are separated by repeats of β-sheet motif (¶¶). These repeats can interact in either an inter-chain (ABP-120) and intra-chain fashion (filamin). Filamin dimerizes through interactions occurring at the C-terminal end of the molecule. The ABDs of filamin are widely separated in space, and free to move at the ends of long flexible chains. This permits high angle branching of F-actin filaments into 3D-networks. The C-terminal domain of filamin (¶) binds glycoprotein 1b (Von Willibrand's factor) receptor of platelets. (Modified from Matsudaira, 1991).
Class I

Class II

Class III

Alpha-actinin.

Spectrin

Dystrophin

ABP-120

Filamin

GP1b/IX binding domain
spectrin subunits contain approximately 20 repeats (~106 residues in length) similar to those found in α-actinin and dystrophin (Byers et al., 1989; Wasenius et al., 1989). The N-terminus of β-spectrin (but not α-spectrin) contains an ABD homologous with α-actinin. The positioning of ABDs at opposite ends of the spectrin tetramer may enable spectrin to cross-link F-actin in a similar fashion to the α-actinin antiparallel dimer (Byers et al., 1989). α-Spectrin contains two EF-hand structures at its C-terminal end (Wasenius et al., 1989), and brain spectrin has been shown to bind calcium (Wallis et al., 1992). This may explain why sea urchin egg spectrin binds to actin in a calcium-sensitive fashion (Fishkind et al., 1987). The extreme C-terminal domain of β-spectrin may be involved in spectrin self-association (Dhermy et al., 1982; Pothier et al., 1987; Kennedy et al., 1991). Somatic cell hybrid and in situ hybridisation analyses have shown that the non-muscle/smooth muscle α-actinin gene is situated on human chromosome 14, bands q22-q24 (Youssoufian et al., 1990). The gene encoding human erythroid β-spectrin is also located on chromosome 14, bands q22-q24 (Prchal et al., 1987; Winkelmann et al., 1988). Furthermore, in Southern blots of human genomic DNA, separated by pulsed-field gel electrophoresis, similarly sized bands are detected with both α-actinin and β-spectrin labelled cDNA probes (Youssoufian et al., 1990). These results suggest that the genes encoding β-spectrin and α-actinin are physically linked (Youssoufian et al., 1990), and are likely to have arisen from the duplication of a common ancestral gene (Dubreuil et al., 1989). Following this intrachromosomal event, the two genes may have evolved independently on the same chromosome.

Dystrophin is a very large protein (427-kDa) that is absent (or present at very low levels) in individuals suffering from Duchenne Muscular Dystrophy (DMD), an X-linked recessive disorder characterized by sarcolemmal lesions due to the stress of contraction (Hoffman et al., 1987). From sequence analysis dystrophin is predicted to be a long (150nm) rod-shaped molecule, composed of four separate domains (Koenig et al., 1988; Lemaire et al., 1988). The N-terminal 240 amino acids are homologous with the ABD of α-actinin. The next domain contains 26 repeats homologous with those found in spectrin and α-actinin (Davison and Critchley, 1988). The middle domain probably adopts a rod shape, as does the middle domain of α-actinin and spectrin (Koenig et al., 1988; Davison and Critchley, 1988). The 3rd domain of dystrophin is rich in cysteines and is homologous with entire C-terminal domain of α-actinin, containing two EF-hand motifs (Koenig et al., 1988). The final domain of dystrophin (~420 residues) is of unknown function, but homologous with the C-terminus of a chromosome 6-encoded protein DRP (Love et al., 1989). Electron microscopy has revealed that tissue-purified dystrophin molecules can form side-by-side and end-to-end dimers and higher order structures (Pons et al., 1990). This oligomerization is necessary
for F-actin cross-linking activity, because monomeric ABDs can bind but not cross-link F-actin filaments (Mimura and Asano, 1986).

Dystrophin has been localised to the sarcolemma of adult muscle fibres (Matsumara et al., 1991; Khurana et al., 1991), where it is found in elements overlying both I bands and M bands and in strands running parallel with the longitudinal axis of the myofiber (Porter et al., 1992). At these sites dystrophin (along with vinculin and spectrin) may function to link the contractile apparatus to the sarcolemma (Porter et al., 1992). In accordance with this proposal, dystrophin has been found to associate tightly with a complex of integral membrane glycoproteins (Campbell and Kahl, 1989; Ervasti et al., 1990; Matsumara et al., 1991; Ervasti and Campbell, 1991). These proteins are also present at significantly lower levels in DMD due to the absence of dystrophin (Ohlendieck and Campbell, 1991). The lack of dystrophin and its associated proteins may cause the sarcolemmal membranes to become destabilised and less capable of withstanding the stresses associated with contraction (Zubryzycka-Gaarn et al., 1988; Mandel, 1989; Byers et al., 1991; Turner et al., 1991). This situation may resemble some hemolytic diseases in which defective spectrin molecules weaken the erythrocyte plasma membrane, causing the cells to collapse in the face of the shear forces encountered during circulation (Marchesi et al., 1987). Symptomatic patients with Duchene muscular dystrophy have severely depleted levels of fast twitch glycolytic fibres, suggesting that dystrophin may play a key role in these particular cells (Minetti et al., 1991). These skeletal muscle fibres can produce large amounts of force quickly but fatigue rapidly (Minetti et al., 1991). Preclinical patients with DMD have normal amounts of fast-twitch glycolytic fibres, but symptomatic patients between 3 and 5 years have depleted amounts of these fibres, and no fibres are present after 5 years (Minetti et al., 1991). Interestingly, an anti-dystrophin antibody raised against the rod domain of the molecule was found to cross-react with a specific variant of α-actinin (90-kDa) found only in fast-twitch glycolytic skeletal muscle fibres (Hoffman et al., 1989). Significantly, in symptomatic patients with DMD little or no fast-twitch glycolytic α-actinin can be detected in the few remaining fibres (Minetti et al., 1991). The mechanism underlying the specific depletion of this variant of α-actinin has yet to be determined, but the phenomenon is clearly a secondary effect of DMD (Minetti et al., 1991).

1.7.iii. β-Sheet Proteins.

Proteins in this third class include Dictyostelium ABP-120 (Noegal et al., 1989) and filamin (Gorlin et al., 1989). These proteins contain, an N-terminal ABD homologous with α-actinin, a spacer segment containing tandem repeats of a β-sheet motif, and a C-terminal dimerization/membrane attachment site (Noegal et al., 1989;
ABP-120 has 6 copies of the repeat unit, whereas filamin has 24. The filamin and ABP-120 repeats are similar in length (~100 residues) and composition, and are separated from one another by ~10 residues. *Dictyostelium* ABP-120 is a rod-shaped homodimer, with ABDs arranged at the N-termini of its antiparallel subunits (Noegal et al., 1989). In this protein, the hydrophobic faces of repeats derived from one subunit may interact, in an inter-chain manner, with the hydrophobic regions of the apposing subunit (Noegal et al., 1989). Filamin also forms a dimer but the long subunit chains are only joined together at their extreme C-terminal ends (Gorlin et al., 1990). The hydrophobic faces of the filamin repeats associate in an intrachain fashion within each subunit (Gorlin et al., 1990). In cross-linking experiments, the dimerization site has been localised to the last 65 residues of the molecule (Gorlin et al., 1990). This site occurs at the end of the 24th repeat, where the regular repeat sequence is interrupted (Gorlin et al., 1990). The N-terminal ABDs at the ends of these long subunits are well separated in space, and are free to move at the end of the long flexible polypeptide chain (Gorlin et al., 1990). This arrangement permits high angle branching of F-actin filaments, and the F-actin networks cross-linked by filamin are considerably elastic (Janmey et al., 1990).

α-Actinin, spectrin and dystrophin are likely to have arisen through the duplication of an ancestral α-helical segment (Davison et al., 1989; Koenig et al., 1988; Bennett, 1990; Dubreuil et al., 1990), whereas filamin and ABP-120 may have arisen from the duplication of an ancestral cross-β sheet segment (Noegal et al., 1989). In this way, two or more classes of actin-cross linking proteins have evolved using a common N-terminal ABD. Although all of these proteins contain a homologous ABD, the distinctive repeat character, as well as other distinguishing aspects of their subunit organisation, may give these proteins their specialised biological functions (Bresnik et al., 1990). For example, ABP-120 inhibits, whilst α-actinin stimulates the actin-activated Mg^{2+}-ATPase of myosin (Condeelis et al., 1984). Furthermore, unlike non-muscle α-actinin *Dictyostelium* ABP-120 is not sensitive to calcium (Condeelis et al., 1984).

### 1.8 Aims of the Project.

The fundamental aims of this project were to establish the complete nucleotide sequence and domain structure of chicken non-muscle α-actinin. This was achieved using two different approaches. The first approach involved sequencing several polypeptides derived from a chicken non-muscle α-actinin. Brain was chosen as the source of chicken non-muscle α-actinin for two main reasons: firstly, because there was already a published protocol for the purification of α-actinin from this tissue; and
secondly, because this protein has clearly been shown to bind to F-actin in a calcium-sensitive manner (Duhaiman and Bamburg, 1984). Chicken brain \( \alpha \)-actinin was cleaved with the protease thermolysin, which cleaves \( \alpha \)-actinin into two major polypeptides: a 27-kDa polypeptide containing the ABD; and a 53-kDa containing the repeats (Mimura and Asano, 1986). The chick brain \( \alpha \)-actinin thermolytic polypeptides were then analysed by SDS-PAGE alongside similar digests of purified chicken smooth muscle and chicken skeletal muscle \( \alpha \)-actinins, so that the peptide maps of the 3 major isoforms of \( \alpha \)-actinin could be compared. The N-terminal ends of the chick brain \( \alpha \)-actinin thermolytic polypeptides were then sequenced to enable a direct comparison to be made with the sequence of the muscle isoforms of chick \( \alpha \)-actinin. The second approach involved using this information to design DNA probes for the isolation of chick non-muscle \( \alpha \)-actinin cDNAs from two independent chick embryo brain cDNA libraries. The complete deduced sequence of chick brain \( \alpha \)-actinin was then compared with the sequence of chicken smooth muscle and chicken skeletal muscle \( \alpha \)-actinins, to give an indication of the mechanism by which these isoforms originate from the chick genome (Baron et al., 1987b; Arimura et al., 1988). In addition, we were interested in the level of amino acid identity between non-muscle \( \alpha \)-actinins derived from different species. For this reason a human placental cDNA library (Clontech) was also screened for \( \alpha \)-actinin cDNAs. The complete human non-muscle \( \alpha \)-actinin sequence was then compared with the complete deduced sequence of chick brain \( \alpha \)-actinin as well as with *Dictyostelium* \( \alpha \)-actinin (Noegal et al., 1987).

Chicken brain \( \alpha \)-actinin has been shown to bind actin in a calcium-sensitive manner (Duhaiman and Bamburg, 1984). The sequence of the EF-hands of chick embryo brain \( \alpha \)-actinin enables us to predict the stoichiometry by which calcium binds to this isoform. However, we also attempted to confirm these predictions by studying calcium-binding to the chick brain protein. Initially, purified chicken brain \( \alpha \)-actinin was analysed using a rapid dot-blot \( ^{45}\text{Ca}^{2+} \)-overlay procedure (Koch et al., 1986; Reinach et al., 1986). This method has recently been used to determine the calcium-binding stoichiometry of several deletion mutants of gelsolin, and was found to give results comparable with those obtained by equilibrium dialysis (Way et al., 1989). Calcium-binding was also evaluated using a stopped-flow spectrophotometric analysis on fragments of chick brain \( \alpha \)-actinin expressed as fusion proteins in *E. coli*. The fluorescent indicator Quin-2 was utilized in this analysis (Tsien et al., 1982). This reagent changes fluorescence on binding calcium and has been used to monitor rapid calcium-binding reactions (Martin et al., 1985). The fusion proteins used in this study were expressed in *E. coli* using the pGEX-1 expression plasmid (Smith and Johnson, 1988). This vector contains the coding sequence for glutathione-S-transferase and the
resultant fusion proteins can therefore be rapidly purified by affinity chromatography on glutathione-agarose.

Complementary cDNAs encoding the smooth muscle isoform of α-actinin have been isolated from a chick embryo fibroblast cDNA library suggesting that non-muscle cells may express smooth muscle α-actinin in addition to the non-muscle isoform (Baron et al., 1987b). However, the precise roles of the smooth and non-muscle α-actinins in these cells is unclear. One possibility is that the calcium-insensitive smooth muscle isoform might be required for the formation of stress fibres which are thought to contain a muscle-like sarcomeric arrangement of cytoskeletal proteins (Baron et al., 1987b). In contrast, non-muscle-type calcium-sensitive α-actinin may be targeted to the filament/membrane junctions of non-muscle cells. To investigate this hypothesis, cDNAs encoding the complete sequence of chick brain or chick smooth muscle α-actinin were cloned into the eukaryotic expression vector pECE (Ellis et al., 1986). The resultant constructs were then transfected into monkey (non-muscle) COS cells (Gluzman, 1981). The α-actinins expressed in these cells were then probed with an antibody specific for chicken α-actinin (characterized by Jackson et al., 1988).
2.1 Chemicals, Enzymes, Resins and other Reagents.

The most important of these are listed below. PMSF (phenylmethyl-sulphonyl fluoride), TEMED (N, N, N', N'-tetramethyl-ethylene-diamine), Tris ([hydroxymethyl] aminomethane), ampicillin, calf thymus DNA (sodium salt), reduced glutathione, glutathione-agarose beads (sulphur linkage), quin-2, BSA (bovine serum albumin), chicken skeletal muscle actin, chicken cardiac muscle cytochrome-C, IPTG (isopropyl β-D-thiogalactopyranoside), DTT (dithiothreitol), PEG 6000 and 8000 (polyethylene glycol 6000 and 8000), Coomassie Brilliant Blue R stain, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, NBT (nitro-blue-tetrazolium), BCIP (5-bromo-4-chloro-indolylphosphate) were supplied by the Sigma Chemical Company, Poole, England. Sepharose resins, deoxyribonucleotides (dNTPs), dideoxyribonucleotides (ddNTPs), hexadeoxyribonucleotides (for oligolabelling), and DNA polymerase 1 (Klenow fragment) were supplied by Pharmacia, Milton Keynes, England. T4 DNA ligase, Hybond-N, and all radionucleotides were obtained from Amersham International plc, Little Chalfont, England. Calf intestinal phosphatase was supplied by Boehringer Corporation (London) plc, Lewes, England. Acrylamide and urea were supplied by Serva, Heidelberg, West Germany. N, N'-methylene-bisacrylamide was supplied by Uniscience, Cambridge, England. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was supplied by Anglian Biotechnology, Colchester, England. Restriction enzymes were supplied by Gibco-BRL plc, Paisley, Scotland; the Boehringer Corporation; Amersham International and Pharmacia LKB Biotechnology. Phenol, and PVP (polyvinylpyrrolidine) were supplied by Fisons of Loughborough, England. Normal melting point agarose was obtained from the FMC Corporation, Rockland, Maine, USA. Ammonium persulphate, hydroxylapatite resin, Ultra-pure CsCl and low melting point (LMP) agarose were supplied by Bio-Rad Laboratories, Watford, England. Filter paper and DEAE-cellulose (DE-52) were obtained from Whatman. Ponceau S, dimethyl dichlorosilane solution (used for siliconising glassware), bromophenol blue and xylene cyanol were supplied by BDH, Poole, England. Synthetic oligonucleotides, including forward and reverse primers, were synthesised by J. Kyte, Biochemistry Department, University of Leicester, England. Alkaline phosphatase conjugated goat anti-rabbit antibody was supplied by Promega. Media for the growth of bacteria were obtained from Oxoid Ltd., Basingstoke, England, except for BBL trypticase (Becton Dickinson Ltd., Oxford, England), Difco Tryptone and Bacto Yeast Extract (Difco Ltd., East Molesley, England.). Anti-chicken smooth muscle α-actinin antibody was raised in rabbit by Baron et al. (1987a). All other chemicals were of analytical grade.
2.2 cDNA Libraries.

The chick brain λgt10 cDNA library constructed using RNA from the whole brains of 12-, 14-, and 16-day-old chick embryos was a kind gift of Dr. Mark G. Darlison (Molecular Neurobiology Unit, Cambridge). The chick brain λgt10 cDNA library constructed using RNA from the whole brains of 14-day-old embryos was a kind gift of Dr. Wasenius (The University of Helsinki). The Human placental λgt11 cDNA library was obtained from Clontech.

2.3 Plasmids and Bacterial Strains.

Bluescript (Stratagene Cloning Systems, San Diego, USA) was used as a general purpose vector for the subcloning, restriction mapping, and sequencing of DNA fragments. The plasmid pGEX-1 (Smith and Johnson, 1988) was used for the expression of cDNA fragments in E.coli. The eukaryotic plasmid expression vector pECE (Ellis et al., 1986) was used for the expression of α-actinin cDNAs in monkey COS cells (Gluzman, 1981).

E.coli Y1090 (Young and Davis, 1983) and JM101 (Messing, 1981) were used as host strains for lambda phage and bacterial plasmids respectively.

2.4 Bacterial Culture Media.

BT-plates contained 10g Bacto-tryptone, 5g yeast extract, 10g NaCl, 1.0g of D-glucose, 15g of Bacto-agar per litre of distilled water. BBL trypticase plates contained 10g BBL trypticase, 5g NaCl, 2.5g MgSO₄, 15g bacto-agar, and 2.5g MgSO₄ per litre of distilled water. 2 x TY contained 15g of Bacto-tryptone, 10g of yeast extract and 10g of NaCl per litre of distilled water. Top agarose contained 5g high-melting point agarose, 10g BBL trypticase, 5g NaCl and 2.5g MgSO₄ per litre of distilled water. All media was autoclaved. An appropriate antibiotic was added when the media had cooled sufficiently (~60°C). For example, when E. coli was transformed (see Section 2.12.vi) with a plasmid conferring resistance to ampicillin (such as Bluescript, pGEX-1 or pECE), this antibiotic was added to the media at a concentration of 50μg/ml.

2.5 Protein Purification Procedures.

To help minimise the amount of protein degradation taking place during these preparations, all manipulations (except where indicated) were carried out on ice or in a cold room at 4°C.
2.5.i Purification of Chicken Brain α–Actinin

Chicken brain α–actinin was purified using a slightly modified version of the method presented by Duhaiman and Bamburg (1984). This method routinely took 4 days to complete:

Day 1. 50g of frozen adult chicken brains (free from connective tissue) were chopped into small pieces and homogenised in a high speed blender (2 x 10s bursts) in 7 to 10 volumes of double de-ionised water containing 0.5mM PMSF. The resultant frothy white suspension was centrifuged at 12,000g for 15 minutes. The pelleted material was then extracted in 10 volumes of buffer B (2mM Tris/acetate (pH 9.0), 1mM EGTA, 2mM NaN₃, and 0.5mM PMSF) at 22°C for 30 minutes with stirring. Care was taken to maintain the pH at 9.0 throughout this extraction procedure. Following centrifugation of the extract at 12,000g for 30 minutes, the supernatant was saved and the pelleted material was re-extracted and re-centrifuged. The supernatants were combined and the pH of the resultant suspension was adjusted to 7.0. The extract was then subjected to ammonium sulphate fractionation. The 0-50% cut recommended by Duhaiman and Bamburg (1984) was replaced by two separate cuts: the first using 0-25% ammonium sulphate (13.4g of solid per 100ml of solution), and the second 25-35% (a further 5.6g of solid per 100ml of solution). At each stage, the solution was stirred for 30 minutes and then centrifuged at 12,000g for 20 minutes. The pellet obtained following the 0-25% cut was discarded. The 25-35% pellet (rich in α–actinin) was dissolved in, and exhaustively dialysed against, buffer A (20mM Tris (pH 7.6), 150mM NaCl, 1mM EDTA, 2mM NaN₃ and 0.1mM DTT).

Day 2. The dialysed material was clarified (24,000g for 30 minutes) and the supernatant (typically 30ml) was applied to a column of DEAE-cellulose (25 x 0.9cm) equilibrated in buffer A. The column was then washed with buffer A until the A₂₈₀nm and conductivity of the eluate had become equal with that of the starting wash buffer. The bound proteins were then eluted with a gradient of sodium chloride in buffer A (150-500mM NaCl; total volume 200mls). The eluate was collected in 2ml fractions and analysed by SDS-PAGE (8% acrylamide). The α–actinin containing fractions were exhaustively dialysed against buffer C (50mM KH₂PO₄/K₂HPO₄ (pH 7.1) containing 2mM NaN₃ and 0.5mM DTT).

Day 3. The pooled dialysed fractions were applied to a hydroxylapatite column (9 x 0.9cm) equilibrated in Buffer C. The column was washed with buffer C until the A₂₈₀nm and conductivity of the eluate had become equal with that of the starting wash buffer. The bound proteins were then eluted with a gradient (total volume 240mls) of potassium phosphate (50-200mM KH₂PO₄/K₂HPO₄ (pH 7.1) containing 2mM NaN₃.
and 0.5mM DTT). The eluate was collected in 2ml fractions and analysed by SDS-PAGE (8% acrylamide).

Day 4. The α-actinin containing fractions were concentrated to 0.4ml by ultrafiltration using either Amicon XM-50 membranes or Centricon 30 centrifugation cones (see Chapter 6). The concentrated material was clarified (12, 500 g for 10 minutes) and chromatographed on a Superose 6 FPLC column (Pharmacia), equilibrated in buffer D (20mM Tris (pH 7.6), 20mM NaCl, 1mM EDTA, 0.5mM PMSF, 0.5mM DTT and 0.5mM NaN3). The fractions (0.4mls) were then analysed by SDS-PAGE (8% acrylamide). Those fractions containing brain α-actinin were stored for up to 1 week at 4°C. The yield of pure protein (>99% pure by densitometry) obtained using this protocol was approximately 0.2-0.3 mg/50g (wet weight) of starting tissue.

2.5.ii Purification of Chicken Gizzard α-Actinin.

Chicken smooth muscle α-actinin was prepared essentially according to Feramisco and Burridge (1980). This protocol routinely took 3 days to complete:

Day 1. 50g of frozen chicken gizzard tissue (free from connective tissue) was chopped into small pieces and homogenised in a high speed blender (3 x 10s) in 7 to 10 volumes of de-ionised water containing 0.5mM PMSF. The resultant frothy suspension was centrifuged at 12, 000g for 15 minutes. The pelleted material was extracted in 10 volumes of buffer A (2mM Tris/acetate (pH 9.0), 1mM EGTA, and 0.5mM PMSF) at 37°C for 30 minutes with stirring. Care was taken to maintain the pH at 9.0 throughout this extraction procedure. The extract was centrifuged at 12, 000g for 30 minutes. The pelleted material was discarded and the supernatant was saved, the pH being adjusted to 7.0-7.2 with glacial acetic acid. MgCl2 was added to a final concentration of 10mM, causing a cloudy white precipitate to form. After stirring for 15 minutes at room temperature the suspension was centrifuged (10 minutes at 12, 000g) and the pellet was discarded. 14.9g of ammonium sulphate was then added per 100ml of supernatant, followed by stirring for 20 minutes. The suspension was centrifuged at 12, 000g for 15 minutes. The pellet was discarded and a further 5.6g of ammonium sulphate was added per 100ml of supernatant. The suspension was stirred for 20 minutes and the precipitate was collected by centrifugation at 12, 000g for 15 minutes. The resultant pellet was dissolved in, and exhaustively dialysed against, buffer B (20mM Tris/acetate (pH7.6), 20mM NaCl, 0.1mM EDTA, and 15mM mercaptoethanol).

Day 2. The dialysed solution was clarified by centrifugation (24,000g for 30 minutes) and applied to a column of DEAE-cellulose (25 x 0.9cm). The column was then washed with buffer A until the absorbance and conductivity of the eluate at 280nm had become equal with that of the starting wash buffer. The proteins bound to the
column were eluted with a gradient of sodium chloride in buffer B (0-370mM NaCl: total volume 250mls). The eluate was collected in 5ml fractions, and analysed by SDS-PAGE (8% acrylamide).

Day 3. The α-actinin containing fractions were pooled and concentrated by adding an equal volume of saturated ammonium sulphate solution. The suspension was then stirred for 20 minutes. The α-actinin precipitate was collected by centrifugation (24,000g for 15 minutes) and dissolved in 5mls of buffer B. The resultant cloudy solution was centrifuged at 80,000g for 2 hours to remove any remaining actin. The supernatant was then applied to a column of Sepharose 6B-CL (2.5 x 90cm) equilibrated in buffer B. The eluate was collected in 3ml fractions and analysed by SDS-PAGE. The α-actinin containing fractions were pooled and stored for up to two months in buffer B at 4°C. The yield of pure protein (>99% pure by densitometry) obtained using this protocol was approximately 15mg/50g of starting tissue.

2.5.iii Purification of Chicken Skeletal Muscle α-Actinin.

Chicken skeletal muscle α-actinin was purified according to Langer and Pepe, 1980. This protocol routinely took 3 days to complete:

Day 1. 50g of frozen chicken skeletal muscle was stripped of connective tissue and cut into small pieces. These pieces were then homogenised in a high speed blender (4 x 10s bursts) in 500mls of wash solution one (50mM Tris/acetate (pH 8.25), 250mM sucrose, 1mM EDTA, 1mM NaN3, 0.5mM PMSF). The homogenate was stirred for 2 hours and then centrifuged at 12,000g for 15 minutes. The supernatant was discarded and the pellet was re-homogenised (1 x 10s burst in high speed blender) in 500ml of wash solution one. The homogenate was stirred for two hours and re-pelleted. The supernatant was discarded and the pellet was re-homogenised in 500ml of wash solution one (1 x 10s burst in high speed blender). The homogenate was stirred for 30 minutes and re-pelleted. The supernatant was discarded and the pellet was homogenised (1 x 10s burst in high speed blender) in 500mls of wash solution two (50mM Tris [pH7.6], 1mM EDTA, 1mM NaN3, and 0.5mM PMSF). The homogenate was stirred for 40 minutes and pelleted as above. The pellet was re-suspended (in small batches) in 650-700mls of de-ionised water, and left to stand overnight at 4°C.

Day 2. The extract was centrifuged at 12,000g and the supernatant was saved at 4°C. The pelleted material was re-extracted twice more (each for two hours at 4°C) in 500ml of de-ionised water. The resultant supernatants were pooled together and PMSF and NaN3 were added to final concentration of 5mM and 1mM respectively. The insoluble material was removed by centrifuging the supernatant at 12,000g for 15 minutes. 13.4g of solid ammonium sulphate was added per 100ml of supernatant, and
the supernatant was stirred for 30 minutes at 4°C. Following centrifugation at 12,000g for 20 minutes, a further 7.4g of solid ammonium sulphate was added per 100ml of supernatant. The supernatant was stirred for 30 minutes at 4°C and centrifuged at 12,000g for 20 minutes. The pellet was dissolved in and exhaustively dialysed against potassium phosphate buffer (0.02M at pH 7.2).

Day 3. The dialysed material was clarified at 12,000g for 20 minutes and applied to a column of hydroxylapatite column (0.9 x 9cm) equilibrated in potassium phosphate buffer. The column was washed in potassium phosphate buffer until the absorbance and conductivity of the eluate at 280nm had become equal with that of the starting wash buffer. The bound proteins were then eluted with a gradient (total volume 240mls) of potassium phosphate buffer (0.02-0.50M at pH 7.2). The eluate was collected in 3ml fractions and analysed by SDS-PAGE (8% acrylamide). The fractions containing the purest α-actinin were pooled and dialysed against 20mM Tris/acetate (pH7.6), 20mM NaCl, 0.1mM EDTA, and 15mM mercaptoethanol. Chicken skeletal muscle α-actinin was stored for up to two months in this buffer at 4°C. The yield of pure protein (>99% pure by densitometry) obtained using this protocol was approximately 10mg/50g of starting tissue.

2.6 General Procedures used to Handle Proteins.

2.6.i Protein Determination.

Chicken skeletal muscle α-actinin has been shown to have an extinction coefficient E^280nm of 10 (Langer and Pepe, 1980). A solution of skeletal muscle α-actinin at a concentration of 1mg/ml would therefore have an absorbance of 1 when measured at 280nm (in a cell of light path 1cm). Furthermore, Duhaiman and Bamburg (1984) have shown that solutions containing identical concentrations of chicken brain, skeletal and smooth muscle α-actinins are identical in absorbance at 280 nm. Therefore, the extinction coefficient of chicken skeletal muscle α-actinin was also used to estimate the concentration of solutions containing chicken brain or chicken smooth muscle α-actinin. Rabbit skeletal muscle F-actin was quantitated by absorption at 290nm assuming a value of E^280nm= 6.5 (Houk and Ue, 1974).

2.6.ii Gel Electrophoresis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to analyse the composition of various protein samples. The running gel solutions were mixed according to Table 2.1a. The gel was formed using either a Bio-
Rad Protean II vertical slab gel electrophoresis apparatus or a LKB 2050 Midget Electrophoresis Unit. The gel surface was then overlaid with water saturated butanol. Once the running gel had set, the excess butanol was flushed out with de-ionised water. The stacking gel solution (see Table 2.1b) was then poured onto the surface of the running gel. A comb was inserted into the stacking gel to produce the sample wells. Once the stacking gel had set, the comb was removed and the wells were flushed out with de-ionised water.

The samples were prepared by adding an equal volume of sample buffer (180mM HCl (pH 6.8), 5.7% SDS, 29% glycerol, 0.005% bromophenol blue) and 5μl of 2-mercaptoethanol, followed by boiling for 2-3 minutes. The samples were added to the wells, which were then topped up with running buffer (25mM Tris, 192mM glycine, 0.1%SDS). The gels were run at 80mA per gel in the Bio-Rad Protean II apparatus, or at 50mA per gel in the LKB 2050 Midget Electrophoresis Unit.

Table 2.1a. Composition of SDS-PAGE Running Gels

<table>
<thead>
<tr>
<th>% Acrylamide</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>12</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide (mls)</td>
<td>5</td>
<td>7.6</td>
<td>9</td>
<td>10</td>
<td>12</td>
<td>13.6</td>
<td>17.2</td>
<td>21.5</td>
</tr>
<tr>
<td>1M Tris pH 8.8 (mls)</td>
<td>16.8</td>
<td>16.8</td>
<td>16.8</td>
<td>16.8</td>
<td>16.8</td>
<td>16.8</td>
<td>16.8</td>
<td>16.8</td>
</tr>
<tr>
<td>Water (mls)</td>
<td>20</td>
<td>19</td>
<td>17.6</td>
<td>16</td>
<td>14.5</td>
<td>13</td>
<td>9.3</td>
<td>5</td>
</tr>
<tr>
<td>20% SDS (μl)</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
</tr>
<tr>
<td>10% AMPS (μl)</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
<td>225</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 2.1b. Composition of the SDS-PAGE Stacking Gel.

| 30% acrylamide stock (ml) | 1.33 |
| 1M Tris pH 6.8 (ml) | 1.25 |
| Water (ml) | 7.36 |
| 20% SDS (μl) | 50.00 |
| 10% AMPS (μl) | 50.00 |
| TEMED (μl) | 15.00 |
After electrophoresis, the gels were placed in stain solution (made by mixing 1g Coomassie Brilliant Blue R, 75ml of glacial acetic acid, 405mls of methanol, and 520mls of water) for 30 minutes with gentle agitation. The gels were then immersed in de-stain solution (10% acetic acid, 10% methanol and 80% water) and left to rock gently until they had fully de-stained.

2.6.iii. Densitometric Analysis of SDS-PAGE Gels.

The purity of the protein preparations was estimated by densitometry using a LKB Uiltroscan Laser Densitometer, and the Gelscan program of P. Heilmann.

2.7 Thermolytic Digestion of α–Actinin.

Purified α–actinin was thoroughly dialysed against 100mM ammonium bicarbonate, 5mM CaCl₂ (pH 7.6) immediately prior to digestion. Thermolysin (Sigma) was pre-activated at room temperature by the tenfold dilution of a 20mg/ml protease stock with 1mM CaCl₂. Thermolysin was added to the protein (1 : 50 ratio of protease to protein) and the digest was allowed to proceed at 37°C for various lengths of time, as indicated in the legends of appropriate figures. Digestion was stopped by the addition of EDTA to a final concentration of 10mM. The samples were then subjected to SDS-PAGE as described in Section 2.6.ii.

2.8 Protein Sequencing.

α–Actinin was digested with thermolysin and the resulting polypeptides separated by SDS-PAGE as described in Figure 3.5. The fragments were then electroblotted onto polyvinylidene difluoride (PVDF) membranes essentially as described by Matsudaira (1987), except that the bloting buffer consisted of 50mM Tris (pH 10), 50mM glycine and 10% methanol. The blotted fragments were sequenced by Dr. Mathew D. Davison (Department of Biochemistry, University of Leicester) using an Applied Biosystems 470A automated gas phase sequencer. The proteins were sequenced direct from PVDF membrane, without the use of any support, such as a glass fibre disk or polybrene.

The chemical process used by the sequencer is derived from the Edman degradation process developed by Pehr Edman in the 1950s (Geisow and Aitken, 1989). Each cycle of this process involves the following stages.

Firstly, the coupled protein is reacted with phenylisothiocyanate (PITC). In the presence of free base (trimethylamine), the PITC reacts quantitatively with the free
N-terminal group (and amino side chains) to form the phenylthiocarbamyl-protein (PTC-protein). Residual PITC and PITC-by-products are removed by extraction with the organic solvents n-heptane and ethyl acetate.

Secondly, the highly volatile organic acid, trifluoracetic acid (TFA) is used to cleave away the PITC-coupled amino acid residue from the N-terminus of the protein, producing the anilinothiazolinone (ATZ) derivative of the amino acid. This process reveals a fresh amino acid at the N-terminus of the protein, ready for the next degradation cycle.

Thirdly, the liberated ATZ-amino acid is converted to the phenylthiohydantoin (PTH)-amino acid in the presence of 25% TFA. The PTH-amino acid is then recognised and quantitated using an amino acid analyser.

At each stage of the process solvents are delivered to the reaction chamber in the vapour phase (hence gas-phase sequencer), which minimises washout of protein by polar reactants during cycling.

2.9 Analysis of the Calcium-Binding Properties of the α-Actinin Isoforms.

2.9.i Rapid 'Dot-Blot' Calcium-binding Assay.

The calcium-binding properties of the purified α-actins were measured using a rapid 'dot-blot' calcium-binding assay developed by Koch et al., 1986 (described in more detail in Chapter 5).

Three 9cm filter circles (No.54 Whatman Inc., Clifton N. J.) were placed in a Buchner funnel and washed under vacuum in 10mM imidazole (pH 7.0), 0.1 M NaCl, and 1.0mM MgCl₂ (wash buffer). Wash buffer and all other solutions used in this assay were made up using BDH Analar water (<1.25μM Ca²⁺), and stored in plastic to avoid contaminating calcium from glassware. A gridded nitrocellulose filter (BA 85/20 Schliecher and Schull, inc., Keene, NH) was placed on top of the filter circles, and rinsed in wash buffer. 50-200 pmols aliquots of protein were spotted onto alternate squares of the gridded nitrocellulose filter under vacuum. The proteins were either thoroughly dialysed against wash buffer before applying onto the nitrocellulose filter, or were applied in their usual storage buffers, and rinsed briefly in wash buffer (2 x 50ml aliquots). After applying protein, the nitrocellulose filter was immersed in 10ml of wash buffer containing 3.2μCi/ml ⁴⁵Ca²⁺ and 0.04-1.00mM carrier 'cold' Ca²⁺. Excess buffer was removed from the filter under vacuum. The filter was then immersed, face down, into 50ml of wash buffer and washed for 20s on an orbital shaker. The filter was
then dried under vacuum and cut into squares. The radioactivity of each square was then measured in a scintillation counter (Model 2000CA, Liquid Scintillation Analyser, United Technologies Packard). Filter squares to which no protein had been added were used as blanks, and chicken heart cytochrome-C (Sigma) was used as a negative control protein. Expressed human plasma gelsolin (a kind gift from Alan Weeds, LMB Cambridge) was used as a positive control protein as it is known to bind calcium.

2.9.ii Stopped-flow Spectrophotometry.

The α-actinin EF-hand fusion proteins expressed in E. coli (see Section 2.15) were analysed by stopped-flow spectrophotometry (also see Chapter 5). Bacterially expressed human plasma gelsolin was used as a positive control protein. The stopped-flow apparatus was built according to a design devised by Professor H. Gutfriend, and is described in detail by Jackson and Bagshaw, (1988). The fluorescent calcium-chelating reagent Quin-2 was used as indicator (Tsien et al., 1982). This reagent is a tetracarboxylic acid which binds Ca$^{2+}$ with a 1:1 stoichiometry ($K_d=115$nm). The fluorescence signal of this reagent increases about fivefold on going from Ca-free to Ca-saturated forms. This reagent was excited at 336.5nm (emission at approximately 500 nm). The emitted light was selected using a 420nm filter and a light green celluloid filter (no. 23A Rank Strand Cinema Safety Colour Filters) with maximal transmission at 500nm. The experiments were performed at 20°C in a buffer containing 20mM Tris/acetate (pH 7.6), 20mM NaCl, in the presence or absence of 0.5mM DTT. All of the proteins assayed using stopped-flow spectrophotometry were dialysed against this buffer overnight at 4°C prior to use. The proteins were allowed to reach room temperature (20°C) before use. The data were fitted using the non-linear regression programme supplied with the Applied Photophysics stopped-flow unit.

2.10. Isolation of cDNAs Encoding Chick Brain and Human Placental α-Actinin.

α-Actinin cDNAs were isolated from lambda phage cDNA libraries following standard procedures (Maniatis et al., 1982), and using radioactively labelled cDNAs as probes (see Chapter 4). The cDNA library was diluted in SM buffer (5.8g NaCl, 2g MgSO$_4$, 50ml 1M Tris/HCl (pH 7.5), 10mg of gelatin per litre of distilled water) to give approximately 10,000 to 15,000 plaques per 100μl. The host strain, E.coli Y1090, was prepared as follows: firstly, the cells were grown overnight in 2 x TY containing ampicillin at 50 μg/ml at 37°C; the cells were then pelleted at 4,000 rpm for 10 minutes in a MSE chillspin. The supernatant was discarded and the cells were re-suspended in
4mls of 10mM MgSO₄ using a pipette. 200 μl of aliquots of host cells were mixed with 100 μl of phage solution in sterile universals, and the mixtures were incubated at 37°C for 5 minutes. 16ml of molten top agarose (at 56°C) was added to each tube. The tubes were then inverted several times to mix the cells evenly throughout the agarose. The contents of the tubes were then poured across the surface of BBL trypticase plates (15cm diameter). The top agarose was allowed to set at room temperature. The plates were then transferred to a 37°C incubator to allow the plaques to develop to a suitable size (several hours). The plates were stored until required, in sterile bags at 4°C. The agarose surface of each plate was covered with a hybond-N filter (diameter 132mm, Amersham). The filter was keyed to the surface of the agarose using a syringe needle. The filters were applied for 5 minutes (primary lifts) or 10 minutes (duplicate lifts). The filters were then removed from the surface of the agarose using forceps, and transferred (DNA side up) into denaturing solution (0.5M NaOH, 1.5M NaCl). After 5 minutes the filters were transferred (DNA side up) into neutralising solution (1.5M NaCl, 0.5M HCl). Five minutes later, the filters were rinsed in 2 x SSC and dried at room temperature on Whatman 3MM chromatography paper. The filters were baked for 10 minutes at 80°C, wrapped in Saran Wrap (Dow Chemical Company) and placed (DNA face down) onto an ultraviolet light transilluminator. The DNA was fixed to the hybond-N membrane by exposing to ultraviolet light for 20s. The filters were then transferred to 100ml of pre-hybridisation solution (Table 2.2) at 65°C in a large hybridisation chamber, and incubated for 1-2 hours at the same temperature. The pre-hybridisation solution was drained away, and replaced with 100ml of hybridisation solution at 65°C. The 3²P-dATP labelled DNA probe (see Section 2.12.vii) was denatured by heating for 5 minutes to 100°C. The denatured probe was added to the hybridisation solution, and the chamber was incubated overnight at 65°C. Following the hybridisation, the filters were transferred into 500ml of wash solution (2 x SSC and 0.1% SDS at 65°C). The filters were rocked gently for 1 minute and the procedure was repeated. The filters were transferred into a further 500mls of wash solution, and incubated in a 65°C water bath for 30 minutes with continual agitation. The filters were then briefly monitored to examine the background. If the background was too high; the filters were transferred into more stringent wash buffer (down to 0.1 x SSC, 0.1% SDS) and incubated for a further 30 minutes at 65°C. The filters were then dried at room temperature on Whatman 3MM paper. The filters were taped (DNA face up) onto a fresh piece of Whatmann 3MM paper.
The paper was marked with radioactive ink (made by mixing a small aliquot of $^{32}$P-dATP with a dilute solution of bromophenol blue) to enable an alignment with the developed autoradiograph. The Whatmann 3MM paper was covered in a single layer of Saran Wrap, applied to X-ray film (Kodak XR), and exposed overnight at $-70^\circ$C with an intensifying screen. The film was developed, and aligned with the Whatman 3MM paper. The Whatman 3MM paper was in turn aligned with the BBL-trypticase plates. Positive plaques (appearing on primary and duplicate lifts) were picked from the BBL-trypticase plate, using the basal end of a 1ml Gilson tip. The phage plug was eluted into 1ml of SM buffer, overnight at $4^\circ$C. The eluted phage were re-titred and re-screened at lower density (approximately 2,000 phage per plate). Positive plaques were again picked, re-titrated and re-screened at successively lower density, until the positive phage were pure.

Table 2.2 Pre-hybridisation and hybridisation Solutions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Hybridisation solution</th>
<th>Pre-hybridisation solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 x SSC*1 (ml)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>50 x Denhardts*2 (ml)</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>20% SDS (μl)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>36% PEG 6000(ml)</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>at 10mg/ml*3 (μl)</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>De-ionised water (ml)</td>
<td>11.2</td>
<td>12.4</td>
</tr>
<tr>
<td>Total (ml)</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

*1 20 x SSC contained 175.3g sodium chloride, and 88.2g of sodium citrate per litre of de-ionised water.

*2 50 x Denhardt's contained 10g Ficoll, 10g polyvinylpyrolidine, 10g of BSA per litre of de-ionised water.

*3 Calf thymus DNA was prepared by dissolving the DNA in de-ionised water to a concentration of 10mg/ml. The DNA was stirred for several hours at room temperature followed by several minutes of sonication. When a watery consistency was achieved, the DNA was boiled for 10 minutes and stored in 400μl aliquots at $-20^\circ$C. Just before use, the DNA was boiled for 5 minutes and then chilled for 30s on ice.
2.11 Large-scale Preparation of Lambda Phage DNA.

Positive phage produced in Section 2.10 were plated out at a low enough density (100-200 plaques per plate) to enable single plaques (surrounded by a lawn of E. coli Y1090) to be picked with the basal end of a sterile 1ml Gilson tip. 5 plaques were picked, and were used to inoculate 250ml of 2 x TY containing ampicillin (50 μg/ml) and MgSO4 (10mM). The cultures were then incubated at 37°C for 9-12 hours. The agar plugs usually contained a suitable multiplicity of phage to cells to enable the complete lysis of the culture during this period. After lysis was evident, 5ml of chloroform was added to each flask. The flasks were then shaken for a further 30 minutes at 37°C and chilled to room temperature. Pancreatic DNase and RNase were each added to a final concentration of 1μg/ml. The flasks were incubated for 30 minutes at room temperature, and then sodium chloride was added to a final concentration of 1M. The sodium chloride was dissolved by swirling the flasks gently. The flasks were allowed to stand for 1hr on ice. The cellular debris was collected by centrifugation (11,000g for 15 minutes), and solid polyethylene glycol 6000 was added to each supernatant to give a final concentration of 10% w/v. The PEG was dissolved by stirring gently on a magnetic stirrer at room temperature. The flasks were allowed to stand on ice for at least an hour, to enable the phage particles to precipitate. The phage precipitates were collected by centrifugation (11,000g for 15 minutes) and re-suspended in 2ml of SM buffer. A step-gradient of CsCl in SM buffer was prepared by layering 3 solutions (see Table 2.3) of successively lower density, one on top of another, in 10.5ml polyallomer tubes. 3ml of each gradient solution was added per tube. The phage solutions were layered onto the CsCl step gradient and the tubes were spun at 22,000 rpm (Sorval Ultracentrifuge, TH-641 rotor) for 2 hours at 20-30°C.

Table 2.3. CsCl Gradient Solutions used in the Large Scale Lambda Phage DNA Preparation.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Density</th>
<th>CsCl (g)</th>
<th>SM (ml)</th>
<th>Refractive Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.45</td>
<td>20.00</td>
<td>28.30</td>
<td>1.3768</td>
</tr>
<tr>
<td>B</td>
<td>1.50</td>
<td>22.33</td>
<td>27.33</td>
<td>1.3815</td>
</tr>
<tr>
<td>C</td>
<td>1.70</td>
<td>31.60</td>
<td>25.00</td>
<td>1.3990</td>
</tr>
</tbody>
</table>
Following centrifugation, the phage particles were visible as a blue band at the interface of gradient solutions A and B. The phage bands were collected using a 1ml Gilson, and dialysed overnight against 5 litres of 50mM HCl (pH 8.0), 10mM NaCl, 10mM MgCl$_2$ at 4°C. The phage were then re-dialysed against a fresh batch of the same buffer at room temperature for 2 hours. The phage suspensions were transferred to 15ml corex tubes and EDTA (pH 8.0), pronase and SDS were added to final concentrations of 20mM, 0.5mg/ml, and 0.5% respectively. The tubes were incubated at 37°C for 1 hour with intermittent shaking. The mixtures were then extracted with phenol (see Section 2.12.ii), and the resultant aqueous phases were dialysed against TE buffer (10mM Tris (pH 8.0), 1mM EDTA) for 2 hours at room temperature. The DNA was ethanol precipitated (see Section 2.12.i ) and re-dissolved in sterile water.

**2.12 General Procedures used to Handle DNA.**

2.12.i Ethanol Precipitation.

Unless stated otherwise DNA solutions were precipitated from aqueous solution by adding a tenth of a volume of 3M/5M potassium acetate (see section 2.13.i) and two volumes of ice-cold absolute ethanol. The DNA solution was then incubated at -70°C for 30 minutes, or overnight at -20°C. The DNA was pelleted at 13,000g for 10 minutes. The supernatant was discarded and the pellet was washed with 1ml of 80% ethanol to remove any remaining salt. The tube was then re-centrifuged at 13,000g for 2 minutes. The supernatant was discarded and the pellet was dried briefly in a vacuum desiccator. The pellet was then re-suspended in a suitable volume of sterile water (or TE buffer) to obtain DNA at a desirable concentration.

2.12.ii Phenol Extraction.

Phenol extraction was performed to remove contaminating protein from DNA solutions. The phenol was prepared (equilibrated) by adding an equal volume of 1M Tris pH 8.0 followed by vigorous mixing. The aqueous phase was removed and the procedure was repeated twice more, this time using 0.1M Tris pH 8.0 instead of 1M Tris pH 8.0. Hydroxyquinoline and 2-mercaptoethanol were added to final concentrations of 0.1 and 0.2% respectively. The phenol was stored saturated in TE-buffer (10mM Tris pH 8.0, 1mM EDTA). An equal volume of phenol was added to the DNA solution, followed by thorough vortexing. The mixture was then centrifuged at 13,000g for 2 minutes. The aqueous phase was removed, and to it was added an equal volume of phenol/chloroform solution, prepared by mixing equal volumes of phenol and
chloroform (containing 1/24 of a volume isoamyl alcohol). The mixture was re-vortexed and re-centrifuged. The aqueous layer was removed, and an equal volume of chloroform was added to it. The mixture was re-vortexed and re-centrifuged. The aqueous layer was removed and the DNA was recovered by ethanol precipitation (Section 2.12.i).

2.12.iii Restriction Enzyme Digests.

Restriction digests containing up to 1µg of DNA were performed in a total volume of 20µl. Digests involving larger amounts of DNA were scaled up accordingly. 1/10 of the total digest volume was made up from a 10 x stock of the manufacturers recommended digestion buffer. 1-2 units of enzyme were added to the mixture, (one unit of enzyme is defined as the amount required to digest 1-2µg of DNA to completion in 1 hour using the manufacturers recommended buffer at the recommended temperature). The volume of enzyme in the restriction digest did not exceed 0.1 volume of the final reaction mixture, since the activity of some enzymes may be inhibited by glycerol. The mixture was incubated at the required temperature for the required time. The digests were either analysed immediately or stored at -20°C.

2.12.iv Alkaline Phosphatase Treatment of Linearised Plasmid DNA.

Alkaline phosphatase treatment was performed to render plasmid DNAs resistant to ligation, by the removal of phosphate residues from their 5' termini. The DNA was digested as usual (Section 2.12.iii), and the volume was made up to 100µl. The DNA solution was then phenol extracted (Section 2.12.ii) and ethanol precipitated (Section 2.12.i). The DNA was dissolved in 45µl of sterile water. 5µl of 10 x CIP buffer (0.5M Tris (pH 9.0), 10mM Mg acetate, 1mM ZnSO₄, 10mM spermidine) and 0.5µl of calf intestinal phosphatase were added, and the reaction was incubated at 37°C for 30 minutes. The volume was then increased by adding 40µl water, 10µl of 10 x STE (100mM HCl [pH 8.0], 1M NaCl, 10mM EDTA) and 5µl of SDS. The mixture was incubated at 68°C for 15 minutes. After this period had elapsed the DNA solution was phenol extracted (Section 2.12.ii) and ethanol precipitated (Section 2.12.i). The DNA was then dissolved in sterile water, at a concentration of approximately 10 ng/µl.
2.12.v Ligation of DNA Fragments into Plasmids.

The insert DNA (20-30ng) and linearised and phosphatased vector DNA (10ng), both in sterile water were mixed to give a maximum volume of 9μl. 1μl of 10 x ligation buffer (0.66M HCl [pH 7.5], 100mM MgCl₂, 10mM ATP, 15mM DTT) and 1-2 units of T4 DNA Ligase were added and the mixture was incubated either at 37°C for 30 minutes, or at room temperature for several hours, or overnight at 13°C.

2.12.vi Transformation of E.coli JM101 with Plasmid DNA.

10ml of 2 x TY was inoculated with E.coli JM101 and grown overnight at 37°C with shaking. The overnight culture was back-diluted into 2 x TY (100μl into 10ml), and grown to mid-log phase at 37°C with shaking (approximately 2 hours). The cells were then harvested at 4, 000 r.p.m. in a MSE ChilSpin at 4°C. The supernatant was discarded and the pellet was re-suspended in 5mls of ice cold 50mM CaCl₂ solution. The cells were re-pelleted, and re-suspended in 0.5ml of 50mM CaCl₂. The cells were now "competent", i.e. ready for transformation. The DNA ligation mixture (Section 2.12.v) was added to a 100μl aliquot of JM101, and left to stand on ice for a minimum of 30 minutes. The cells were then subjected to 'heat shock' i.e. they were transferred from ice into a 37°C water bath and incubated for 5 minutes. The cells were then transferred back onto ice and left to stand for a further 5 minutes. 900μl of 2 x TY was added to the cells which were then incubated for 1 hour at 37°C with shaking. After this time had elapsed, 25-100μl aliquots of the transformation mix were spread out onto BT-plates (Section 2.4). The plates were left to dry at room temperature for 30 minutes, and then incubated overnight at 37°C. Colonies were picked with a sterile toothpick, streaked onto fresh BT-plates, and grown overnight at 37°C. The plates were stored at 4°C until required. When the transformation procedure involved the Bluescript vector (Stratagene), recombinants were selected using a "blue/white" selection procedure. In this situation the chromogenic substrate X-gal (0.002% w/v) and the inducer IPTG (1mM) were incorporated into the BT-plate agar. The polylinker sequence of Bluescript lies within the lacZ gene of the vector. Recombinant plasmids can therefore be identified by the inactivation of the β-galactosidase gene. Colonies containing recombinant plasmids were white on blue/white selection plates, whereas wild-type plasmids gave blue colonies. Transformations involving plasmids which did not facilitate blue/white selection, were plated onto ordinary BT-plates (Section 2.4) and screened by colony hybridisation (see Section 2.12.viii).
2.12.vii Preparation of Radiolabelled DNA Probe.

DNA probes labelled with $^{32}$P-dCTP were synthesised using the random priming method of Feinberg and Vogelstein (1982). The DNA (10 ng in 9.8 µl) was first denatured by boiling for 7 minutes in a screw capped tube. The DNA was chilled on ice for 30 seconds, and then added to a tube containing 3 µl of Oligo-Labeling Buffer\(^1\), 0.6 µl of enzyme grade BSA (Pharmacia, 1 mg/ml), and 1 µl of $^{32}$P-dCTP. 0.6 µl of Klenow fragment of DNA polymerase 1 (2-3 units) was added, and the tube was incubated for 2 hours at 37°C. 85 µl of sterile water was added, and the probe was either stored at -20°C or used immediately. To check for labelling, 1 µl of the reaction mix was run on a 1% agarose gel (Section 2.12.ix). The gel was then dried, using a hair dryer, and exposed to X-ray film (Kodak XR) for 15 minutes. The presence of a distinct band, at the correct position, indicated that the labelling reaction had worked. The incorporation of dCTP was very efficient, and so the unincorporated dCTP was not removed. The probe was boiled for 7 minutes before adding to the hybridisation mix.

2.12.viii Colony Hybridisation.

This method was used to detect transformants containing recombinant plasmid in situations where the cloning vector (pECE and pGEX) did not allow a blue/white selection to be performed (see Section 2.12.vi). Gridded Hybond-N circles (82 mm, Amersham) were placed onto the surface of two BT-plates. The gridded squares were numbered for identification purposes. Colonies were picked from the bacterial transformation plate using a sterile toothpick. The toothpick was used to inoculate a single square on one of the Hybond-N covered plates. The same toothpick was used to inoculate an equivalent square on the duplicate Hybond N covered plate. This process was repeated until a sufficient number of colonies had been sampled. Both Hybond-N plates were then incubated overnight at 37°C. The Hybond-N filter from one of the plates was removed, and immersed (DNA face up) in a petri dish containing several ml

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\(^1\) Oligo-labelling buffer was made by mixing solutions A, B and C in the ratio of 2 : 5 : 3.

Solution A consisted of 625 µl of 2M HCl, pH 8.0, 25 µl of 5M MgCl\(_2\), 350 µl of H\(_2\)O, 18 µl of 2-mercaptoethanol, 5 µl of each dNTP (10 mg/ml of 3mM HCl pH 7.0, 0.2 mM EDTA).

Solution B consisted of 2M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) titrated to pH 6.6 with NaOH.

Solution C consisted of hexadeoxyribonucleotides (Pharmacia) evenly suspended in 3mM HCl pH 7.0, 0.2 mM EDTA at 90 OD units/ml.
of denaturing solution (1.5M NaCl, 0.5M NaOH). The duplicate plate was stored at 4°C. After 7 minutes the filter was removed from denaturing solution and immersed (DNA face up) in a petri dish containing neutralising solution (1.5M NaCl, 0.5M HCl pH 7.2). After 2-3 minutes, the filter was removed and placed in 2 x SSC. Cell debris was removed from the surface of the filter by pipetting a stream of buffer across its surface. The filter was then dried at room temperature and baked for 10 minutes at 80°C. The filters were wrapped in Saran wrap, placed (DNA face down) on an ultraviolet light transilluminator, and fixed for 20s. The filters were then pre-hybridised, hybridised, washed and exposed to X-ray film as described in Section 2.10. Positive colonies were identified by aligning the autoradiograph with the hybridised filter. The positive colonies were picked from the duplicate plate, streaked onto fresh BT-plates, grown overnight at 37°C, and stored at 4°C until required.

2.12.ix. Agarose Gel Electrophoresis.

The gel size varied from 7.5 x 2.5 to 20 x 20cm dependent upon the use of the gel, the resolution required, and the number of samples to be run. The gels were run in 1 x TAE buffer (40mM Tris, 1mM EDTA, 30mM sodium acetate) containing ethidium bromide at 0.5 µg/ml. The concentration of the agarose varied between 0.5 and 2% (w/v) according to the anticipated molecular weight of the DNA samples. To the gel sample was added a tenth of a volume of DNA loading buffer (30% glycerol, 0.1M EDTA [pH 8.0], 0.1% bromophenol blue, 0.1% xylene cyanol). Once loaded, the samples were run at 120 volts for 1-2hrs, or 10-30 volts overnight. The DNA was visualised by placing the gel on a Ultra-Violet light transilluminator.

2.12.x Gel Purification of DNA Fragments

Fragments of DNA released during restriction enzyme digestion (Section 2.12.iii) were purified from low melting point agarose (BRL) by phenol extraction. The fragment of interest was excised in the minimal possible volume of gel (approximately 100µl) and melted at 65°C for 5 minutes. 3 volumes of sterile water at 65°C were added to the gel, and the mixture was vortexed vigorously. The diluted gel solution was placed in a 65°C waterbath for 5 minutes, followed by further vortexing. The sample was then extracted once with phenol and once with phenol chloroform (as described in Section 2.12.2). During this procedure care was taken to avoid the precipitous material at the interface between aqueous and non-aqueous layers. The DNA was then ethanol precipitated (Section 2.12.1) and re-suspended in sterile water.
2.12.xi Quantification of DNA in Solution.

DNA was quantitated by measuring the optical density of the solution at 260nm, where an absorbance of 1 is equivalent to 50\mu g/ml of double stranded DNA or 37\mu g/ml single stranded DNA (eg. oligonucleotide DNA). Where the amounts of DNA were low the concentrations were estimated by the intensity of staining with ethidium bromide (relative to DNA standards of known concentration) after separation by agarose gel electrophoresis (Section 2.12.ix).

2.13 Isolation of Plasmid DNA.

Two methods were used for the preparation of plasmid DNA, dependent on the yield and quality of DNA required. The 'mini-prep' method was used for small scale plasmid isolations, (1.5-3.0ml). In situations where a greater quantity, or higher quality, of plasmid DNA was required, the DNA was prepared by equilibrium centrifugation in CsCl-ethidium bromide gradients (Maniatis et al., 1982).

2.13.i Plasmid DNA Mini-Preparations.

A bacterial colony picked with a sterile toothpick was used to inoculate 10ml of 2 x TY containing ampicillin at 50 \mu g/ml. The culture was grown overnight at 37\degree C with shaking. A 1.5ml aliquot of the culture was centrifuged at 13,000g for 2 minutes. The supernatant were removed by pipette, leaving the cell pellet as dry possible. The pellet was re-suspended in 100\mu l of icy cold solution 1 (25mM HCl [pH8.0], 50mM glucose, and 10mM EDTA) using a Gilson pipette. After 5 minutes at room temperature, 200\mu l of freshly prepared solution 2 (0.2M NaOH, 1% SDS) was added, and the tube was inverted several times to mix the contents. The tube was immediately transferred onto ice, and left to stand for 5 minutes. 150\mu l of ice-cold solution 3 (3M potassium/5M acetate) was added, and the tube was inverted several times. Solution 3 (3M potassium/5M acetate) was made by adding 11.5ml glacial acetic acid and 28.5ml of sterile water to 60ml of 5M potassium acetate (pH 4.8). After 5 minutes on ice, the tube was centrifuged at 13,000g for 10 minutes. The supernatant was transferred to a fresh microfuge tube, taking care not to disrupt the pellet (consisting of chromosomal DNA, protein, and cell debris). 10\mu l of DNase free RNase was added to the supernatant, which was then incubated at 37\degree C for 30 minutes. DNase free RNase was prepared by making a 50mg/ml solution of Ribonuclease A (Sigma) in 10mM HCl (pH 7.5), 15mM NaCl. The Ribonuclease A solution was heated in a boiling water bath for 15 minutes, and then allowed to cool slowly to room temperature, before being dispensed into
aliquots and stored at -20°C. The supernatant was then extracted with phenol (Section 2.12.ii) and precipitated by adding two volumes of ethanol. The tubes were left to stand for two minutes at room temperature, and then centrifuged at 13, 000g for 10 minutes. The pellet was washed with 1ml of 70% ethanol, and re-centrifuged for 2 minutes. The supernatant was discarded and the pellet was dried briefly in a vacuum desiccator. The pellet was then re-suspended in 20μl of sterile water. 1-2μg of plasmid DNA was produced routinely using this protocol.


A bacterial colony was picked and used to inoculate 10ml of 2 x TY containing 50 μg/ml ampicillin. This culture was then grown overnight at 37°C with shaking. The overnight culture was then used to inoculate 500ml of the same medium. This culture was also incubated overnight at 37°C overnight. The culture was centrifuged at 12, 000g for 10 minutes. The supernatant was discarded, leaving the cell pellet as dry as possible. The supernatant was then re-suspended in 10ml of solution 1 (see Section 2.13.i) containing 5mg/ml lysozyme. After 5 minutes standing at room temperature, 20ml of freshly prepared solution 2 (see Section 2.13.i) was added. The solution was mixed gently, and left to stand on ice for 10 minutes. After this time had elapsed, 15ml of ice cold solution 3 (see Section 2.13.i) was added, and the solution was left to stand for 10 minutes on ice. The precipitate was collected by centrifugation at 12, 000g for 20 minutes. The supernatant was transferred, using a pipette, into a 30ml corex tube, and 0.6 volumes of propan-2-ol was then added. The solutions were mixed and allowed to stand at room temperature for 30 minutes. The supernatant was discarded, and the pellet was dried by vacuum desiccation. The pellet was then re-suspended in 8ml of TE-buffer (10mM Tris pH 8.0, 1mM EDTA). 8.8g of Ultra-Pure CsCl (BRL) was added, and the solid was dissolved by mixing gently. 800μl of ethidium bromide solution (10 mg/ml in water) was added, and the solution was mixed thoroughly. The solution was transferred, using a drawn out pasteur pipette, into centrifuge tubes (Beckman, Quick-Seal pots). The tubes were then centrifuged overnight in an ultracentrifuge (Beckman) at 45, 000 rpm. Under longwave ultra-violet light, 2 bands of DNA could be observed in the centrifuge tube, the upper band consisting of linear bacterial DNA and nicked circular plasmid DNA, and the lower band consisting of closed circular plasmid DNA. RNA and protein was deposited along the side of the tube. The top of the centrifuge tube was pierced with a fine gauge needle, to release the pressure. The side of the tube was then punctured, just beneath the lower DNA band, with a 25-gauge needle attached to a 1-2ml syringe. The DNA solution was then
withdrawn from the tube, and extracted with an equal volume of water-saturated butanol. The mixture was vortexed briefly and centrifuged at 4,000 rpm for 5 minutes. The upper non-aqueous layer (now containing most of the ethidium bromide) was removed and discarded. The aqueous layer was then subjected to a further two or more rounds of butanol extraction, until the aqueous phase had lost all of its pink colouration. The aqueous layer was then filtered through an acrodisc (0.2μm). An equal volume of isopropanol was added to the DNA solution, which was then incubated at room temperature for 30 minutes. The DNA was pelleted at 12,000g for 20 minutes, and dried in a vacuum dessicator. The DNA was then re-suspended in 4ml of TE, ethanol precipitated (Section 2.12.i), and re-suspended in a suitable volume of sterile water.

2.14 DNA Sequencing.

The double-stranded plasmid Bluescript (Stratagene) was used as sequencing vector. DNA fragments were cloned into Bluescript as described in Sections 2.12.v. and 2.12.vi.

2.14.i Preparation of Sequencing Template.

Plasmid DNA mini-preparations were used for sequencing (Section 2.13.i). The DNA pellet was re-dissolved in 16.8μl of sterile water. 21.3μl of 13% PEG 8,000 and 4.5μl of 5M NaCl were added, and the mixture was left to stand on ice for at least 30 minutes. The PEG precipitate was collected at 13,000g for 10 minutes. The supernatant was discarded and the pellet was washed with 1ml of 80% ethanol. The tube was spun at 13,000g for 2 minutes. The supernatant was discarded, and the pellet was dried in a vacuum dessicator. The pellet was then re-suspended in 22μl of sterile water. 2μl of the DNA solution was analysed on an agarose gel (Section 2.12.ix), to check the yield. 2-10μg of plasmid DNA, in a total volume of 20μl was denatured by adding 2μl of freshly prepared 2M NaOH, 2mM EDTA. The solution was left to stand at room temperature for 5 minutes. After this time had elapsed, the tube was transferred onto ice and 6μl of 2M sodium acetate pH 5.6 and 75μl of absolute ethanol were added sequentially. After 20 minutes incubation at -70°C, the DNA was collected by centrifugation at 13,000g for 10 minutes. The supernatant was discarded, and the pellet was washed with 0.5ml of 80% ethanol. The tube was centrifuged at 13,000g for 2 minutes. The supernatant was discarded, and the pellet was dried in a vacuum dessicator. The DNA pellet was then re-suspended in 7μl of sterile water in preparation for sequencing.
2.14.ii The Sequencing Reaction.

DNA was sequenced using the dideoxy chain termination method of Sanger et al. (1977). The chain termination method involves the in-vitro synthesis of a synthetic DNA strand from a single-stranded DNA template. This synthesis is catalysed by DNA polymerase, and is initiated at sites where a synthetic oligonucleotide primer has annealed to the template. The reaction is performed in the presence of dNTPs (one of which has been radioactively labelled) and the chain terminating nucleotide analogs, the 2', 3'-dideoxynucleoside 5'-triphosphates (ddNTPs). The ddNTPs (unlike the dNTPs) do not contain the 3'OH group necessary for chain elongation. If the correct ratio of ddNTP : dNTP is chosen, a series of labelled strands will result, the lengths of which will vary according to the distance of the terminator base from the initiation site. The resultant labelled fragments are separated on an acrylamide gel, and the pattern of fragments revealed by autoradiography, gives the DNA sequence.

The United States Biochemical Corporation Sequenase Kit was used for all sequencing reactions. This kit utilizes a genetic variant of T7 polymerase, which completely lacks the 3'-5' exonuclease activity of the native, wild-type T7 DNA polymerase. It has been observed that Sequenase gives radioactive bands of more uniform intensity and less background radioactivity than other DNA polymerases, such as E.coli DNA polymerase 1 (Tabor and Richardson, 1987).

Using the Sequenase kit, DNA sequencing was carried out in two stages. The first step, the labelling reaction, involved primer extension using a limited amount of dNTPs and radioactively labelled dATP. During this stage [α-35S]dATP was incorporated into DNA chains which varied randomly in length from several to hundreds of nucleotides. To the 7µl of denatured DNA solution (Section 2.14.i) was added 2µl of 5 x Sequenase buffer (200mM HCl pH 7.5, 100mM MgCl2, 250mM NaCl) and 1µl of the oligonucleotide primer solution (at 1ng/µl). The primer was annealed to the template at 37°C for 30 minutes. 1µl of 0.1M dithiothreitol and 2µl of appropriately diluted labelling mixture (7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP) were then added. When reading sequences very close to the primer (30-100 nucleotides), the labelling mixture was diluted 1/15 in sterile water. For reading up to 300-400 nucleotides the labelling mixture was diluted 1 in 5 in sterile water. Immediately after the addition of labelling mix 1µl of [α-35S]dATP (at >1000 Ci/mmol, from Amersham) was added to the labelling reaction. Immediately before use, the Sequenase Version 2 enzyme was diluted in ice-cold TE buffer at a ratio of 1 : 7. 2µl of diluted sequenase was added the contents of the sequencing reaction tube, and the contents were mixed by pipetting up and down. The labelling reaction was allowed to proceed for 2-5 minutes at room temperature. When reading sequences very close to the primer (30-100 nucleotides) the labelling reactions
were allowed to proceed for 2 minutes. For 300-400 nucleotides of sequence the labelling reactions were allowed to proceed for 5 minutes.

The second stage of the sequencing process, the termination reaction, involved the continued chain extension in the presence of increased dNTPs and a ddNTP. In this stage the chains were only extended on the average only several dozen nucleotides, before being terminated with the incorporation of a ddNTP. 2.5\mu l aliquots of ddATP, ddCTP, ddGTP, and ddTTT termination mixes were dispensed into 4 tubes labelled A, C, G, and T respectively. Each termination mixture contained dATP, dCTP, dGTP, and dTTP (each at 80\mu M) and 50mM NaCl. Each termination mix also contained the appropriate dideoxynucleotide at 8\mu M. For example, the ddATP termination mix contained 8\mu M ddATP. 3.5\mu l of the labelling reaction was then added to each of tubes A, C, G and T, and the contents of the tube were mixed by pipetting up and down. The termination reaction was allowed to proceed for 2-5 minutes at 37°C. When reading sequences very close to the primer (30-100 nucleotides) the termination reactions were allowed to proceed for 2 minutes. For 300-400 nucleotides of sequence the labelling reactions were allowed to proceed for 5 minutes. The reactions were stopped by adding 4\mu l of stop solution (95% formamide, 20mM EDTA, 0.05% Bromophenol blue, 0.05% xylene cyanol FF). The samples were either stored at -20°C until required or used immediately. Before use, the samples were boiled for 2-3 minutes and loaded onto a 6% acrylamide gel. 6% acrylamide was made by mixing 21g urea, 5ml of 10 x TBE, 7.5ml of 40% acrylamide (38% acrylamide/25% N, N'-methylene-bisacrylamide) and distilled water to a total volume of 50ml. 400\mu l of 10% ammonium persulphate and 70\mu l of TEMED were added immediately prior to pouring the gel. The gel was run in 0.5 x TBE (10 x TBE: 108g Tris, 55g Boric acid and 9.5g EDTA per litre of distilled water) at 35 Watts for 2-4 hours. The sequencing gel plates were then separated and the plate to which the gel was stuck was immersed in 10% acetic acid/12% methanol for 10 minutes to remove the urea. The gel was then transferred to Whatman 3MM paper and dried down under vacuum at 80°C. The gel was then exposed to Fuji X-ray film overnight at room temperature. Intensifying screens were not necessary. To help eliminate sequencing errors, due to secondary structure, sequencing was performed on both strands of the plasmid DNA. Furthermore, sequencing was also performed using labelling and termination mixes containing dITP instead of dGTP. This substitution has been observed to eliminate the secondary structures which produce gel artefacts (Barnes et al., 1983, Gough and Murray, 1983).

Both DNA and deduced protein sequences were analysed using the sequence-analysis software packages of the University of Wisconsin Genetic Computer Group (Devereux et al., 1984)
2.15. The Expression of $\alpha$-Actinin Fragments in *E. coli* using the Prokaryotic Expression Vector pGEX-1.

Fusion proteins, containing fragments of $\alpha$-actinin fused to glutathione-S-transferase, were prepared using the bacterial expression vector pGEX-1 (Smith and Johnson, 1988). The fusion proteins were purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilised glutathione.

Complementary DNA fragments spanning both EF-hands of chick brain and smooth muscle $\alpha$-actinins were prepared by polymerase chain reaction (PCR)-amplification (Sambrook *et al.*, 1989). This technique involves the use of successive rounds of DNA replication (usually 30-35 cycles), using oligonucleotide primers designed against short stretches of sequence derived from the coding and non-coding strands of the DNA of interest. This technique utilises thermostable DNA polymerase (Taq polymerase), derived from *Thermus aquaticus*, which can withstand repeated exposure to high temperatures (94-95°C) required for strand separation (Sambrook *et al.*, 1989). With each cycle of DNA replication the number of DNA strands increases exponentially, and this technique can be used to amplify DNA fragments by at least $10^5$ fold. This technique is therefore useful for obtaining manageable quantities of a specific DNA fragment from sources where DNA is scarce. When designing cDNA fragments for expression studies, PCR is particularly useful in that the synthetic primers can be designed against any region of interest. Furthermore, a convenient restriction site can be inserted for cloning purposes (see below). This can be particularly useful when the amplified fragment is to be cloned into a vector with few cloning sites. In this experiment cDNA fragments were obtained by PCR-amplification using the primers: EF1, 5'-CTACACCATGGATCCTATTCGTGTGGGCTGGGAGC-3' and EF2, 5'-GGGAGGGTTGCGTGGGAGGATCCAAAGTTAAAG-3'. EF1 is almost identical with nucleotides 2238 to 2272 in the sense strand of the complete chick smooth muscle $\alpha$-actinin cDNA (Baron *et al.*, 1987b) and nucleotides 2142 to 2176 in the complete chick brain $\alpha$-actinin sequence (Chapter 4). EF-1 therefore primes in the region encoding the end of the fourth spectrin-like repeat. Two basepair mismatches were included in the EF-1 primer sequence (at nucleotides: 13 (G to T) and 15 (A to C)), so as to introduce a Bam HI site between nucleotides 10 to 15 (GGATCC). EF2 is almost identical with the antisense of nucleotides 2776 and 2811 in the complete chick smooth muscle $\alpha$-actinin cDNA (Baron *et al.*, 1987b) and the antisense of nucleotides 2695 to 2730 in the complete chick brain $\alpha$-actinin cDNA sequence (Chapter 4). EF-2, therefore, primes in the region encoding the stop codon of the chick brain and smooth muscle $\alpha$-actinin cDNAs. 6 mismatches were included in the EF-2 primer sequence between nucleotides 21-26 (changing the sequence from 5'-CAGAAA-3' in the
antisense sequence of the complete chick brain and smooth muscle α–actinin cDNAs to
5'-GGATCC-3' in EF-2) to introduce a Bam HI site at this position.

The chick brain and chick smooth muscle α–actinin cDNAs (C17 and 7a/9a respectively, Baron et al., 1987b and Chapter 4) in Bluescript SK were prepared for amplification by equilibrium centrifugation in CsCl-ethidium bromide gradients. When setting up the PCR-reaction, extreme care was exercised at all times to ensure that no extraneous material, that could act as a source of amplifyable DNA, came into contact with the stock reagents or the interior of the reaction vessels. Pipette tips and microfuge tubes were used direct from the suppliers' bags, and gloves were worn at all times. The reaction mixes was set up in 0.5ml polypropylene microfuge tubes as shown in Table 2.4. The surface of the reaction mixture was sealed with 2 drops of liquid paraffin to prevent evaporation. The tubes were placed in the PCR machine (Perkin Elmer Cetus) and the heating/cooling cycles were set as follows: denaturation, 94°C for 1.5 minutes; annealing, 65°C for 2.5 minutes; extension, 72°C for 2 minutes. The machine was set for 30 cycles, plus an additional step of 72°C for 10 minutes to inactivate the enzyme at the end of cycling. The tubes were then left to cool to 4°C in the PCR machine (usually overnight). The bulk of the liquid paraffin was removed by pipette, and the remainder by extraction with an equal volume of ether.

Table 2.4 The Composition of the PCR Reaction Mixtures

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled water</td>
<td>16μl</td>
<td>- - - -</td>
</tr>
<tr>
<td>10 x PCR buffer</td>
<td>5μl</td>
<td>10mM Tris (pH 8.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50mM KCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5mM MgCl₂</td>
</tr>
<tr>
<td>dA, dC, dG and dTTP mix (each at 1.25mM)</td>
<td>8μl</td>
<td>Each at 200μM</td>
</tr>
<tr>
<td>Primer EF-1 (2μM)</td>
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<td>0.2μM</td>
</tr>
<tr>
<td>Primer EF-2 (2μM)</td>
<td>5μl</td>
<td>0.2μM</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>10μl</td>
<td>1.5 units per 50μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1μl</td>
<td>1ng/50μl</td>
</tr>
<tr>
<td>Total mix</td>
<td>50μl</td>
<td></td>
</tr>
</tbody>
</table>
The amplified DNA was digested with Bam HI (Section 2.12.iii) and separated on a 1% LMP-agarose gel (see Section 2.12.ix). The digested DNA was gel purified (see Section 2.12.x) and cloned into the plasmid pGEX-1 (see Sections 2.12.iv-viii). The pGEX-1 constructs containing the EF-hand containing fragments of either chick brain or chick smooth muscle α–actinins were referred to as EF-brain or EF-smooth respectively.

The bacterial transformants containing recombinant plasmids EF-brain, EF-smooth or the control plasmid pGEX-1 (negative control) were used to inoculate 5mls of 2 x TY containing 50 μg/ml ampicillin. The cultures were grown overnight at 37°C with shaking. The overnight cultures were diluted tenfold in 2 x TY, containing 50 μg/ml ampicillin. The diluted cultures were grown at 37°C, with shaking, until the optical density had reached 0.5-0.8 (mid-log phase). The cultures were then induced by adding 1M IPTG to a final concentration of 1mM. Following a 1-2hr incubation at 37°C, the induced cultures were pelleted at 4,000 rpm in a MSE ChilSpin. The supernatants were discarded and the pellets were re-suspended in 1/50 to 1/100 of the culture volume in MTPBS (150mM NaCl, 12mM NaH₂PO₄/Na₂HPO₄ [pH 7.3]). Triton X-100 (BDH chemicals) and PMSF were then added to final concentrations of 1% w/v and 0.1mM respectively. The cells were vortexed briefly, and sonicated until a noticeable change in colour had occurred (approximately 45 seconds). The insoluble fraction was pelleted in a high speed microfuge for 5 minutes. The supernatant was added to an equal volume of glutathione/agarose beads (sulphur linkage, Sigma), and the tube was inverted several times to mix the contents. The tubes containing the beads were left for 10 minutes at room temperature, with intermittent shaking. 20 volumes of ice-cold MTPBS was added and the tubes were inverted several times to mix the contents. The glutathione/agarose beads were pelleted in a MSE ChilSpin. The supernatants were discarded and the pelleted glutathione/agarose beads were washed twice more in 20 volumes of ice-cold MTPBS. The fusion proteins were eluted from the beads by adding an equal volume of freshly prepared 50mM Tris (pH 8.0) containing 5mM reduced glutathione (Sigma). The tubes were inverted several times to mix the contents, and left at room temperature for 10 minutes. The glutathione/agarose beads were then pelleted in a high speed microfuge for 30 seconds. The supernatants were removed and kept on ice. The glutathione/agarose beads were re-extracted with an equal volume of 50mM Tris (pH 8.0) containing 5mM reduced glutathione (Sigma). The respective supernatants were combined and thoroughly dialysed against 20mM Tris (pH 7.6), 20mM NaCl (with or without 0.5mM DTT (see Chapter 5)). Chick brain and chick smooth muscle α–actinin fusion proteins were produced with similar yield (approximately 2mg of each fusion protein per 250ml of induced culture) and purity (>99% homogeneous by densitometry). The protein solutions were used immediately following dialysis.
2.16 Western Blotting.

5-10 μg aliquots of the protein to be analysed were subjected to SDS-PAGE as described in Section 2.6.ii. The gel was then blotted onto nitrocellulose membrane (Sartorius) at 80V for 2 hours (large gel) or 30 minutes (mini gel) in Transfer buffer (192mM glycine, 12.5mM Tris, 10% methanol). Following transfer, the filter was stained in Ponceau-S (BDH) for 5 minutes with agitation. The filter was de-stained briefly in de-ionised water. The position of the protein was marked with a ballpoint pen. The filter was then thoroughly de-stained in TBS (25mM HCl [pH 7.5], 0.9% NaCl), and incubated in 50ml of blocking buffer (TBS containing 3% Marvel Milk [Cadburys]) for 1 hour at room temperature (or overnight at 4°C). The filter was then transferred into 10ml of blocking buffer containing the appropriately diluted rabbit primary antibody. After a 2 hour incubation at room temperature, the filter was washed 3 times (each wash lasting 3 minutes) in wash buffer (TBS/0.1% Tween 20). After the final wash the filter was transferred into 10ml of blocking buffer containing a 1/7500 dilution of the secondary antibody (goat anti-rabbit antibody with alkaline phosphatase conjugate [Promega]). After a 30 minute incubation at room temperature, the filter was washed, as above, and transferred to 10ml of freshly prepared developing solution (100mM Tris/HCl [pH9.5], 100mM NaCl, 5mM MgCl2) containing 66μl of NBT (100mg/ml in 70% DMF) and 33μl of BCIP (100mg/ml in 100% DMF). The filter was then developed at room temperature, until until the required intensity of staining had been reached. The reaction was then stopped by diluting away the developing solution with large volumes of de-ionised water.

2.17 Expression of Chick α-Actinin cDNAs in Monkey COS Cells.

2.17.1 Transfection

Complete cDNAs encoding chick brain (Chapter 4) or smooth muscle α-actinin (Baron et al., 1987b) were cloned (see Sections 2.12.iv-viii) into the eukaryotic expression vector pECE (Ellis et al.,1986). Closed circular plasmid DNA was then prepared for transfection using the CsCl method (Section 2.13.ii). Monkey COS cells (Gluzman, 1981) were then transfected by Dr. Paul Jackson (Department of Biochemistry, University of Leicester) using the method of Cullen (1987). COS cells were grown in 9cm tissue culture dishes (Nunclon) in DMEM (Dulbeccos Modified Essential Medium from Gibco plc.) supplemented with 10% NCS (heat inactivated Newborn Calf Serum). Actively growing cells were used for transfection. The medium was aspirated from the cells, and each dish was washed with 5ml of serum-free DMEM
at 37°C. The DMEM was aspirated from the dish and the cells were washed with 2ml of Trypsin/Versene solution at 37°C. The solution was aspirated from the dish and 1ml of fresh Trypsin/Versene solution (CellTech) was added to the cells, which were then incubated for 3-5 minutes at 37°C. The cell suspension was collected from the dishes and placed into sterile test tubes. Each dish was rinsed with 3-4ml of DMEM containing 10% NCS, and this was added to the tube containing the cell suspension. The cells were pelleted for 5 minutes at 1-2,000g and the media was aspirated from the pellet. The cells were then evenly re-suspended in 10ml of DMEM containing 10% NCS. The cells were aliquoted at the rate of 1 x 10^6 cells per 9cm dish in 10ml of DMEM containing 10% NCS. If the cells were to be examined by immunofluorescence then sterile glass coverslips were placed in the dish. The cells were then incubated overnight at 37°C in a 5% CO2 humidified atmosphere. The medium was aspirated from the cells and replaced with 10ml of serum-free DMEM at 37°C. The solution was then aspirated from the cells and replaced with a further 10ml of serum free DMEM at 37°C. 20μg of closed circular plasmid DNA was dissolved in 4ml of DMEM/Tris solution (consisting of 20ml of serum free DMEM and 5ml of 0.25M Tris/HCl [pH7.5]). The DNA solution was then mixed with 1ml of freshly prepared DEAE-dextran solution (at 1mg/ml in Tris-buffered Saline: 25mM Tris [pH 7.4], 138mM NaCl, 5mM KCl, 0.7mM CaCl2, 0.5mM MgCl2, and 0.6mM Na2HPO4). The media was aspirated from the cells and 5ml of the DEAE-dextran/DNA complex was added to each dish. The dishes were left for 6-8 hours at 37°C in 5% CO2. The DEAE-dextran/DNA complex was aspirated from the dish and 5ml of 10% DMSO in HBS (HEPES buffered saline) was added. The cells were incubated for 2-5 minutes at 37°C. HBS was made by dissolving 8g of NaCl, 375mg of KCl, 100mg of Na2HPO4, 1.08g glucose, and 5g of HEPES in 1 litre of distilled water, and the pH adjusted to 7.1 with NaOH. The DMSO solution was removed by aspiration, and the cells were rinsed with 10ml of serum-free DMEM. The wash solution was removed and replaced with 10ml of DMEM containing 10% NCS. The cells were incubated for 48 hours at 37°C and fixed for immunofluorescence, or for 24 hours for radiolabelling and immunoprecipitation.

2.17.ii Fixation and Permeabilization of Transfected COS Cells.

COS cells were fixed and permeabilised by Dr. P. Jackson (Department of Biochemistry, University of Leicester). The media was aspirated from the COS cells grown on coverslips (2.17.i), and the cells were washed twice with 5ml of PBS (10g of NaCl, 0.25g of KCl, 1.44g of Na2HPO4, and 0.25g of KH2PO4 per litre of distilled water) at 37°C. The PBS was aspirated from the dishes, and to each dish was added 10ml of 0.5% Triton X-100 in 50mM MES [pH 6.0], 3mM EGTA, and 5mM MgCl2 at
0°C. The cells were left for a maximum of 2 minutes, before the Triton X-100 solution was removed by aspiration. The cells were then washed twice in 5ml of PBS at 37°C. The PBS was removed by aspiration and a solution of 3.7% formaldehyde in PBS was added. The cells were incubated in this fixative solution for 15 minutes at room temperature. The fixative solution was removed and the cells were washed twice in 5ml of PBS at 37°C. The PBS was then completely removed by aspiration.

2.17.iii Immunofluorescence of Fixed and Permeabilised COS Cells.

All immunofluorescence experiments were performed by Dr. P. Jackson (Department of Biochemistry, University of Leicester). Jackson et al. (1988) made an antibody specific for chick α-actinin, by adsorbing a rabbit anti-chick gizzard α-actinin antiserum against fixed and permeabilized monolayer cultures of monkey COS cells. The resulting antibody was used to detect chick α-actinin expressed in COS cells specifically, and not endogenous monkey COS cell α-actinin. The coverslips containing the fixed and permeabilized COS cells (Section 2.17.ii) were transferred to small plastic petri dishes. For the double-labelling of α-actinin and actin, cells were first stained with the adsorbed rabbit anti-chick α-actinin antiserum followed by a Texas red-labelled donkey anti-rabbit serum (Miles). Each coverslip was covered by 100μl of antibody in PBS containing 0.1% BSA, and incubated for 30 minutes at 37°C. Each coverslip was then given 4 brief washes with 1ml aliquots of PBS at 37°C. Before staining the cells were further labelled with nitrobenzoxadiazole (NBD)-phallicidin (Molecular probes, Eugene, OR/USA) to stain F-actin. 50-100μl of NBD-phallicidin solution in PBS was added to the cells on the coverslip, which were then incubated at room temperature for 20 minutes. The cells were given two brief washes with 1ml aliquots of PBS at 37°C. The cells were mounted, cell-side-down, on a drop of glycerol/PBS (50 : 50 or 90 : 10) on a slide. The edges of the coverslip were sealed with clear nail varnish before viewing. Photographs were taken with a Zeiss Axiohot photomicroscope with epifluorescence, using Ilford HP5 film (ASA 400) uprated to 1600 ASA during processing.

2.17.iv Radiolabelling and Immunoprecipitation of Transfected COS Cells.

The radiolabelling and immunoprecipitation of transfected COS cells was performed by Dr. P. Jackson (Department of Biochemistry, University of Leicester). Cells were grown in 9cm dishes for 24 hours after transfection (2.17.i) in 10ml of DMEM containing 10% NCS, and then rinsed twice in 10ml of methionine-free-DMEM. The medium was removed from the cells and replaced with 10ml of methionine-free DMEM containing 10% NCS and 25μCi/ml [35S]-methionine (>800 mCi/mmol,
Amersham International, Bucks. [UK]). The cells were then incubated for 18 hours at 37°C, and the radioactive medium was removed. The cells were then given 3 rapid washes in 5ml of ice-cold PBS. 0.5ml of ice cold RIPA buffer (0.01M Tris/HCl [pH 7.0], 0.15M NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, 2mM EDTA, 50μM sodium orthovanadate, and 2mM PMSF, added fresh in 100% ethanol) was then added to the cells. The cells were scraped into the RIPA buffer, mixed carefully, and left on ice for 10 minutes. The samples were then centrifuged at 13,000g for 15 minutes at 4°C. The supernatant was first pre-extracted with pre-immune serum by adding 5μl of pre-immune serum and incubating at 0°C for 1 hour. 30μl of a 50% suspension of protein A-Sepharose (Sigma Chemicals, St. Louis, MO/USA) in NET-wash buffer (50mM Tris/HCl [pH 7.0], 150mM NaCl, 5.0mM EDTA, 0.1% BSA, 0.1% SDS, 0.5% Nonidet P-40, 0.1% sodium deoxycholate) was then added to the supernatant, which was then incubated for 1 hour at 4°C. The samples were centrifuged at 6,000g for 15s to remove insoluble material. Either 5μl of rabbit pre-immune serum or 20μl of adsorbed rabbit anti-chick α-actinin (see Section 2.17.iii) was added to the supernatant, which was then incubated for 2 hours on ice. 30μl of of the 50% solution of protein A-sepharose was then added to the supernatant for 1 hour on ice. The immune complexes were pelleted at 6,000g for 15s, rinsed 3 times in NET buffer, re-suspended in 100μl of SDS-PAGE sample buffer and analysed by SDS-PAGE (see Section 2.6.ii). The Coomassie stained gel was incubated for 1 hour in 1M sodium salicylate, dried under vacuum, and then exposed to Fuji NR x-ray film for 4 days.
Chapter 3. Isolation, Purification and Characterization of Chicken Brain α-Actinin.
3.1 Introduction.

As detailed in Chapter 1, the fundamental aims of this work were to establish the complete nucleotide sequence and domain structure of chicken non-muscle α-actinin. It was decided that this information would be obtained by following two approaches. The first approach involved sequencing several peptides derived from a chicken non-muscle α-actinin. Primarily, this was done to give us an early indication of the degree of isoform relatedness between the chick smooth and non-muscle α-actinins. The second approach then involved the isolation and sequence analysis of several chicken non-muscle α-actinin cDNAs (Chapter 4). This chapter will cover the data accumulated using the first approach of this strategy, i.e. the isolation of a non-muscle α-actinin and subsequent analysis of its protease cleavage products.

Brain was chosen as the source of non-muscle α-actinin largely because there was a published protocol for the purification of α-actinin from this tissue (Duhaaiman and Bamburg, 1984). In the same study, chicken brain α-actinin was shown to be distinct from the chicken skeletal and chicken smooth muscle isoforms by binding to F-actin in a calcium-sensitive fashion. Furthermore, chicken brain α-actinin was found to give a similar but distinct total amino acid composition and V8-protease cleavage pattern. On the basis of these results, Duhaaiman and Bamburg (1984) suggest that chicken brain α-actinin might not be encoded by the same gene as either the chicken smooth or chicken skeletal muscle isoforms of the protein. The data obtained from the polypeptide mapping and sequence analysis of chicken brain α-actinin in this study will help to clarify this interpretation.

Thermolysin was used to generate the α-actinin polypeptides. This protease cleaves at positions C-terminal to bulky hydrophobic residues, such as: leucine; phenylalanine; isoleucine; valine; methionine; and alanine (Davison et al., 1989). This protease has already been shown to liberate two major polypeptides from α-actinin, which are common to both the non-muscle and muscle isoforms of the protein (Mimura and Asano, 1986). The first is a 53-kDa polypeptide which has been shown to be rod-shaped and dimeric (Mimura and Asano, 1986; Imamura et al., 1989). Limited polypeptide sequence from this smooth muscle fragment has located its N-terminus as Leu$_{267}$ within the complete deduced smooth muscle α-actinin sequence$^{1}$ (Blanchard et al., 1989). This is close to the start of the first spectrin-like repeat at Phe$_{246}$ (Blanchard et al., 1989). Each repeat is predicted to have a molecular weight of approximately

$^{1}$ Numbering is based on the complete deduced amino acid sequence of chick smooth muscle α-actinin (Baron et al., 1987b) and includes the initiating methionine.
Frozen adult chicken brains were homogenised in water containing 0.5mM PMSF.

\[ \text{Centrifugation: 12,000g for 15 minutes} \]

Alpha-actinin was extracted from the pelleted material by stirring in 10 volumes of buffer B for 30 minutes at 22°C.

\[ \text{Centrifugation: 12,000g for 30 minutes} \]

The supernatant was subjected to ammonium sulphate fractionation (25-35% cuts). The 25-35% pellet was resuspended in and exhaustively dialysed against buffer A.

\[ \text{Centrifugation: 24,000g for 20 minutes} \]

The supernatant was applied to a column of DEAE-Cellulose equilibrated in buffer A. The bound proteins were eluted with 150-500mM NaCl.

\[ \text{Alpha-actinin fractions were dialysed against buffer C.} \]

The pooled and dialysed fractions were applied to a column of hydroxylapatite equilibrated in buffer C. The bound proteins were eluted with 50-200mM potassium phosphate.

\[ \text{Alpha-actinin fractions were concentrated down to 0.4ml} \]

The concentrated sample was chromatographed on a Superose 6 FPLC column (Pharmacia) in buffer D.
12-kDa (Davison et al., 1989) and the 53-kDa fragment is therefore large enough to contain all 4 repeats. The second major thermolysin resistant fragment derived from α-actinin is a 27-kDa polypeptide, which has been found to contain the ABD (Mimura and Asano, 1986). In smooth muscle α-actinin this polypeptide has been found to start at residue Leu$_{225}$ within the complete deduced sequence (Blanchard et al., 1989).

Interestingly, other proteases will also liberate polypeptides equivalent to the 53- and 27-kDa released by thermolysin. For example, a 55-kDa polypeptide (starting at Val$_{266}$ within the complete deduced smooth muscle sequence) was generated in partial tryptic digests of chicken smooth muscle α-actinin (Davison et al., 1989). Chymotryptic digests of chicken smooth muscle α-actinin were found to contain a 31-kDa fragment containing the ABD of the molecule, and a 55-kDa fragment, starting at Ala$_{268}$ within the first repeat of the smooth muscle sequence (Imamura et al., 1989). This suggests that the actin-binding and repeat domains of α-actinin form fairly stable structures which are relatively resistant to protease activity.

With these considerations in mind, it was assumed that thermolytic digests of purified chicken brain α-actinin would also produce fragments of similar size to those liberated from the muscle α-actinins. N-terminal sequence of these brain α-actinin fragments would therefore enable a direct sequence comparison to be made with the muscle α-actinins, giving an immediate indication of the degree of similarity between these isoforms. It was decided that the polypeptides would be separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. These polypeptides would then be sequenced directly from the PVDF-membrane (Matsudaira et al., 1987), using an Applied Biosystems 470A sequencer. This strategy was adopted to obviate the need for the purification of polypeptides by FPLC or HPLC.

### 3.2 The Isolation of Chicken Brain α-Actinin.

α-Actinin was purified from freshly frozen adult chicken brains using a slightly modified version (Figure 3.1) of the protocol presented by Duhaiman and Bamburg (1984). The modifications were as follows:

1. Duhaiman and Bamburg (1984) recommended a 0-50% ammonium sulphate cut following the low ionic strength extraction. However, we found that the ammonium sulphate fractionation range had to be narrowed down (25-35% cuts) in order to achieve preparations which were of sufficient purity for our requirements. This amendment was based upon a step used in the purification of rat liver α-actinin (Kuo et al., 1982).

2. To improve the purification of α-actinin from the contaminating proteins, the gradients used in the ion-exchange chromatography steps (DEAE-cellulose and
Figure 3.2 Stages in the Purification of Chicken Brain α-Actinin.

α-Actinin was purified from 50g of freshly frozen chicken brains using the procedure described in Chapter 2.5.i. The samples shown are as follows: lane A, contained a 100μl aliquot (derived from a total 31ml) of the chicken brain extract obtained after Day 1 of the purification procedure; lane B, contained a 100μl aliquot (derived from a total of 30ml) of the pooled DEAE-cellulose fractions; lane C, contained a 100μl aliquot (derived from a total of 28ml) of the pooled hydroxylapatite fractions; and Lane D, contained a 50μl aliquot (derived from a total of 3ml) of the pooled Superose-6 fractions. The samples were subjected to SDS-PAGE (7% acrylamide) as described in Section 2.6.ii. The numbers along the left hand side of the figure indicate the sizes of the molecular weight markers in kDa.
hydroxylapatite) were narrowed down to concentrate on the \( \alpha \)-actinin containing fractions (see Chapter 2).

3. The material eluted from the hydroxylapatite column was subjected to gel filtration chromatography as described by Duhaiman and Bamburg, 1984. However, in order to obtain a better resolution (and a smaller elution volume) an FPLC apparatus Superose 6 column (Pharmacia) was used instead of conventional Sepharose CL-6B. Superose 6 is a cross-linked agarose based medium, with an optimal separation range of 5\( \times 10^3 \)-5\( \times 10^6 \) Daltons.

This protocol takes approximately 4 days to complete, and Figure 3.2 shows the relative degree of purity after each day of a typical purification. Lane A shows the level of purification of the protein following the initial extractions (i.e. the homogenization of the tissue in water containing 0.5mM PMSF, the low ionic strength extraction, and the ammonium sulphate fractionation). Brain \( \alpha \)-actinin could be detected at this stage using SDS-PAGE, but it only represented a minor proportion of the total protein. The major contaminants at this stage, included polypeptides at approximately 45-kDa (possibly actin), 130-kDa and 180-kDa. DEAE-chromatography (Figure 3.2, lane B) did not remove these major contaminant proteins completely, but brain \( \alpha \)-actinin was enriched significantly during this stage. Hydroxylapatite chromatography resulted in the near complete removal of the remaining contaminating proteins (Figure 3.2, lane C). However, a trace of the 45-kDa contaminant protein could still be detected at this stage in some preparations. Subsequent gel filtration chromatography on a Superose 6 (FPLC) column was found to give homogeneous brain \( \alpha \)-actinin (Figure 3.2 (lane d) and Figure 3.3). Densitometric analysis of the stained gels indicated that the homogeneity of the protein was greater than 99%.

The yield of \( \alpha \)-actinin obtained using this protocol was 0.2-0.3mg/50g wet weight of tissue. This yield is far lower than the 2mg/50g wet weight of tissue obtained by Duhaiman and Bamburg (1984). My preparations were probably of lower yield because a narrower ammonium sulphate fractionation range (25-35%) had to be used to obtain protein of sufficient purity for our requirements.

3.3 Characterization of Chicken Brain \( \alpha \)-Actinin.

3.3.1 Peptide Mapping.

As part of the characterization of the purified chicken brain \( \alpha \)-actinin we decided to compare its proteolytic cleavage patterns with those of the smooth and skeletal muscle isoforms. Chicken brain, smooth and skeletal muscle \( \alpha \)-actinins were digested with thermolysin (at a 1 : 50 w/w ratio of protease to protein) for varying times, and the
**Figure 3.3 Superose-6 Chromatography of Chicken Brain α-Actinin.**

α-Actinin was purified from 50g of freshly frozen chicken brains using the procedure described in Section 2.5.i. The concentrated fractions from the hydroxylapatite column were clarified by centrifugation at 13,000g for 10 minutes. The supernatant was then applied to a FPLC Superose-6 column (Pharmacia) equilibrated in 20mM Tris (pH 7.6), 20mM NaCl, 1mM EDTA, 0.5mM DTT, 0.5mM PMSF and 0.5mM NaN3. The column was run at a flow rate of 0.3ml/min, and 0.4ml fractions were collected. 50μl aliquots of each fraction were analysed by SDS-PAGE (8% acrylamide) as described in Section 2.6.ii. The inset shows the α–actinin containing fractions (fractions 33 to 37). The numbers along the right hand side of the inset indicate the sizes of the molecular weight markers in kDa.
cleavage products were analysed by SDS-PAGE (Figures 3.4 and 3.5). All three isoforms generated a similar pattern of polypeptides in the molecular weight range 27-32-kDa, a major polypeptide of approximately 53-kDa, and a cluster of polypeptides between 32- and 53-kDa. This suggested that the three major isoforms of α-actinin might contain similar domain structures. However, when the pattern of α-actinin proteolytic fragments were examined in more detail a number of clear differences were also observed. Firstly, there were a greater number of high molecular weight polypeptides (53-103-kDa) in the brain α-actinin digest at any one time point. One of these high molecular weight brain polypeptides (64-kDa) was particularly prominent and retained its intensity throughout the time course of digestion (Figure 3.5). This polypeptide was hardly evident in the digests of either smooth or skeletal muscle α-actinin (Figures 3.4 and 3.5). Secondly, the major polypeptide produced by the smooth muscle and skeletal α-actinin was a 53-kDa polypeptide. However, brain α-actinin was found to give a 51-kDa peptide in addition to the 53-kDa peptide. Unlike the 53-kDa fragment of smooth muscle α-actinin, both the 51- and 53-kDa fragments of brain α-actinin were found to retain their intensity during the time course of digestion (Figure 3.5). Thirdly, all 3 isoforms generated a cluster of polypeptides of between 32- and 53-kDa, but the brain α-actinin polypeptides were smaller than those produced in digests of chicken smooth or chicken skeletal muscle α-actinin (Figures 3.4 and 3.5). Finally, the chicken brain and chicken smooth α-actinins were each found to produce fragments of 27-, 29-, and 32-kDa, but the rates at which these fragments accumulated were different for each isoform (Figure 3.5). The skeletal isoform did not produce a 32-kDa fragment (Figure 3.4), but one of slightly lower molecular weight (31-kDa).

3.3.ii Peptide Sequencing.

Thermolytic fragments derived from chicken brain α-actinin were separated by SDS-PAGE and electro-blotted onto PVDF-membrane (Matsudaira, 1987). The 64-, 53-, 51-, 32-, 29-, and 27-kDa polypeptides were then sequenced directly from PVDF-membrane by Dr. M. D. Davison (Department of Biochemistry, University of Leicester) using a gas phase amino acid sequencer (Applied Biosystems, model 470A). Two stretches of contiguous amino acid sequence were generated (Figures 3.6 and 3.7), and compared with the complete deduced sequence of chicken smooth muscle α-actinin (Baron et al., 1987b).

The first stretch (A) was obtained from polypeptides derived from the N-terminal ABD (32-, 29-, and 27-kDa), and the second stretch (B) was obtained from polypeptides derived from the central domain containing the spectrin-like repeats (64-, 53-,
Figure 3.4 Thermolysin Digests of the Chicken Brain, Chicken Skeletal Muscle, and Chicken Smooth Muscle $\alpha$-Actinins.

The $\alpha$-actinins were purified as described in Chapter 2.5 and dialysed thoroughly against 100mM ammonium bicarbonate, 5mM CaCl$_2$ (pH 7.6) at 4°C. Lanes a, c and e show an aliquot of the intact purified chicken smooth muscle, chicken brain and chicken skeletal muscle $\alpha$-actinins respectively. Lanes b, d and f show thermolysin digests of the purified chicken smooth muscle, chicken brain and chicken skeletal muscle $\alpha$-actinins respectively. Thermolysin (Sigma) was activated by diluting a 20mg/ml stock of the protease tenfold in 1mM CaCl$_2$. 30$\mu$g of each $\alpha$-actinin (at 1mg/ml) was digested with thermolysin (1 : 50 ratio w/w of protease to protein) at 37°C for 90 minutes. The samples were subjected to SDS-PAGE (10-17.5% acrylamide) as described in Chapter 2.6.ii. The numbers along the left hand side of the figure indicate fragment sizes in kDa. (Th) indicates the position of thermolysin. For clarity, the words SMOOTH, BRAIN, and SKELETAL are written above those lanes containing chicken smooth muscle, chicken brain and chicken skeletal muscle $\alpha$-actinins respectively.
53-, and 51-kDa). The actual numbers of amino acids sequenced per polypeptide is summarised in Table 3.1.

The 64-, 53-, and 51-kDa thermolytic fragments of chick brain α-actinin fragments were found to contain the same N-terminal residue (aligning with Leu267 in the complete deduced sequence of chick smooth muscle α-actinin). These fragments might therefore show a precursor-product relationship, whereby the 64-kDa fragment is cleaved at its C-terminal end to give the 53-kDa fragment (Figure 3.7). The 53-kDa fragment might then be cleaved to give the 51-kDa fragment. Similarly, the 29-kDa fragment (starting at Met14 within the complete deduced smooth muscle α-actinin sequence) might be a precursor of the 27-kDa peptide (which starts at Leu25). The chicken smooth muscle α-actinin 32-kDa fragment is refractory to sequencing, as is the intact smooth muscle α-actinin (Davison et al., 1989), suggesting that the 32-kDa fragment contains the N-terminus of the protein. This might also be the case for chicken brain α-actinin, because the 32-kDa fragment of this isoform was also found to be refractory to sequencing. All of the brain polypeptides sequenced at their N-terminal end were found to display total identity with the smooth muscle α-actinin sequence (Figure 3.6). This suggests that the amino acid sequence of chicken brain α-actinin may be completely identical with the smooth muscle isoform in the region of the ABD and the repeats.

Table 3.1 The Number of Amino Acid Residues Sequenced in Each of the Major Thermolytic Polypeptides Derived from Chicken Brain α-Actinin.

<table>
<thead>
<tr>
<th>Stretch.</th>
<th>Thermolytic peptide (kDa)</th>
<th>Number of residues sequenced.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stretch A.</td>
<td>32</td>
<td>R*1</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>33</td>
</tr>
<tr>
<td>Stretch B.</td>
<td>64</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>5</td>
</tr>
</tbody>
</table>

*R*1 - Polypeptide proved refractory to sequencing.
The purified α-actinins were dialysed thoroughly against 100mM ammonium bicarbonate, 5mM CaCl₂ (pH 7.6) at 4°C. Thermolysin (Sigma) was activated by diluting a 20mg/ml stock of the protease tenfold in 1mM CaCl₂. 300μg of each α-actinin (at 1mg/ml) was digested with thermolysin (at a 1:50 w/w ratio of protease to protein). Digestion was allowed to proceed at 37°C, and at the time points indicated (in minutes) at the top of the figure, aliquots of the digest (50μl) were removed and the reaction was stopped by the addition of EDTA to a final concentration of 10mM. The samples were then subjected to SDS-PAGE (10-17.5% acrylamide) as described in Chapter 2.6.ii. The numbers along both sides of the Figure indicate fragment sizes in kDa. (Th) indicates the position of thermolysin. For clarity, the words SMOOTH and BRAIN are written above those lanes containing chicken smooth muscle and chicken brain α-actinin respectively.
Figure 3.8 shows the sequence of four stretches of chicken skeletal muscle α-actinin sequence, aligned with the deduced sequence of chicken smooth muscle α-actinin (Drs. A. D. Blanchard and M. D. Davison, Department of Biochemistry, University of Leicester, unpublished data). These sequences were obtained using a similar strategy to that described for chicken brain α-actinin. Stretches A and B were derived from the ABD of the protein, whereas stretches C and D were derived from the repeat domain. The chicken skeletal muscle and smooth muscle α-actinins are particularly divergent at the extreme N-terminal end of the molecule. Indeed, the first 9 residues of the skeletal muscle 31-kDa fragment are totally distinct from the smooth muscle sequence. Outside of this region these isoforms were only found to be divergent at 9 amino acid positions scattered throughout the polypeptides sequenced (Figure 3.8).

Thermolytic peptides derived from the C-terminal domain (containing the EF-hands) of chicken brain and skeletal α-actinin were not identified using this approach.

3.4 Discussion.

α-Actinin was first isolated from chicken brain by Duhaiman and Bamburg (1984). It was found to be distinct from the chicken skeletal and chicken smooth muscle α-actinins in several respects. For example, it was found to contain a similar but distinct total amino acid composition and V8-protease cleavage map. Furthermore, in contrast to the chicken skeletal and smooth muscle isoforms chicken brain α-actinin was found to bind to F-actin in a calcium-sensitive fashion (Duhaiman and Bamburg, 1984). On the basis of these observations it was suggested that the chicken brain isoform might be the product of a unique gene (Duhaiman and Bamburg). In an attempt to further clarify the relationship between the three major isoforms of α-actinin, I purified chicken brain α-actinin to homogeneity and compared its thermolytic cleavage products with the chicken smooth and chicken skeletal muscle isoforms.

The brain protein was purified using a modified version of the protocol presented by Duhaiman and Bamburg, 1984 (see Chapter 2). The modifications to the protocol had to be made in order to obtain α-actinin of sufficient purity for our purposes. Considering the nature of these modifications, it would seem unlikely that the α-actinin purified using this protocol should differ in activity from the α-actinin prepared by Duhaiman and Bamburg, 1984. However, our yield of brain α-actinin (0.2-0.3mg per 50g of wet tissue) was far lower than that obtained by Duhaiman and Bamburg (1984). This may be because a narrower range of ammonium sulphate fractionation (25-35%) was used in our protocol. Indeed, at an equivalent stage in the preparation of chicken skeletal muscle α-actinin a large proportion of the α-actinin is
Figure 3.6 Alignment of the Limited Polypeptide Sequence of Chicken Brain α-Actinin with the Complete Deduced Sequence of Chicken Smooth Muscle α-Actinin.

Purified chicken brain α-actinin (100μg) was digested with thermolysin (at a 1:50 w/w ratio of protease to protein) as described in Chapter 2.7. The polypeptides were then separated by SDS-PAGE (10-17.5% acrylamide) as described in Chapter 2.6.11, and transferred to polyvinylidene difluoride (PVDF) membrane, essentially as described by Matsudaira, 1987. Chicken brain α-actinin polypeptides (64-, 53-, 51-, 32-, 29-, and 27-kDa) were sequenced directly from PVDF-membrane by Dr. Mathew D. Davison (Department of Biochemistry, University of Leicester) using an Applied Biosystems 470A gas phase sequencer. Two stretches of contiguous sequence were generated and aligned with the complete deduced sequence of chicken smooth muscle α-actinin (Baron et al., 1987b). The residue number within the complete deduced smooth muscle α-actinin sequence is indicated below each alignment. The arrows mark the N-termini of the polypeptides sequenced. The molecular weight of the fragment sequenced (Da) is indicated above each arrow.

A.

29k.

CBr. Met-Gln-Pro-Glu-Glu-Asp-Trp-Asp-Arg-Asp-Leu-Leu-Leu-Asp-Pro-Ala-
CSm. Met-Gln-Pro-Glu-Glu-Asp-Trp-Asp-Arg-Asp-Leu-Leu-Leu-Asp-Pro-Ala-
14

CBr. Trp-Glu-Lys-Gln-Gln-Arg-Lys-Thr-Phe-Thr-Ala-Trp-Cys-Asn-Ser-His-
CSm. Trp-Glu-Lys-Gln-Gln-Arg-Lys-Thr-Phe-Thr-Ala-Trp-Cys-Asn-Ser-His-
30

CBr. Leu-Arg-Lys-Ala-Gly-Thr-Gln-Ile-Glu-Asn-Ile-Glu-
CSm. Leu-Arg-Lys-Ala-Gly-Thr-Gln-Ile-Glu-Asn-Ile-Glu-
46

B.

64, 53, and 51k.

CBr. Leu-Ala-Val-Asn-Gln-Glu-Asn-Glu-Gln-Leu-Met-Glu-Asp-Tyr-Glu-Lys-
CSm. Leu-Ala-Val-Asn-Gln-Glu-Asn-Glu-Gln-Leu-Met-Glu-Asp-Tyr-Glu-Lys-
267

CBr. Leu-Ala-Ser-Asp-Leu-Leu-Glu-Trp-Ile-
CSm. Leu-Ala-Ser-Asp-Leu-Leu-Glu-Trp-Ile-
283

27k.

29
lost during a 16-24% ammonium sulphate fractionation (Andrew D. Blanchard, University of Leicester, personal communication).

Chicken brain, smooth and skeletal muscle α-actinins were digested with thermolysin and analysed by SDS-PAGE (Figures 3.4 and 3.5). All three isoforms of α–actinin were found to produce similar, but distinct, one dimensional peptide maps. The similarities observed suggest that all three α–actinins may possess a similar domain structure. However, the observed differences in peptide maps indicated that there are also likely to be regions of amino acid sequence divergence. The regions of amino acid sequence divergence which occur may contribute to the observed differences in peptide cleavage profiles, either directly by creating (or eliminating) proteolytic cleavage sites, or indirectly by inducing a change in conformation which affects the degree of exposure of given cleavages sites. Either way, this data supports the results of Duhamain of Bamburg (1984), in that chicken brain α-actinin appears to have a distinct structure from the chicken muscle α-actinins.

In order to establish the precise degree of sequence similarity between the brain and smooth muscle isoforms of α–actinin, we obtained N-terminal sequence from 5 thermolytic fragments derived from the brain isoform, consisting of a total of 69 amino acids (Figures 3.6 and 3.7; and Table 1). This sequence represented approximately 8% of the total brain α–actinin sequence, and was derived from the ABD and the first of the spectrin-like repeats of brain α-actinin. All of the brain polypeptides were found to display total identity with the smooth muscle α-actinin sequence (Figure 3.6). This data is consistent the non-muscle/smooth muscle α-actinin splicing model described in Chapter 1. The actin-binding and repeat domains of the smooth muscle and non-muscle α-actinins may be identical in sequence and encoded by the same exons. However, these isoforms differ in their sensitivity to calcium (Duhamain and Bamburg, 1984), and may contain distinct EF-hand domains. If this were the case then the region(s) of divergence between the non-muscle and smooth muscle α-actinins would be confined to the C-terminal regions of the molecules. The 64-, 53-, and 51-kDa thermolytic fragments of brain α-actinin all have the same N-terminus (Leu267). The 64-kDa polypeptide is large enough to extend to the EF-hand domain, and it must be cleaved within this domain to yield the 53- and 51-kDa fragments. The relative resistance of the brain 64-kDa polypeptide to further proteolysis, when compared with that of the smooth muscle isoform, suggests that brain and smooth muscle α-actinin must differ in the EF-hand region of the protein. Thermolytic polypeptides derived from the EF-hand domain of chicken brain α-actinin were not identified during this analysis. Indeed, these polypeptides have never been identified on SDS-PAGE gels, regardless of the source of α-actinin or the protease used (Davison et al., 1989; Imamura et al., 1989; Blanchard and Davison, unpublished data; and also this Chapter). The C-terminal domain may be
3.7 The Major Thermolytic Digestion Products of Chicken Brain \( \alpha \)-Actinin.

Polypeptide mapping and sequence analysis has revealed that thermolytic polypeptides are liberated from two major sites within chick brain \( \alpha \)-actinin molecule. The smallest polypeptides (27-, 29-, and 32-kDa fragments) were derived from the N-terminal ABD of \( \alpha \)-actinin. The 51-, 53-, and 64-kDa fragments were derived from the central domain containing the spectrin-like repeats. The overall domain structure of chicken brain \( \alpha \)-actinin (shown at the top of the Figure) is based upon the complete deduced sequence of chick brain \( \alpha \)-actinin (see Chapter 4).
extremely sensitive to proteases, and cleaved into fragments which are too small to be resolved by conventional SDS-PAGE. Such peptides might only be identified by FPLC or HPLC. This approach can be fairly time consuming, and so it was decided that this sequence would be obtained by isolating cDNAs encoding chick brain α-actinin (see Chapter 4).

Several thermolytic polypeptides derived from chicken skeletal muscle α-actinin have recently been sequenced using a similar strategy to that just described for brain α-actinin (Blanchard and Davison; unpublished results, see Figure 3.8). These isoforms were found to display a very high level of sequence identity over the regions studied. The only region where there was considerable divergence between these isoforms occurred in the N-terminal region of the molecule. The brain and skeletal isoforms of human dystrophin are also quite divergent at their extreme N-termini (Nudel et al., 1989). It would therefore appear likely that this region in α-actinin and dystrophin is not directly involved in binding to F-actin. The recent publication of a complete cDNA sequence encoding chick skeletal muscle α-actinin (Arimura et al., 1988) has revealed that this isoform is divergent with chick smooth muscle α-actinin at many amino acid positions scattered along the length of the protein. This data strongly suggests that the skeletal muscle and smooth muscle isoforms of α-actinin are the products of two separate genes.
Figure 3.8 Alignment of the Limited Polypeptide Sequence of Chicken Skeletal Muscle α-Actinin with the Complete Deduced Sequence of Chicken Smooth Muscle α-Actinin.

Chicken skeletal muscle thermolytic polypeptides were sequenced by Drs. Andrew D. Blanchard and Mathew Davison (Department of Biochemistry, University of Leicester) using a similar strategy to that described for chicken brain α-actinin. 4 stretches of contiguous sequence were generated (Stretches A, B, C, and D) and aligned with the complete deduced sequence of chicken smooth muscle α-actinin (Baron et al., 1987b). The residue number within the deduced sequence of chicken smooth muscle α-actinin is indicated below each alignment. The arrows mark the N-termini of the polypeptides sequenced. The molecular weight of each fragment (Da) is indicated above each arrow. Regions of amino acid divergence have been boxed. Residues that could not be clearly determined have been denoted X.
Chapter 4. Isolation of cDNAs Encoding the Non-muscle Isoform of α-Actinin.
Figure 4.1 Alignment of the Restriction Maps of Eight Chick Brain α-Actinin cDNAs with Two Chick Fibroblast α-Actinin cDNAs.

Restriction maps of: (A) the complete smooth muscle α-actinin cDNA, C17, isolated from a chick fibroblast λgt11 cDNA library by Baron et al. (1987b); (B) a putative non-muscle α-actinin cDNA, C18, isolated from the same chick fibroblast cDNA library by Baron et al. (1987a); (C) the first series of chick brain α-actinin cDNAs, clones 9a, 9b, 9c and 4T isolated during this study using C18 as labelled probe; and (D) the second series of chick brain α-actinin cDNAs, clones 1, 7, 8a, and 8b isolated during this study using the 3' Sma I/Eco R I fragment of C17 as labelled probe. The inset shows a comparison of the deduced amino acid sequences of C17 and C18, in the region of variation (underlined). Residue numbers are indicated. The origin and termination of transcription are represented with arrows (see text). The enzymes are abbreviated as follows: Spe I (Sp); Sst I (Ss); Bam HI (B); Pst I (P); Sma I (Sm); and Dra I (D).
Alignment of the Restriction Maps of the Chick Brain and Chick Fibroblast Alpha-actinin cDNAs

Chick Fibroblast Alpha-actinin cDNAs
A. Complete Chick Fibroblast Alpha-actinin cDNA, C17.

B. Incomplete Chick Fibroblast Alpha-actinin cDNA, C18.

Chick Brain Alpha-actinin cDNAs
A.

B.
4.1 Introduction.

In Chapter 3, I described the purification of chicken brain α-actinin and a characterization of its thermolytic cleavage products. Two stretches of polypeptide sequence were generated, the first derived from the N-terminal ABD, and the second derived from the repeat region of the protein. Both stretches were found to be identical in sequence with the chicken smooth muscle isoform. These results support an alternative splicing model, in which the smooth muscle and non-muscle isoforms of α-actinin are encoded by the same gene. In order to provide more complete evidence in support of this hypothesis it was decided that we should determine the complete cDNA sequence of a chick non-muscle α-actinin.

The polypeptide sequence data suggested that there were likely to be large areas of total sequence identity between chick brain α-actinin and the deduced amino acid sequences of the chick fibroblast cDNAs, C18 (Baron et al., 1987a) and C17 (Baron et al., 1987b). These cDNAs were therefore used to screen two chick embryo brain cDNA libraries. The first library was a kind gift of Dr. Mark G. Darlison (MRC Molecular Neurobiology Unit, Cambridge), and constructed in the vector λgt10 using mRNAs derived from the whole brains of 12-, 14- and 16-day-old chick embryos. The second cDNA library was a kind gift of Dr. V. Wasenius (The University of Helsinki), and constructed in the vector λgt10 using mRNA derived from 14-day-old chick embryo whole brains (Wasenius et al., 1989). In addition, we were interested in determining the level of amino acid sequence identity between non-muscle α-actinins derived from different species. For this reason, I also screened a human placental cDNA library made in the vector λgt11 (Clontech).

It has been proposed that the non-muscle and smooth muscle isoforms of α-actinin may be expressed simultaneously in non-muscle cells, and function at unique sites within the cell (Baron et al., 1987b). To investigate this hypothesis complete cDNAs encoding either chick non-muscle or chick smooth muscle α-actinin were cloned into the eukaryotic expression vector pECE (Ellis et al., 1986). The resultant constructs were then used to transfect monkey (non-muscle) COS cells (Gluzman, 1981; Cullen, 1987). The intracellular localisations of the expressed proteins were then investigated with an antibody specific for chicken α-actinin (characterized by Jackson et al., 1988).
The complete sequence of chick brain α-actinin was determined by sub-cloning several fragments of the clones 9a, 9b and 7a into the vector Bluescript. These fragments were then sequenced on both strands using forward and reverse primer (designed against Bluescript polylinker sequence) and synthetic oligonucleotides (designed against the nucleotide sequence of chick brain α-actinin). The fragments sequenced include: the Eco RI/BamH I fragment of 9a, which was further digested with Eco RV to give 3 smaller fragments (435, 237, and 215bp); the Pst I fragments of clone 9b (710, 514 and 345bp); and the Sma I/Eco RI (996 and 144bp), Hind III/Eco RI(1032 and 108bp), and Dra I/Eco RI (546 and 503bp) fragments of clone 7a. The individual sequence runs are indicated with arrows, and their directions relate to coding (to the right) or non-coding (to the left) sequence. The numbers in brackets refer to the position within the complete nucleotide sequence of chick brain α-actinin (Figure 4.3). The word linker refers to the Eco RI linkers added to the ends of the cDNA during the manufacture of the cDNA library. Internal refers to the natural Eco RI site present at 2329bp within the complete cDNA sequence of chick brain α-actinin (see text).
4.2 Results.

4.2.i The Isolation of α-Actinin cDNAs from Chick Brain cDNA Libraries.

The first chick embryo brain cDNA library was a kind gift from Dr. Mark Darlison (MRC Molecular Neurobiology Unit, Cambridge), and constructed in the vector λgt10 from mRNA derived from the whole brains of 12-, 14-, and 16-day-old chick embryos. Approximately 50,000 plaques derived from this library were screened by Dr. D. R. Critchley (University of Leicester) using a 2.1kb chick fibroblast cDNA probe (called C18: refer to Baron et al., 1987a) using standard procedures (Maniatis et al., 1982). Four positive clones (9a; 9b; 9c; and 4t) were identified and purified to homogeneity (see Figure 4.1). When analysed by agarose gel electrophoresis the inserts derived from clones 9a, 9b, 9c and 4t were found to be approximately 2.3, 1.6, 1.1, and 2.3kb in length respectively. Initially, each insert was sequenced at the 5' and 3' termini after subcloning into the Bluescript plasmid (Stratagene). When compared with the cDNA sequence of smooth muscle α-actinin (Baron et al., 1987b) the two largest of these brain clones, 9a and 4t, were found to cover the same sequence, i.e. nucleotides 97 (within the 5' untranslated sequence) to 2423 (in the region coding for the N-terminal EF-hand). The chick brain library was amplified prior to our receiving it (Mark G. Darlison, personal communication) and so 9a and 4t might have originated from the same original clone. Clone 9b was found to start at nucleotide 855 relative to the smooth muscle cDNA sequence and end at nucleotide 2423. The smallest of these brain clones, 9c, was found to cover nucleotides 1278-2423 relative to the smooth muscle cDNA sequence. Chick brain α-actinin cDNAs extending beyond nucleotide 2423 relative to the smooth muscle cDNA sequence were not obtained using C18 as a probe.

To obtain the remaining 3' sequence of the chick brain α-actinin cDNA, I screened a further 50,000 plaques derived from the same chick brain library, using the 3' Sma I/EcoR I fragment of the chick smooth muscle α-actinin cDNA C17 as probe (Baron et al., 1987b also see Figure 4.1). We did not have any protein sequence from the C-terminal domain of chicken brain α-actinin (Chapter 3), and so there was no guarantee that the 3' Sma I/EcoR I fragment of the chick smooth muscle cDNA would be sufficiently homologous with the equivalent region of the brain α-actinin cDNA for hybridisation to occur. However, I did identify 4 positives using this cDNA fragment as a probe, namely clones 1, 7a, 8a, and 8b (Figure 4.1). Each was found to be approximately 1.1kb in length when analysed by agarose gel electrophoresis. Sequencing of the 5' and 3' ends of these clones showed that each contained the remaining coding
Figure 4.3 The Complete Nucleotide Sequence of Chick Brain α-Actinin Together with its Deduced Amino Acid Sequence.

The numbered line is the chick brain α-actinin nucleotide sequence. The putative initiator methionine starts at nucleotide 19. A stop codon (*) is reached at nucleotide 2698. The translation initiation signal (CCGCCATG) and the polyadenylation signal (ATTAAA) are overlined (Kozak, 1984, and Mclaughlan et al., 1985). The amino acid sequence deduced from this cDNA is shown beneath the numbered line. This sequence is underlined in the regions corresponding to the sequence determined for several chicken brain α-actinin polypeptides (see Chapter 3). The chick brain α-actinin nucleotide sequence was compared with chick smooth muscle isoform (Baron et al., 1987b), using the University of Wisconsin Genetic Computer Group Package (Devereux et al., 1984). Base differences occurring in the smooth muscle α-actinin sequence are shown above the brain α-actinin sequence. Dashes (-) are introduced where there are gaps in the alignment.
T

1201 CACCTGGCAGAGAAGTTCCGGCAGAAAGCATCTATTCACGAGTCCTGGACAGATGGTAAG 1260
HLAEKFRQKAS IHESWTDGK

1261 GAAGCGATGCTGCAGCAGAAGGATTATGAAACTGCTACCCTCTCGGAGATAAAGGCCCTG 1320
EAMLQQKDYETATLSEIKAL

1321 GAAGCGATGCTGCAGCAGAAGGATTATGAAACTGCTACCCTCTCGGAGATAAAGGCCCTG 1380
LKKHEAFESDLAAHQPDRVEQ

1381 ATTTGCTGCTATTGGCAAAAGAGCTGAATGAGCTGGACTATTATGACTCTCCGAGTGTCAAT 1440
AAIAQELNELDYYDSPSVN

1441 ATTTGCTGCTATTGGCAAAAGAGCTGAATGAGCTGGACTATTATGACTCTCCGAGTGTCAAT 1500
ARCMQKICDQWDNLGALTQKR

1501 ATTTGCTGCTATTGGCAAAAGAGCTGAATGAGCTGGACTATTATGACTCTCCGAGTGTCAAT 1560
REALERSEKLLETIDQLYLE

1561 TATGCCAAACAGGCTGCCGCCCTTCAATAACTGGATGGAAGAGGCCCCATGGAGGACCTGCA 1620
YAKRKAAPFNNWMSEGAMEDLQ

1621 GACACGTCATTTGGCAAAAGAGCTGAATGAGCTGGACTATTATGACTCTCCGAGTGTCAAT 1680
DTFIVHTIEEETALHEQ

1681 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 1740
FKATLPDADKERQAILGHN

1741 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 1800
EVSKIVQYTVHVMAGTNPYT

1801 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 1860
TITPQIEINGKWEHVRQQLVPR

1861 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 1920
RDQALMEEHARQQNEALRRLK

1921 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 1980
QPGAGQANVIGPMWIOQTKMEEIC

1981 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 2040
GRISIEMHGLTEDQJNHLRQ

2041 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 2100
YEKSIVNKP IslandLEGDHO

2101 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 2160
QQIQEAFLIDKNKTNYTMHE

2161 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 2220
RVGWEQLLTTTARTINEVEN

2221 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCCCTG 2280
QILTRDAKGGISQEQMQMFRA

AGA GA T T T G A G ATTG A T CG

2281 TTCAAAGGCCACCTTGGAATGACCGAAGAAGGTATCGCCATCTCCGAGATAAAGGCC 2340
SFNHFDRDHSGTLGPPEEPKA

2340
sequence of chick brain α-actinin, extending from nucleotide 2424 relative to the smooth muscle cDNA sequence, to the poly-A tract of the cDNA. The clones 1, 7a, 8a, and 8b were identical in sequence at their 5' end, however at their 3' ends they were distinct in that they contained poly-A tails of different lengths (31-43 nucleotides). This would suggest that each is an independent clone.

The 5' chick brain clones (9a, 9b, 9c and 4t) were each found to end at nucleotide 2423 relative to the complete smooth muscle α-actinin cDNA C17, and the 3' chick brain clones (1, 7a, 8a, and 8b) were each found to begin at nucleotide 2424. This strongly suggests that an internal Eco RI site occurs at this position within the complete sequence of chick brain α-actinin. This was confirmed by the sequence of the 3' end of the gene encoding the non-muscle/smooth muscle isoforms of α-actinin (see Section 4.3) (Waites et al., 1992). The cDNA used to make the Darlison chick embryo brain cDNA library was insufficiently methylated (personal communication, Mark Darlison, MRC Molecular Neurobiology Unit, Cambridge). Internal Eco RI sites within this cDNA were therefore not protected from cleavage with Eco RI during the manufacture of the cDNA library. This may explain why individual cDNAs containing sequence extending in both 5' and 3' directions from the internal Eco RI site of chick brain α-actinin were not isolated during this analysis.

I obtained the complete sequence of chick brain α-actinin using the following strategy (Waites et al., 1992) (Figure 4.2). Firstly, the 5' Eco RV Bam HI fragment (887bp) of the brain α-actinin clone 9a was cloned into Bluescript and sequenced from its 5' and 3' termini. The Eco RV/Bam HI fragment of 9a in Bluescript was then digested with Eco RV to give 3 smaller fragments (435, 237, and 215bp). These smaller fragments were cloned into Bluescript and sequenced on both strands. Secondly, the brain α-actinin clone 9b in Bluescript was digested with Pst I to give 3 smaller fragments (710, 514 and 345bp). These fragments were also subcloned into Bluescript and sequenced on both strands. Thirdly, the Sma I/Eco RI (996 and 144bp), Hind III/Eco RI(1032 and 108bp), and Dra I/Eco RI (546 and 503bp) fragments of brain clone 7a were cloned into Bluescript and sequenced on both strands.

The complete sequence of the chick brain α-actinin cDNA called 7a/9a was 3469bp in length (Figure 4.3). The putative initiating methionine codon at position 19 lies within the sequence CCGCCCATG, which is typical for an active eukaryotic initiation site (Kozak, 1984). Unfortunately, the N-terminus of chick brain α-actinin could not be sequenced (see Chapter 3) and so we were unable to confirm the authenticity of the translation start site directly. The putative initiation codon methionine is followed by a single continuous open reading frame of 2682bp. A stop codon, TAA, is present at position 2698. The complete sequence encodes a protein of 893 amino acids with a deduced molecular mass of 107, 644 Da (excluding the initiating methionine). This is in
Figure 4.4. Alignment of the Deduced Sequences of Chick Brain and Chick Smooth Muscle α-Actinin.

The complete deduced amino acid sequences of chick brain α-actinin (CBA) and chick smooth muscle α-actinin (CSA, Baron et al., 1987b) were aligned using the program Bestfit of the University of Wisconsin Genetic Computer Group (Devereux et al., 1984). The one letter amino acid code is used for clarity, and vertical lines (1) indicate positions of total sequence identity. Positions of amino acid divergence are shown in bold type. The smooth muscle α-actinin sequence is underlined in the regions proposed to contain the ABD and EF-hands. Dots (..) are introduced where there are gaps in the alignment.
close agreement with the estimates of the molecular weight of brain α-actinin deduced by SDS-PAGE (Duhaimean and Bamburg, 1984). The stop codon is followed by 726 bp of 3' untranslated sequence, a polyadenylation signal ATTAAA (McLauchlan et al., 1985), and a poly-A tail of at least 43 nucleotides. The protein sequence deduced from the chick brain α-actinin cDNAs exactly matched the polypeptide sequence derived from the actin-binding and repeat domains of chicken brain α-actinin (Chapter 3).

The cDNA sequence of chick brain α-actinin is almost identical with the sequence of the smooth muscle α-actinin cDNA, C17 (Baron et al., 1987b), except at the following positions:

1. The region encoding the latter half of the N-terminal EF-hand of chick brain α-actinin (residues 762 to 779, see Figure 4.4) was found to be divergent with the corresponding region of the smooth muscle isoform. As a result, the chicken brain and smooth muscle isoforms were found to differ at the Y, Z, -Y, -X and -Z calcium liganding positions of the N-terminal EF-hand (see Figure 4.5).  

2. When compared with the chick smooth muscle sequence the chick brain α-actinin cDNA was found to contain an extra stretch of coding sequence (nucleotides 2367 to 2381, see Figure 4.3) encoding an additional 5 residues between the two EF-hands (residues 784-788, see Figure 4.4 and 4.5).  

3. In addition to the differences mentioned above, there were 10 single bp mismatches distributed throughout the complete chicken brain sequence (nucleotides 261, 482, 834, 1239, 1287, 1341, 1519, 1899, 2004 and 3195, see Figure 4.3). These differences may be attributed to allelic variations, and/or simple misreading by reverse transcriptase during synthesis of the cDNA (Sambrook et al., 1989). The great majority of the single bp mismatches (7 out of 10) were found to occur at the third base of the codon, and caused no variation in amino acid sequence between the isoforms. However, there were two positions where the coding sequence was found to be altered, Cys155 and Ser501, see Figure 4.4.

I also screened a second chick embryo brain cDNA library for α-actinin cDNAs. This library was constructed in the vector λgt10 from mRNA derived from 14-day-old chick embryo whole brains (Wasenius et al., 1989). Approximately 300,000 plaques from this library were screened using the chick brain α-actinin cDNA 7a as labelled probe. A single positively-reactive clone, 8W, was isolated and purified to homogeneity. The insert was found to be approximately 2kb in length. When I sequenced the extreme 5' and 3' terminal ends of 8W in Bluescript, both termini were found to be identical in sequence with the corresponding regions of the chick skeletal α-actinin cDNA isolated by Arimura et al. (1988), extending from nucleotide 908 to the poly-A tail within this sequence (Figure 4.6). The cDNA 8W was then left for Dr. T. Parr (Department of Biochemistry, University of Leicester) for complete sequence analysis. The results
N-terminal EF-hand

<table>
<thead>
<tr>
<th>n</th>
<th>n</th>
<th>nX</th>
<th>Y</th>
<th>Z-Y-X</th>
<th>-Zn</th>
<th>n</th>
<th>n</th>
</tr>
</thead>
</table>

CBR  EFRASFNHFDRDHSGTSLGPEEFKAACLISLGLYGIDNGDAOGGEAFARIMSVDPNRMGVVTQAFIDFMSRE

CSM  EFRASFNHFDRKKTGMDCEDFRACLISMGYNM-----GEAEFARIMSIVDPNRMGVVTQAFIDFMSRE

CSK  DFRASFNHFDRKNGMLDHDDFRACLISMGYDL-----GEAEFARIMSIVDPNGQTGTVTQSFIDFMTRE

HSK1 EFRASFNHFDRRNGLMDHEDFRACLISMGYDL-----GEAEFARIMTLVDPNGQTGTVTQSFIDFMTRE

HSK2 EFRASFNHFDRKRNMMPEDFRACLISMGYDL-----GEVEFARIMTMVDPNAAGVVTQAFIDFMTRE

HPL  EFRASFNHFDRHSGTSLGPEEFKAACLISLGLYGIDNGDPQGEAEFARIMSIVPRLGVVTQAFIDFMSRE

DFA  EFRSSFNHFDKNRTGRLSPEEFKSLVSLGYSIGERQGDLDFQRLAVDPNPNTGYVHFDAFLDFMTRE

DAA  EFKACFSHFDKDNKNLRLEFSSCMLKSIGDEL-----TEEQLNQVISIKIDTDGNTISFEFIDYMVSS

|---|---|

Linker region

Figure 4.5. Alignment of the EF-hand Region of Eight Different α-Actins.

Shown are the amino acid sequences of: (CBR) Chick brain α-actinin (this chapter and Waites et al., 1992); (CSM) Chick smooth muscle α-actinin (Baron et al., 1987b); (CSK) chick skeletal muscle α-actinin (Arimura et al., 1988); (HSK1) human skeletal muscle α-actinin variant HuActSk1 (Beggs et al., 1992); (HSK2) human skeletal muscle α-actinin variant HuActSk2 (Beggs et al., 1992); (HPL) human placental α-actinin (this chapter and Millake et al., 1989); (DFA) Drosophila α-actinin (Fyrborg et al., 1990); (DAA) Dictyostelium α-actinin (Noegel et al., 1987). The one letter amino acid code is used throughout. Pad characters (-) are introduced to accommodate the extra 5 residues between the two EF-hands of CBR, HPL, and DFA α-actins. The positions of the calcium chelating residues of the EF-hand loop are denoted by the letters X, Y, Z, -Y, -X and -Z. The hydrophobic aspects of the helices are located under columns headed n. The region of sequence divergence between the chick brain and chick smooth muscle α-actins is underlined in the brain sequence.
indicate that 8W is identical with the chick skeletal muscle isoform identified by Arimura et al. (1988), except in the region encoding the N-terminal EF-hand and the EF-hand linker peptide (Figure 4.7). The N-terminal EF-hand of the α-actinin encoded by 8W contains an histidine at the -X-vertex (Figure 4.7) and is therefore predicted to be non-functional, as is the C-terminal EF-hand which is identical in amino acid sequence with its chick skeletal muscle counterpart. Unlike the chick skeletal muscle isoform, the variant encoded by 8W contains an EF-hand linker segment of 4 residues (as compared with the 5 residue linker encoded by the cDNA 7a/9a). Chick brain therefore appears to express both smooth muscle- and skeletal muscle-based variants of α-actinin. Complementary DNAs encoding the smooth muscle-based variant of chick brain α-actinin were not isolated from the Wasenius cDNA library during this analysis.

4.2.ii The Isolation of a Human Placental α-Actinin cDNA.

A human placental λgt11 cDNA library (Clontech) was screened with the 3.6kb chick smooth muscle α-actinin cDNA, C17 (Baron et al., 1987b). To take into account the possibility of a low level of conservation between the chick smooth muscle and human non-muscle α-actinin sequences at the DNA level, the hybridization conditions employed in this experiment were of fairly low stringency (3 x SSC at 65°C). The initial stage of screening this library was performed by Mr. Bipin Patel (Department of Biochemistry, University of Leicester) and all subsequent stages were performed by myself. Approximately 150,000 plaques were screened with this probe, and a single positive clone (P6) was identified. The clone P6 was purified to homogeneity, and the insert was found to be approximately 3.0kb in length.

The insert derived from P6 was cloned into Bluescript, and sequenced on both strands using the strategy shown in Figure 4.8. The P6 insert in Bluescript was digested with Bam HI to generate three cDNA fragments of approximately 1.0, 1.1, and 0.9 kb. These fragments were then cloned into Bluescript. The 5' Eco RI/Bam HI and the 3' Bam HI/ Bam HI fragments were initially sequenced at their 5' and 3' termini using forward and reverse primers. The remaining sequence of these fragments was obtained using synthetic oligonucleotides as primers. The central Bam HI/Bam HI fragment in Bluescript was digested with Pst I and the resultant fragments (Bam HI/Pst I and Pst I/Pst I) were cloned into Bluescript. The Bam HI/Pst I and Pst I/Pst I fragments in Bluescript were then given to Dr. Andrew D. Blanchard (Department of Biochemistry, University of Leicester) for sequence analysis.

The complete human placental α-actinin sequence (3022bp) is shown in Figure 4.9 (Millake et al., 1989). The putative initiating methionine, at 199bp, lies within the sequence CCATCATG, which fits the consensus for an active eukaryotic initiation site
Figure 4.6 Alignment of the Partial Chick Brain $\alpha$-Actinin cDNA 8W with the Complete Chick Skeletal $\alpha$-actinin cDNA Pa1.

The partial (2.0kb) chick brain $\alpha$–actinin cDNA 8W is aligned with the complete (3.0kb) chick skeletal $\alpha$–actinin cDNA Pa1 isolated by Arimura et al. (1988). Enzymes are abbreviated as follows: Bam HI (Ba); Bgl II (Bg); Hinc II (H); Pst I (Ps); and Pvu II (Pv). The positions of the initiator methionine (M) and stop codon (S) are also shown.
(Kozak, 1984). Between the initiating methionine and the stop codon (TAA) at nucleotide 2875, there is a single continuous reading frame encoding a protein of 892 amino acids. The predicted molecular weight of human placental α-actinin is 105,365 Daltons (including the initiating methionine). The placental cDNA does not contain either a poly-A adenylation signal or a poly-A tail and so the 3' untranslated sequence is probably incomplete.

The chick brain and human placental α-actinin cDNAs display a striking level of identity across the coding region: 85 and 97% identity at the DNA (Figure 4.9) and protein (Figure 4.10) levels respectively. There are only 400 nucleotide mismatches between these cDNAs across the coding region, and 350 (88%) of these occur at the third nucleotide of the codon. There are in total 24 mismatches at the amino acid level, and most of these are non-conservative substitutions (Figure 4.10, and Table 4.1). Only two substitutions occur within the ABD (Table 4.1), and these are found at the extreme N-terminal end of the domain (Figure 4.10). Most of the amino acid substitutions (16 out of 24) occur in the central domain containing the spectrin-like repeats (Figure 4.10 and Table 4.1). The first EF-hand of the human placental and chick brain α-actinins are identical in sequence at the amino acid level (Figure 4.5). There is a single amino acid mismatch between the C-terminal EF-hand of chick brain α-actinin and the C-terminal EF-hand of human placental α-actinin, and this difference occurs at the Z vertex (Figure 4.5). A leucine is present at this vertex in human placental α-actinin, and a methionine in chick brain α-actinin. There is also a single amino acid mismatch in the EF-hand spacer regions of human placental and chick brain α-actinins (Figure 4.5). An alanine at this site in chick brain α-actinin is replaced with a proline in human placental α-actinin. The sequences of two further human non-muscle (Youssouffian et al., 1990; Nishiyama et al., 1990) and two human skeletal muscle (Beggs et al., 1992) α-actinins have recently been published, and are aligned with human placental α-actinin in Figure 4.11.

Table 4.1 A comparison of the Amino-acid Sequence Identity Between the Deduced Sequences of Human Placental and Chick Brain α-Actinins.

<table>
<thead>
<tr>
<th>Region of the Protein*1</th>
<th>Amino-acid Identity (%)*2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-245 ABD</td>
<td>243/245 (99.2)</td>
</tr>
<tr>
<td>245-712 Central repeats</td>
<td>461/477 (96.6)</td>
</tr>
<tr>
<td>713-892 C-terminal domain</td>
<td>174/180 (96.7)</td>
</tr>
<tr>
<td>750-778 N-terminal EF-hand</td>
<td>29/29 (100)</td>
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<tr>
<td>779-790 EF-hand linker region</td>
<td>11/12 (91.7)</td>
</tr>
<tr>
<td>791-819 C-terminal EF-hand</td>
<td>28/29 (96.6)</td>
</tr>
</tbody>
</table>

*1 Numbering refers to the complete deduced sequence of human placental α-actinin.

*2 Lack of identity is scored for both mismatches and insertion/deletions.
**N-TERMINAL EF-HAND**

<table>
<thead>
<tr>
<th>n</th>
<th>n</th>
<th>nX</th>
<th>Y</th>
<th>Z-X</th>
<th>-Zn</th>
<th>n</th>
<th>n</th>
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</thead>
<tbody>
<tr>
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<td>EFRASFHFDSDKTGMMDCTACSLMGYNM------GEAEFRIMSIVDPMQVENFGVTFQAFIDFMSRE</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SM_{NM}</td>
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**C-TERMINAL EF-HAND**

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<th>Z-X</th>
<th>-Zn</th>
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<th>n</th>
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<tr>
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<tr>
<td>SM_{NM}</td>
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<td>SK</td>
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<tr>
<td>SK_{NM}</td>
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**Figure 4.7. Alignment of the EF-hand Regions of Various α-Actinins.**

Shown are the amino acid sequences of: (SM) chick smooth muscle α-actinin (cDNA C17, Baron et al., 1987b); (SM_{NM}) chick smooth muscle α-actinin-based variant of non-muscle α-actinin (cDNA 7a/9a, Wailes et al., 1992); (SK) chick skeletal muscle α-actinin (cDNA Pa1, Arimura et al., 1988); and (SK_{NM}) chick skeletal muscle α-actinin based variant of non-muscle α-actinin (cDNA 8W, Parr et al., 1992). The one letter amino acid code is used throughout. Pad characters (-) are introduced to accommodate the extra 5 residues between the EF-hands of SM_{NM} and the extra 4 residues between the EF-hands of SK_{NM} α-actinin. The positions of the calcium chelating residues of the EF-hand loop are denoted by the letters X, Y, Z, -Y, -X and -Z. The hydrophobic aspects of the helices are located under columns headed n. The 27 amino acids of the N-terminal EF-hand of SM_{NM} which are distinct from the 22 in SM have been underlined, as have the 26 residues of the N-terminal EF-hand of SK_{NM} which are distinct from the 22 in the SK α-actinin sequence.
The complete human endothelial α-actinin cDNA (3075bp) begins at nucleotide 88 within the human placental α-actinin sequence and contains a poly-A tail (Youssouffian et al., 1990). The human endothelial cDNA is near identical with the human placental α-actinin cDNA (except at 5 nucleotide and two amino acid positions) up until nucleotide 2198. From this position onwards (i.e. across the latter half of the 3' non-coding sequence) these cDNAs become totally divergent. The partial human hepatocellular carcinoma α-actinin cDNA (2341bp) begins at nucleotide 1087 within the human placental α-actinin sequence and contains a poly-A tail (Nishiyama et al., 1990). The human hepatoma and placental α-actinins differ at 8 nucleotide and 6 amino acid positions (Figure 4.11). The 3' untranslated region of the human hepatoma α-actinin cDNA is identical in sequence with the corresponding region of the human placental α-actinin cDNA P6. Beggs et al. (1992) have isolated complete cDNAs encoding two distinct variants of human skeletal muscle α-actinin, HuActSk1 and HuActSk2 (Figure 4.11). HuActSk1 (104-kDa) is the human homologue of the chicken skeletal muscle variant identified by Arimura et al. (1988), but HuActSk2 (103-kDa) is a novel skeletal muscle variant. The two variants of human skeletal muscle α-actinin display ~80% total amino acid identity with each other and with the human placental isoform, and the amino differences between these proteins extend throughout the length of the protein (Figure 4.11).


It has been proposed that the non-muscle and smooth muscle isoforms of α-actinin may be expressed simultaneously in non-muscle cells and function at unique sites within the cell (Baron et al., 1987b). To investigate this hypothesis, we expressed full length chick smooth and non-muscle α-actinin cDNAs in monkey (non-muscle) COS cells (Gluzman, 1981), and used an antibody specific for chick α-actinin (Jackson et al., 1988) to examine the distribution of the expressed protein. This work was done in several stages.

To produce a cDNA containing the complete sequence of chicken brain α-actinin I first cloned the partial, and non-overlapping cDNAs, 7a and 9a into the Eco RI site of the plasmid Bluescript SK (Stratagene). This was done by way of a '3-way-ligation' procedure (i.e. a ligation involving 3 fragments, see Figure 4.12). The cDNAs were cloned into this vector to take advantage of its polylinker sequence, containing a large number of unique restriction sites. The orientation of the cDNAs in Bluescript was checked by restriction enzyme mapping, and by sequencing across the insertion site
Figure 4.8 Strategy used to obtain the Complete cDNA Sequence of Human Placental α-Actinin.

The human placental α-actinin cDNA, P6, was cloned into the vector Bluescript and digested with the restriction enzyme Bam HI to generate three fragments of approximately 1.0, 1.1, and 0.9 kb. These fragments were further sub-cloned into Bluescript. The 5’ Eco RI/Bam HI and the 3’ Bam HI/Bam HI fragments were then sequenced on both strands using forward and reverse primer (designed against Bluescript polylinker sequence) and synthetic oligonucleotides (designed against the sequence of human placental α-actinin determined from previous gel runs). The central Bam HI/Bam HI fragment in Bluescript was digested with Pst I and the resultant fragments (Bam HI/Pst I and Pst I/Pst I) were cloned into Bluescript and sequenced. The individual sequence runs are indicated with arrows, and their directions relate to coding (to the right) or non-coding (to the left) sequence.
**Human Placental Alpha-actinin cDNA Sequencing Strategy**

- cDNA in Bluescript SK
  - 0kb
  - 1kb
  - 2kb
  - 3kb

5' Eco RI
Bluescript polylinker cut site.

- Clone 5' Eco RI/BamHI fragment into Bluescript

- Sequence on both strands using synthetic 17mers.

3' Bam HI

- Clone central Bam HI fragment into Bluescript

- Complete cDNA sequence.
(using forward and reverse primers) and the junction of 7a and 9a (using the synthetic oligonucleotide 5'AGTTCTTATCTCCAGCC3').

Once the orientation of 7a and 9a in Bluescript had been confirmed, the restriction enzymes Kpn I and Xba I were used to liberate the resultant complete brain \(\alpha\)-actinin cDNA (called 7a/9a) from Bluescript. I then cloned 7a/9a into the eukaryotic expression vector pECE cut with the enzymes Kpn I and Xba I (Figure 4.12).

DNA from the resultant construct, p.7a/9a, was transfected into COS cells using the DEAE-dextran method of Cullen (1987). All transfections and immunoprecipitations were performed by Dr. Paul Jackson in our laboratory. The vector pECE contains the SV40 virus early promoter and origin of replication upstream of the cDNA cloning site. The SV40 origin of replication enables these plasmids to become amplified when transfected into COS cells, which contain the SV40 large T antigen in their genome. The expressed chick brain \(\alpha\)-actinin was detected in the following ways:

1. COS cells were transfected with p.7a/9a (or control plasmid) and then labelled with \([^{35}S]\) methionine. Using a chicken \(\alpha\)-actinin-specific antibody, \(\alpha\)-actinin was immunoprecipitated from cells transfected with p.7a/9a (Figure 4.13, lane B), but not cells transfected with control plasmid (Figure 4.13, lane D). \(\alpha\)-Actinin was not immunoprecipitated with rabbit pre-immune serum in cells transfected with p.7a/9a (Figure 4.13, lanes A) or control plasmid (Figure 4.13, Lane C).

2. The intracellular localisation of the expressed chick brain \(\alpha\)-actinin was examined by staining fixed and permeabilized transfected COS cells with the chicken \(\alpha\)-actinin-specific antibody as described in Section 2.17. The expressed chick brain \(\alpha\)-actinin could be detected distributed periodically along stress fibres and also at adhesion plaques (Figures 4.14, A). The smooth muscle-type \(\alpha\)-actinin cDNA C17 (Baron et al., 1987b) was also cloned into pECE and the resultant construct was transfected into COS cells by Dr P. Jackson. The expressed chick smooth muscle \(\alpha\)-actinin could again be detected distributed periodically along stress fibres and also at adhesion plaques using the chick \(\alpha\)-actinin-specific antibody (Figure 4.14, C).

4.3 Discussion.

The major aim of this section was to determine the complete cDNA sequence of a chick non-muscle \(\alpha\)-actinin, and thus establish the mechanism by which the non-muscle and smooth muscle isoforms of \(\alpha\)-actinin originate from the chick genome. The complete cDNA sequence of a chick embryo brain \(\alpha\)-actinin (7a/9a) was found to near identical with the sequence of a chick smooth muscle \(\alpha\)-actinin cDNA (C17) (Baron et al., 1987b). In fact these cDNAs were only divergent across a short span of sequence encoding the latter half of the N-terminal EF-hand and EF-hand linker region. This
The numbered line is the human placental α-actinin sequence. The initiator methionine starts at 199bp. A stop codon (*) is reached at 2875bp. The translation initiation signal (CCATCATG) is overlined (Kozak, 1984). The placental α-actinin cDNA does not contain either a poly-A adenylation signal, AATAAA (McLaughlan et al., 1985), or a poly-A tail and so the 3' untranslated sequence is probably incomplete. The amino acid sequence deduced from this cDNA is shown beneath the numbered line. The human placental α-actinin nucleotide sequence was compared with that of the chick brain isoform using the University of Wisconsin Genetic Computer Group Package (Devereux et al., 1984). Across the coding region these cDNAs display a level of identity of 85%. Base differences occurring in the brain α-actinin sequence are indicated above the human placental α-actinin sequence. The comparison is shown across the coding sequence only (nucleotides 199-2874 in the human placental α-actinin sequence), because the 5' and 3' untranslated sequences were totally divergent (see text). When one compares these sequences, a single codon deletion (-) is found between nucleotides 207 and 208 in human placental α-actinin. In the alignment this codon in chick brain α-actinin, CAC (coding for His4), is represented with a bold X.
T T T A A A A T G T G T A C
2761 GACCAGGCTAGTACTGCATCGCGCGGATGGCCCCCTACACCGCCTGCTGCCAC
2765 D O A E Y C I A R M A P Y T G P D S V P
2776
G C T A T
2821 GGTGCTCTGGACTACATGTCCTTCTCCACGGCGTGTACGGCGAGAGTGACCTCTAATCC
2880 G A L D Y M S F S T A L Y G E S D L *
2886
3000
3001 G T G G C A T C G A T C C T C C C T G C C C
strongly suggests that the brain and smooth muscle isoforms of chick $\alpha$-actinin are derived from the same gene, and a model for this system is presented in Figure 4.15. In this model the smooth and non-muscle $\alpha$-actinin mRNAs are encoded by the same exons of the gene, except for those encoding the latter half of the N-terminal EF-hand and the EF-hand linker regions. These exons are alternatively spliced to give isoform specific mRNA. This model has been confirmed by Dr G. Waites (Department of Biochemistry, University of Leicester) who sequenced the 3' end of the gene encoding chick smooth muscle/non-muscle $\alpha$-actinin (Waites et al., 1992). The organisation of this region of the gene is shown in Figure 4.16. The exon EF1a encodes from residue 713 (the end of repeat 4) to residue 761 in the N-terminal EF-hand, the exact position of sequence divergence between the isoforms. The adjacent exon (EF1b NM) encodes the 27 residues unique to the N-terminal EF-hand of the non-muscle isoform, and is followed by exon EF1b SM encoding the 22 residues unique to the N-terminal EF-hand of the smooth muscle isoform. The C-terminal EF-hand, which is common to both isoforms, is contained entirely within its own exon.

The chick brain and smooth muscle isoforms of $\alpha$-actinin start to become divergent at the Y co-ordinate of the N-terminal EF-hand (Figure 4.5). In chick smooth muscle $\alpha$-actinin there is a lysine residue at this vertex (Baron et al., 1987b). Lysine does not possess an oxygen-containing side chain and thus cannot help co-ordinate a calcium ion. The reverse situation is true at the Y vertex of chick brain $\alpha$-actinin, where there is an aspartate residue. The chicken brain and smooth muscle isoforms also differ at the Z, -Y, -X and -Z calcium liganding positions of the N-terminal EF-hand (see Figure 4.5). The most important of these differences is likely to be the aspartate for glutamate substitution at the -Z vertex of chicken brain $\alpha$-actinin. As mentioned in Chapter 1, glutamate is now considered to be an obligatory residue at this site (Strynadka and James, 1989). The only questionable calcium chelating residue in the N-terminal EF-hand of chick brain $\alpha$-actinin is the glycine residue at the -X vertex (Figure 4.5). Glycine does not possess an oxygen-containing side chain and thus cannot ligand a calcium ion directly. However, it may suffice if it allows a water molecule to co-ordinate the calcium ion, as is the case for carp parvalbumin (Kretsinger, 1980; Seamon and Kretsinger, 1983). The N-terminal EF-hand of chicken brain $\alpha$-actinin (unlike its counterpart in chicken smooth muscle $\alpha$-actinin) might therefore be capable of binding a calcium ion. The C-terminal EF-hand of chick brain $\alpha$-actinin is identical in sequence with the C-terminal EF-hand of chick smooth muscle $\alpha$-actinin (Figure 4.5). This EF-hand has a methionine and an alanine at the Z and -Z positions respectively, and is not predicted to bind calcium (Baron et al., 1987b). Based on these predictions, chick brain $\alpha$-actinin may bind one Ca$^{2+}$ per subunit, and chick smooth muscle $\alpha$-actinin may not bind any. This would appear to explain why chick brain $\alpha$-actinin (unlike chicken smooth muscle
Figure 4.10 Alignment of the Human Placental and Chicken Brain \(\alpha\)-Actinin Amino Acid Sequences.

The complete deduced amino acid sequence of human placental \(\alpha\)-actinin (HPA, cDNA P6) and chicken brain \(\alpha\)-actinin (CBA, cDNA 7a/9a), were aligned using the program Bestfit of the University of Wisconsin Genetic Computer Group (Devereux et al., 1984). The one letter amino acid code is used for clarity, and vertical lines (III) indicate positions of total sequence identity. Positions of amino acid divergence are shown in bold type. The chick brain sequence is underlined in the region containing the ABD and the EF-hands. Dashes are introduced where insertions/deletions occur.
α-actinin) binds to actin in a calcium-sensitive fashion (Duhaian and Bamburg, 1984). In chick myosin regulatory light chain it has been established that only one of the four EF-hands is functional (Reinach et al., 1986), and this EF-hand is the only one containing all of the critical Ca^{2+}-chelating oxygens. This suggests that a functional EF-hand can associate with a non-functional EF-hand and still retain its functional ability. Thus it may also be possible for chick brain α-actinin to function with only one functional EF-hand per subunit (however, see Chapters 5 and 6). Both EF-hands of the Dictyostelium α-actinin subunit are predicted to bind calcium (Noegal et al., 1987). Furthermore, equilibrium dialysis experiments have shown that each subunit of rabbit macrophage α-actinin can bind two calcium ions (Bennett et al., 1984). These results suggest that some non-muscle α-actinins may bind one calcium ion per subunit, whilst others bind two per subunit. The binding of one calcium ion per subunit may be sufficient to facilitate the observed calcium-sensitivity of chick brain α-actinin. However, the mechanism by which calcium brings about inhibition is not known. On binding calcium the C-terminal domain may change its conformation, and this change may be transmitted directly along the subunit to inhibit actin-binding at the N-terminus. However, since α-actinin forms an antiparallel homodimer, there is also the possibility that the binding of calcium by one subunit may disrupt the binding of actin by the apposing subunit (Noegal et al., 1987). At this stage it is not known if regions outside of the two EF-hand motifs can also influence calcium-binding. For example, the ABD of the α-actinin molecule is likely to be intimately associated with the EF-hand domain in the native dimeric molecule, and may supply some calcium-binding co-ordination points. Chick brain α-actinin also contains an additional 5 amino acids in its EF-hand linker region, which are not present in Dictyostelium α-actinin, chick smooth muscle α-actinin, or chick skeletal muscle α-actinin (Figure 4.5). The effect this has on the functional ability of either (or both) EF-hands is not known. The extended linker region may be involved in transmitting the conformational change induced upon binding a calcium ion.

A partial chick fibroblast α-actinin cDNA has recently been isolated by Arimura et al. (1988). The sequence of this fibroblast cDNA, termed Fα1, is identical with 7a/9a and is therefore likely to code for the same variant of non-muscle α-actinin. However, since Fα1 is lacking some 5' coding sequence (starting at nucleotide 130 within the complete chicken brain sequence 7a/9a) one should not overlook the possibility that this cDNA may encode a variant of non-muscle α-actinin that is divergent with 7a/9a at its 5' end. A similar argument can be applied to the partial chick fibroblast cDNA isolated by Baron et al. (1987a), termed C18. This cDNA is also identical in sequence with the chick brain α-actinin cDNA, but is lacking some 5' and 3' coding sequence (covering nucleotides 200-2326 within the complete chick brain α-actinin cDNA sequence).
**Figure 4.11 Alignment of the Amino Acid Sequence Deduced from Five Human α-Actinin cDNAs.**

Amino acid sequence comparison of human placental α-actinin (HPL) with human endothelial α-actinin (HEN, see Youssouffian et al., 1990), human hepatocellular carcinoma α-actinin (HHA, see Nishiyama et al., 1990), and two variants of human skeletal muscle α-actinin, HuActSk1 (HSK1, Beggs et al., 1992) and HuActSk2 (HSK2, Beggs et al., 1992). The one letter amino acid code is used for clarity, and dashes (-) indicate positions of sequence identity. The human placental α-actinin sequence is underlined in the region believed to contain the ABD and the EF-hands. Asterisks (*) are introduced to accommodate the extra 5 residues between the two EF-hands of human non-muscle α-actinin. Numbering refers to the sequence of human placental α-actinin.
In an attempt to uncover novel variants of chick α-actinin, I also screened a second chick embryo brain cDNA library (a kind gift of Dr. Wasenius, University of Helsinki). The chick brain α-actinin cDNA 7a was used as labelled probe and a single positively reactive clone (8W) was isolated. This variant of chick brain α-actinin was found to be identical in sequence with the chick skeletal muscle isoform of α-actinin isolated by Arimura et al. (1988), except in the region encoding the N-terminal EF-hand and the EF-hand linker peptide (Figure 4.7). The chick gene encoding chick skeletal muscle α-actinin has been shown to contain two alternative exons in the region encoding the EF-hands (Parr et al., 1992). One of these alternative exon encodes the residues unique to the N-terminal EF-hand and EF-hand linker peptide peptide of chick skeletal muscle α-actinin, and the other exon encodes the corresponding regions of the chick brain variant encoded by 8W. The exon encoding the 26 residues unique to N-terminal EF-hand of the 8W-type variant precedes the exon encoding the 22 residues unique to the skeletal muscle isoform (Parr et al., 1992). The organisation of the 3' end of this gene is thereby analogous with the chick smooth muscle/non-muscle α-actinin gene system. The variant encoded by 8W is presumably a non-muscle isoform of α-actinin, although this cannot be stated definitively. Unlike the EF-hands of typical non-muscle α-actinins which are expected to bind calcium, those encoded by 8W are predicted to be non-functional. Furthermore, 8W encodes an EF-hand linker peptide (4 residues in length) which is not present in the archetypal non-muscle α-actinin derived from Dictyostelium (Noegal et al., 1987). Complementary DNAs encoding the smooth muscle α-actinin-based variant of chick brain α-actinin were not isolated from the Wasenius chick brain λgt10 cDNA library. This was surprising considering the frequency with which they were isolated from the Darlison chick brain cDNA library (8 clones isolated from a total of 100,000 plaques). The 8W variant might be expressed at specific stages during development and in specific tissues. The quantity of 8W-type cDNA might therefore vary between one non-muscle cDNA library and another (Parr et al., 1992). The thermolytic polypeptides of chick brain α-actinin were found to be identical in sequence with the chick smooth muscle isoform in the region of the ABD and repeats (Chapter 3), and there was no indication of a skeletal muscle α-actinin-related sequence. This suggests that the variant encoded by 8W might be expressed at very low levels in adult non-muscle tissues. However, it may be expressed at far higher levels at certain stages during embryogenesis.

Another major aim of this project was to investigate the degree of conservation between non-muscle α-actinins derived from different species. As part of this analysis a cDNA encoding a variant of human placental α-actinin was isolated and sequenced. Human placental α-actinin was found to display a high level of identity with the major variant of chick brain α-actinin, and these proteins were found to display 85 and 97%
Figure 4.12 Cloning the Complete Chick Brain α-Actinin cDNA (7α/9α) into the Eukaryotic Expression Vector pECE.

A. The partial and non-overlapping chick brain α-actinin cDNAs 7α and 9α were cloned by way of a '3-way-ligation' into the Eco RI site of the vector Bluescript.

B. The complete chick brain α-actinin cDNA (7α/9α) was then liberated from Bluescript by cutting simultaneously with the restriction endonucleases Kpn I and Xba I. The cDNA 7α/9α was then ligated into the vector pECE, which had also been cut with Kpn I and Xba I. The resultant construct was called p.7α/9α.

A.

3-way Ligation of the Partial Chick Brain Alpha-Actinin cDNAs 9α and 7α into Bluescript (SK).

B.

Ligation of the cDNA 7α/9α into the MCS of the Eukaryotic Expression Vector pECE.
identity at the DNA and protein levels respectively. Only 24 amino acid differences were observed between human and chick non-muscle \( \alpha \)-actinins, and the bulk of these were located in the central domain containing the spectrin-like repeats. The function of this domain must therefore be more tolerant to amino acid substitution than the rest of the molecule. The EF-hands of human placental \( \alpha \)-actinin are almost identical with chick brain \( \alpha \)-actinin (Figure 4.5). Only one amino acid difference actually occurs at a calcium-chelating position: vertex Z of the C-terminal EF-hand. A methionine occurs at this site in chick brain \( \alpha \)-actinin, but there is a leucine at the same position within human placental \( \alpha \)-actinin. Neither of these residues would be expected to promote the liganding of calcium ion. Therefore, based upon EF-hand predictions, one would expect human placental \( \alpha \)-actinin (like chick brain \( \alpha \)-actinin) to bind one \( \text{Ca}^{2+} \) per subunit.

Figure 4.17 shows an alignment of the amino acid sequences of \( \alpha \)-actinins from human placenta, Dicyostelium (Noegal et al., 1987), Drosophila (Fyrberg et al., 1990), Nematode (Barstead et al., 1991) and chick brain (this work, Waites et al., 1992). The degree of conservation between human \( \alpha \)-actinin and those from more primitive organisms is far lower than that observed between human and chick (Figure 4.17). Across their entire amino acid sequences, Nematode, Drosophila, and Dicyostelium \( \alpha \)-actinins are 36-63% identical with the sequence of human placental \( \alpha \)-actinin. Most amino acid substitutions in this alignment preserve amino acid character (Figure 4.17). The most highly conserved region of the molecule is the ABD and this may reflect the high degree of conservation of the actin molecule (Hightower and Meagher, 1986; Mclean et al., 1990). For example, residues 24-244 in the human placental \( \alpha \)-actinin sequence display 64% total identity with residues 15-238 of the most primitive \( \alpha \)-actinin in this alignment, Dicyostelium \( \alpha \)-actinin. Outside of this region, the level of absolute amino acid identity between these \( \alpha \)-actinins is reduced to 27% (Figure 4.17), suggesting a probable boundary for the ABD. However, the extreme sequence conservation between human and chick \( \alpha \)-actinin extends throughout the whole molecule. This strongly suggests that every portion of vertebrate \( \alpha \)-actinin is likely to perform an important structural or functional role. These may include binding sites for F-actin (Hemmings et al., 1992); vinculin (Wachstock et al., 1987), zyxin (Crawford et al., 1992), lipids (Burn et al., 1985; Kahana and Gratzer, 1991), \( \text{Ca}^{2+} \) (Bennett et al., 1984), the fibronectin receptor (Otey et al., 1989) and possibly other ligands which have not yet been identified. The adhesion plaque protein vinculin is also very highly conserved between human and chick (>95% identity at the amino acid level), suggesting that each portion of this molecule is also likely to perform an important function in vertebrate cells.

Two regions of exceptional variation stand out in the alignment presented in Figure 4.17. Firstly, the extreme N-terminal region is virtually unique to each species of \( \alpha \)-actinin. This region of molecule may not bind F-actin directly, but it may bind to other
Figure 4.13 Expression of Radiolabelled Chick Brain α-Actinin in COS Cells.

$^{35}$S methionine labelled extracts of COS cells transfected with: the complete chick brain α-actinin cDNA (7a9a) in the vector pECE (lanes A and B); or with the control plasmid (lane C and D), were immunoprecipitated using either chick specific antiserum (lanes B and D) or rabbit pre-immune serum (Lanes A and C). These experiments were performed by Dr. P. Jackson using the methodology described in Section 2.17.iv.
cytoskeletal proteins. This may provide a mechanism for the specific localisation of different isoforms α-actinin to unique locations within the same cell. For example, the extreme N-terminal sequence of skeletal muscle α-actinin may contain sites which target the protein specifically to the Z-disc (Arimura et al., 1988; Endo and Masaki, 1984). The extreme N-terminal region of the smooth muscle isoform differs markedly in sequence from the skeletal muscle isoform and may contain binding sites for proteins associated with the myotube membrane (Endo and Masaki, 1984) (see Section 1.3).

Secondly, within the N-terminal region of the first spectrin-like repeat of Nematode α-actinin there is a 27 amino acid insertion (Figure 4.17). This insert is rich in the residue proline, an imino acid frequently found in regions of turn/coil. The supercontractile isoform of Drosophila α-actinin has also been shown to contain a proline-rich insert (22 residues) in this region of the molecule (Roulier et al., 1992). The precise function of the insert is not known, but it may act as a flexible arm linking the actin-binding and repeat domains. Alternatively, it may contain sites for binding to other cytoskeletal proteins.

The human placental and chick brain α-actinins show substantial sequence diversion at both their 5' and 3' untranslated sequences. This discrepancy is unlikely to be due to a cloning artifact because Nishiyama et al. (1990) have isolated a partial non-muscle α-actinin cDNA from a human hepatoma cDNA library which is identical in sequence with our cDNA in the 3' non-coding region. Using the 3' non-coding region of HCC cDNA as labelled probe these workers detected a 3.5kb transcript in RNA from human hepatoma, fibroblast, macrophage and monocyte cell lines in northern blots. However, this probe failed to detect a transcript in rat tissue RNA. The untranslated sequences of non-muscle α-actinin may therefore differ markedly between species. However, there is also evidence that the non-coding sequences may vary from one non-muscle α-actinin to another within the same species. The human endothelial and human placental α-actinin cDNAs are totally divergent across the latter half of their 3' untranslated sequences (Millake et al., 1989; Youssofian et al., 1990). This difference may arise via alternative splicing of the exon(s) encoding the 3' untranslated region, and may produce α-actinin mRNAs with distinct intracellular localisation sites. This may restrict the expression of α-actinin to specific sites in some non-muscle cells. Good examples of localized mRNAs are the maternal mRNAs of flies and frogs, which are important for the development of axial polarity. In the case of the mRNAs for the bicoid gene in Drosophila and the Vgl gene in Xenopus the essential sequence directing internal localization lies not in the 5' region but in the 3' untranslated region of the mRNA (Yisraeli and Melton, 1988; MacDonald and Struhl, 1988; Gottlieb, 1992). Complementary DNAs encoding two distinct variants of human skeletal muscle α-actinin have recently been isolated (Beggs et al., 1992). Both variants are as dissimilar with one
Figure 4.14 Double Fluorescence Staining of COS Cells Expressing the Chick Brain and Smooth Muscle isoforms of α-Actinin with a Chick-Specific Antiserum.

Full length cDNAs encoding the chick brain (A,B) or chick smooth muscle (C, D) isoform of α-actinin were cloned into the eukaryotic expression vector pECE (Ellis et al., 1986) and transfected into monkey COS cells (Gluzman, 1981). The expressed proteins were then detected by immunofluorescence using a chick-specific α-actinin antiserum, as described in Section 2.17. (A, C) cells stained with a chick-specific polyclonal α-actinin antibody (Jackson et al., 1988). (B, D) the same cells stained with NBD-phallicidin to label actin filaments. Arrows indicate the co-alignment of α-actinin with actin filaments. Magnification x 1100. The transfection experiments were performed by Dr. P. Jackson (Department of Biochemistry, University of Leicester).
another as they are with the human placental isoform, displaying ~80% total amino acid identity. These proteins are particularly divergent with one another at the extreme N-terminal end of the molecule, but there are also many amino acid differences taking place throughout the length of the molecule indicating that each isoform is the product of a unique gene. The human smooth muscle/non-muscle α-actinin gene is linked to the gene encoding β-spectrin on chromosome 14q22-24 (Youssoufian et al., 1990), whereas the genes encoding the skeletal muscle variants HuActSk1 and HuActSk2 have been localised to chromosomes 1q42-q43 and 11q13-q14 respectively (Beggs et al., 1992). In fact most genes encoding structural proteins (with the exception of the myosin heavy chain genes) are also scattered with respect to one another (Beggs et al., 1992). HuActSk1 is the human homologue of the chick skeletal muscle variant identified by Arimura et al. (1988) and is expressed in skeletal and cardiac muscles, whereas HuActSk2 is a novel variant expressed only in skeletal muscle (Beggs et al., 1992). Northern blot studies have shown that there are 3 major transcripts encoding the HuActSk1 variant of skeletal muscle α-actinin (~3.3, 3.8, and 5.6kb) (Beggs et al., 1992). These transcripts are produced by alternate usage of polyadenylation signals and do not affect the encoded protein product (Beggs et al., 1992).

The isolation of a smooth muscle α-actinin cDNA from a chick fibroblast cDNA library gave rise to the hypothesis that non-muscle cells may express smooth muscle α-actinin in addition to the non-muscle isoform (Baron et al., 1987b). This has been confirmed using a quantitative assay based on the polymerase chain reaction (Waites et al., 1992). Chick kidney and chick lung express equal ratios of the two transcripts, but the non-muscle transcript is expressed at a higher level in chick liver, brain and spleen (Table 4.2). MRC-5 fibroblasts and HeLa cells express mRNAs encoding both isoforms, whereas Namalwa lymphoblastoid cells which lack actin stress fibres only express the non-muscle mRNA (Waites et al., 1992). The adhesion plaques of HeLa cells are more prominent than those of MRC-5 fibroblasts, which contain more tightly organised and 'muscle-like' actin stress fibres (Waites et al., 1992). This observation may reflect of the proportion of the smooth muscle isoform expressed in MRC-5 cells (25%) when compared with HeLa cells (3%), where the non-muscle isoform predominates (97%) (Waites et al., 1992). It has also been proposed that non-muscle and muscle isoforms may be differentially localised in non-muscle cells where they perform unique functional roles (Baron et al., 1987b). For instance, the non-muscle isoform may be incorporated into the adhesion plaques, whereas the smooth muscle isoform may be incorporated into the actin stress fibres which are muscle-like in structure (see Chapter 1). Previous attempts to address this question have involved the microinjection of fluorescently labelled α-actinins into cells.
Figure 4.15 A Model for the Chick Non-Muscle and Smooth Muscle α-Actinin Splicing System.

This model is based on the complete cDNA sequences of chick brain α-actinin (7a/9a) and chick smooth muscle α-actinin (C17) (Baron et al., 1987b). The non-muscle and smooth muscle isoforms are encoded by the same exons of the gene, except for the one encoding the latter half of the N-terminal EF-hand and the EF-hand linker region (exon EF1b). The non-muscle (NM) and smooth muscle (SM) exons EF1b are alternatively spliced at the level of the primary transcript to give isoform specific mRNA. These mRNAs then give rise to α-actinin isoforms with distinct EF-hand domains, and different sensitivities towards calcium.
Table 4.2 The Relative Proportions of the Smooth Muscle and Non-muscle (Type 7a/9a) α-Actinin mRNAs in Chick (Waites et al., 1992)

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<td></td>
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</table>

For example, when non-muscle α-actinin from Dictyostelium was microinjected into chick embryo fibroblasts it was found to be incorporated into both the stress fibres and adhesion plaques of these cells (Schulze et al., 1989). A similar distribution was observed when α-actinin from calf thymus was microinjected into cardiac fibroblasts (Meigs and Wang, 1986) and α-actinin from chicken smooth muscle was microinjected into monkey kidney BSC-1 cells and chick fibroblasts (Feramisco, 1979, and McKenna et al., 1985). These results suggest that a differential localisation of non-muscle and smooth muscle isoforms does not occur in non-muscle cells. However, such data may be misleading since the purified protein used for microinjection may have consisted of a mixture of isoforms. To overcome this limitation the complete chicken brain (7a/9a) and smooth muscle (C17) cDNAs were cloned into the eukaryotic expression vectors pECE (Ellis et al., 1986) and the recombinant vectors were transfected into monkey (non-muscle) COS cells. The distribution of the expressed proteins was then analysed with an antibody specific for chicken α-actinin. Both chick brain and smooth muscle α-actinins were found to be localised at both the adhesion plaques and stress fibres of COS cells (Figure 4.14), confirming the results of the microinjection studies. Taken together these results suggest that α-actinin may function as a mixed population of isoforms at each site in non-muscle cells. However, one must not exclude the possibility that the high levels of intracellular α-actinin achieved by microinjection into or through
Figure 4.16 The Sequence of the Smooth Muscle/Non-Muscle α-Actinin Gene in the Region Encoding the EF-hands.

The nucleotide sequence of the exons is underlined. The amino acid sequence of each exon is shown in one-letter code beneath the nucleotide sequence. The intron sizes in kb are indicated. Flanking intron sequences are shown in lower case letters. Exons labelled EF1a, EF2 and 3' exon are common to both smooth (SM) and non-muscle (NM) α-actinin. Exon EF1b NM and EF1b SM encode sequence unique to each isoform. All genomic clones were isolated and sequenced by Dr. Gillian Waites (Department of Biochemistry, University of Leicester).
transient expression in non-muscle cells may have lead to aberrant localization of the protein.
Figure 4.17 Complete Amino Acid Alignment of α-Actinins Derived from Five Species.

The complete amino acid sequences of α-actinins derived from: chick brain (CBA, cDNA 7a/9a); human placenta (HPA, cDNA P6); Nematode (NAA, Barstead et al., 1991); Drosophila (DFA, Fyrberg et al., 1990); and Dictyostelium (DAA, Noegal et al., 1987), were aligned using the Staden CLUSTAL multiple alignment program. A match across all species is indicated with an asterisk (*), and a conservative substitution is indicated with a dot (•). The pad character (-) is introduced at certain sites to facilitate maximal alignment. The one letter amino acid code is used throughout.
Chapter 5. Calcium-Binding Properties of Chick Brain α-Actinin.
5.1 Introduction.

Non-muscle α-actinin is distinct from its muscle counterparts in that it binds to F-actin in a calcium-sensitive fashion (Bennett et al., 1984; Duhaiman and Bamburg, 1984; Landon et al., 1985). However, a detailed analysis of the calcium-binding properties of non-muscle α-actinin has not yet been carried out, and very little is known about the molecular basis by which calcium brings about an inhibition of actin-binding. For example only one study, that of Bennett et al. (1984), has actually reported a quantitative value for the binding of Ca$^{2+}$ to a non-muscle α-actinin. Using equilibrium dialysis, these workers have shown that rabbit macrophage α-actinin binds 4 calcium ions per dimer with a K$_d$ of 4x10$^{-6}$M. This suggests that macrophage α-actinin contains two functional EF-hands per subunit. This calcium-binding stoichiometry is in agreement with the predicted number of functional EF-hands per subunit of the non-muscle α-actinin from Dictyostelium (Noegel et al., 1987). However, α-actinins from chick brain (Chapter 4, Waites et al., 1992), chick fibroblast (Arimura et al., 1988), human placenta (Millake et al., 1989), human endothelia (Youssofian et al., 1990), and human hepatoma (Nishiyama et al., 1990) are each predicted to bind a single calcium ion per subunit (at the N-terminal EF-hand). It is therefore possible that some non-muscle α-actinins may bind a single calcium ion per subunit whilst others bind two. The fact that chick brain α-actinin is sensitive to calcium (Duhaiman and Bamburg, 1984), suggests that only a single calcium ion per subunit need be bound to facilitate this property. As yet no quantitative calcium-binding data has been presented for the muscle α-actinins, but because they bind to F-actin in a calcium-insensitive fashion one might expect them not to bind Ca$^{2+}$. In support of this, the EF-hands of chick smooth (Baron et al., 1987b) and chick skeletal muscle α-actinin (Arimura et al., 1988) are predicted to be non-functional.

In this Section, I have attempted to determine the calcium-binding stoichiometries of the chicken brain, skeletal, and smooth muscle isoforms α-actinin. Two strategies were adopted. Firstly, the calcium-binding properties of tissue-purified chicken α-actinins were measured using a rapid dot-blot $^{45}$Ca$^{2+}$-overlay procedure (Koch et al., 1986; Reinach, 1986; Way et al., 1989). In this procedure, small aliquots (50-200 pmols spots) of the protein under investigation are pipetted onto a gridded nitrocellulose circle. The filter is then immersed in a solution containing $^{45}$Ca$^{2+}$. After a 20 minute incubation, the filter is washed briefly, and the spots are excised and analysed by scintillation counting. The advantage of using this method is that it is quick, requires relatively small quantities of protein, and enables a large number of samples to be analysed simultaneously. Secondly, the calcium-binding properties of the fusion proteins containing the EF-hand regions of chick brain or smooth muscle α-actinin were
Figure 5.1 Calcium Binding to the Brain, Smooth Muscle, and Skeletal Muscle Isoforms of Chicken α–Actinin.

The calcium binding properties of chicken smooth muscle α–actinin (SM), chicken skeletal muscle α–actinin (SK), chicken brain α–actinin (BR), gelsolin (GEL), and cytochrome-C (CYT-C) were measured using a rapid dot-blot $^{45}\text{Ca}^{2+}$-overlay procedure developed by Koch et al. (1986). See text for the conditions applying to each of the individual experiments (a) to (e). CPM-counts per minute.
a). This experiment was performed exactly as described in Section 2.9.i.

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b). This experiment was performed exactly as described in Section 2.9.i except that the chicken brain alpha-actinin was freshly prepared, and the proteins were applied in their usual storage buffers (see Chapter 2).

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c). This experiment was performed exactly as described in Section 2.9.i, except the wash step was removed altogether, and the filter was dried immediately under vacuum following incubation in $^{45}$Ca$^{2+}$-buffer.

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<th>MEAN CPM - BLANK</th>
<th>TOTAL Ca$^{2+}$ (p moles)</th>
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d). This experiment was performed exactly as described in Section 2.9.i, except that the wash time was significantly reduced. The filter was rapidly drawn through 50ml of wash buffer following incubation in $^{45}$Ca$^{2+}$-buffer, and then dried immediately under vacuum.

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e). This experiment was performed exactly as described in Section 2.9.i, except a higher total concentration of calcium (1mM) was used in the $^{45}$Ca$^{2+}$-overlay and wash buffers. The filter was washed and dried as in experiment 5.1.d

<table>
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<tr>
<th>PROTEIN</th>
<th>QUANTITY (p moles)</th>
<th>NO. OF SPOTS</th>
<th>CPM</th>
<th>MEAN CPM - BLANK</th>
<th>TOTAL Ca$^{2+}$ (p moles)</th>
<th>Ca$^{2+}$ BOUND (mol/mol)</th>
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<td>-896</td>
<td>0.00</td>
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analysed by stopped-flow spectrophotometry (Eccleston, 1987). In this procedure, two solutions are pumped through a mixing chamber at constant pressure, into an observation tube. The first solution contains the protein of interest, and the second contains the indicator. The indicator used in these experiments was the fluorescent compound Quin 2 which binds calcium with high affinity (K\textsubscript{d} = 100nM) (Tsien et al., 1982). The fluorescence signal of this indicator increases about fivefold on going from the calcium-free to the calcium-saturated state (Tsien et al., 1982). It can therefore be used to monitor the release of calcium ions remaining associated with the protein of interest. Indeed, Quin-2 has been used to monitor rapid kinetic measurements, such as those described for calmodulin (Martin et al., 1985). A large excess of Quin 2 over protein (50-100 fold excess) is used to achieve total release of calcium from the protein to the indicator. Stopped-flow spectrophotometry using Quin 2 requires several millilitres of protein solution at micromolar concentrations (personal communication, Dr. C. Bagshaw, Department of Biochemistry, University of Leicester). Consequently, this technique was not used to assay chicken brain α-actinin purified from tissue where the yield obtained was too low (0.2-0.3mg per 50g of wet tissue). To produce sufficient protein for this assay, α-actinin EF-hand fusion proteins were expressed in E. coli using the plasmid pGEX-1 (Smith and Johnson, 1988). This vector enables cDNA inserts to be expressed as fusion proteins containing the enzyme glutathione-S-transferase, which can be purified by affinity chromatography using a glutathione-agarose resin.

5.2 Results.

5.2.1 Rapid 'Dot-blot' 45Ca\textsuperscript{2+}-Overlay Analysis.

The calcium-binding properties of the purified whole α-actins were analysed using a rapid dot-blot 45Ca\textsuperscript{2+}-overlay procedure developed by Koch et al. (1986). Aliquots (50-200pmols) of chicken smooth muscle, skeletal muscle, and brain α-actins, gelsolin (positive control), and cytochrome C (negative control) were spotted onto a gridded nitrocellulose membrane. The filter was then immersed in wash buffer (10mM imidazole [pH 7.0], 0.1M NaCl, and 1.0mM MgCl\textsubscript{2}) containing 3.2μCi/ml 45Ca\textsuperscript{2+}, and cold Ca\textsuperscript{2+} to a final concentration of 40μM. This concentration of calcium should saturate the calcium-binding sites of chicken brain α-actinin, assuming that they have an affinity constant in the micromolar range. This assumption was based on the following observations. Firstly, rabbit macrophage α-actinin has been shown, by equilibrium dialysis, to have a K\textsubscript{d} of 4.5μM for calcium (Bennett et al., 1984). Secondly, a free [Ca\textsuperscript{2+}] of about 1μM was found to give a 50% inhibition of the
Figure 5.2 Purification of the EF-hand Containing Fragments of Chick Brain or Chick Smooth Muscle α-Actinin Expressed as Glutathione-S-Transferase Fusion Proteins in E. coli

Cell cultures (final volume 50ml) were grown as described in Section 2.15. Cells were induced by adding IPTG to a final concentration of 1mM, followed by incubation at 37°C for 90 minutes. The fusion proteins were purified as described in Section 2.15. Lanes A-F, 12% acrylamide SDS-PAGE of samples followed by Coomassie blue staining. Lanes A and B show 1/100th of the total cell extract after induction with IPTG and incubation at 37°C. Lanes C and D show 1/20th of the total insoluble fraction. Lanes E and F show 1/50th of the total material purified on glutathione-agarose (see section 2.15). Lanes A, C and E show the results obtained with EF-brain and lanes B, D and F show the results obtained with EF-smooth. The position and sizes (kDa) of the molecular weight markers are shown along the left hand side of this figure.
maximal viscosity increase brought about by chicken brain α-actinin on a solution of F-actin (Duhaimean and Bamburg, 1984).

Mg\(^{2+}\) was included in the wash buffer (at a concentration of 1mM) to eliminate low-affinity non-specific divalent cation binding (Reinach et al., 1986). After a brief wash (20s) the filter was dried, cut into squares, and the amount of \(^{45}\)Ca\(^{2+}\) bound measured by scintillation counting. The amount of Ca\(^{2+}\) bound mol per mol of protein was calculated using the following equations:

1. \[
\text{SPECIFIC ACTIVITY} = \frac{\text{SCINTILLATION COUNT OF BUFFER ALIQUOT (CPM)}}{\text{AMOUNT OF CALCIUM IN BUFFER ALIQUOT (pmols)}}
\]

2. \[
\text{TOTAL CALCIUM BOUND TO FILTER SQUARE (pmols)} = \frac{(\text{MEAN SAMPLE SCINTILLATION (CPM)} - \text{MEAN BLANK (CPM)})}{\text{SPECIFIC ACTIVITY OF INCUBATION BUFFER (CPM/pmols)}}
\]

3. \[
\text{CALCIUM BOUND TO PROTEIN SPOT (mol/mol)} = \frac{\text{TOTAL CALCIUM BOUND TO FILTER SQUARE (pmols)}}{\text{AMOUNT OF PROTEIN (pmols)}}
\]

Gelsolin is known to bind two Ca\(^{2+}\) ions per subunit and cytochrome C does not bind Ca\(^{2+}\) (Way et al., 1989). Figure 5.1(a) shows the result of the first calcium-binding analysis. Gelsolin and cytochrome C were found to bind 1.5 and 0.15 mols of Ca\(^{2+}\) per mol of protein respectively, confirming that this assay system can give reasonably accurate Ca\(^{2+}\)-binding data (Koch et al., 1986; Reinach et al., 1986; and Way et al., 1989). The smooth, skeletal, and brain α-actinins were found to bind 0.36, 0.28, and 0.34 mols of Ca\(^{2+}\) per mol of protein respectively. These binding stoichiometries were not much higher than those obtained with cytochrome C, suggesting that none of the α-actinins bound to Ca\(^{2+}\). Increasing the quantity of gelsolin (50-200pmols) gave a corresponding increase in \(^{45}\)Ca\(^{2+}\) bound. However, no such trend could be detected for the α-actinins (or for cytochrome C), suggesting that \(^{45}\)Ca\(^{2+}\)-binding to these proteins was at a background level.

In an effort to verify the results of the first experiment, the experiment was repeated under essentially the same conditions, except:

(a) In the first experiment, the chicken brain α-actinin had been stored for 1 week at 4°C prior to use. To investigate the possibility that the brain α-actinin had become functionally inactive during this period, freshly prepared protein was used in the second experiment.

(b) In the first experiment the proteins were dialysed overnight against assay wash buffer. To investigate the possibility that this prolonged exposure to wash buffer may have caused the α-actinins to become functionally inactive, the proteins (in their usual storage buffers) were applied directly onto the nitrocellulose membrane. After their
Figure 5.3 Identification of the α-Actinin EF-hand Fusion Proteins by Western Blotting.

Overnight Cultures (each 5ml) of *E. coli* JM101, and *E. coli* JM101 transformed with EF-brain, EF-smooth, or the control plasmid pGEX-1 were diluted 1/10 in fresh medium to a final volume of 50ml. The cells were grown to mid-log phase at 37°C induced by adding IPTG to a final concentration of 1mM, followed by incubation for 90 minutes at 37°C. Samples (500μl) were pelleted for 1 minute in a high speed bench centrifuge. The supernatants were removed and replaced with an equal volume of 1xSDS-PAGE sample buffer. The samples were boiled for 1 minute, and 50μl of each was then subjected to 12% SDS-PAGE. Lanes A, E, and I show cells transformed with EF-brain. Lanes B, F and J show cells transformed with EF-smooth. Lanes C, G and K show cells transformed with the control plasmid pGEX-1. Lanes D, H and L show wild-type *E.coli* JM101. Lanes A to D show a gel stained with Coomassie. Lanes E to H show a western blot with whole rabbit anti-chicken smooth muscle α-actinin antibody, diluted 1/500. Lanes I to L show a western blot with whole rabbit pre-immune serum, diluted 1/500. The position and sizes (kDa) of the molecular weight markers are shown along the left hand side of the figure.
application onto the filter, the proteins were rapidly equilibrated in wash buffer by applying 2 x 50ml aliquots of buffer under vacuum.

The results of the second experiment (Figure 5.1[b]) essentially confirmed the findings of the first. The calcium-binding stoichiometries of the α-actinins were similar to those obtained for cytochrome C, further suggesting that these proteins do not bind calcium under these conditions. The amount of $^{45}\text{Ca}^{2+}$ bound in this experiment was generally lower than that obtained in the first experiment. This may have been due to the removal of the dialysis step, and its replacement with a brief washing step following the application of the proteins onto the filter.

If the EF-hands of chicken brain α-actinin bind calcium with very low affinity then the dissociation rates are also likely to be very rapid. If so, then all of the $^{45}\text{Ca}^{2+}$ associated with the protein may be removed during the wash procedure. To investigate this possibility, it was decided that the experiment would be repeated with a number of modifications (Figures 5.1[c]-[e]). Firstly, the washing procedure was removed altogether. The filter was immersed in $^{45}\text{Ca}^{2+}$-buffer as usual, but was then dried immediately under vacuum. The filter was then cut into squares and analysed by scintillation counting (Figure 5.1[c]). As expected this amendment resulted in a higher background. However, reasonable estimates of calcium-binding by gelsolin were observed (2.6 Ca$^{2+}$/mol). But once again, no significant degree of binding was observed for any of the α-actinins. Secondly, the wash procedure was included but the time was reduced significantly. Following, incubation in $^{45}\text{Ca}^{2+}$-buffer, the filter was drawn rapidly through 50ml of wash buffer and then dried under vacuum. The filter was then cut into squares and analysed by scintillation counting (Figure 5.1[d]). The results of this experiment essentially bore out the findings of the previous experiments, i.e. calcium-binding was not detected for any of the α-actinins, despite obtaining reasonable results with gelsolin (2.95 Ca$^{2+}$/mol). Thirdly, we tried using a higher concentration of calcium (1mM) in the overlay buffer. In theory, this amendment should help binding if the sites are of very low affinity, and should also reduce the possibility that the divalent cation Mg$^{2+}$ (at 1mM) had competed for the binding sites. In this experiment, the filter was rapidly drawn through the wash buffer, as in the previous experiment. Calcium was also included in the wash buffer at a concentration of 1mM. However, calcium-binding still could not be detected for any of the α-actinins (Figure 5.1[e]). It was also of concern that this assay system gave far less accurate results for the control proteins under these conditions. For example, the stoichiometry of binding by the positive control protein, gelsolin, was found to be overestimated by tenfold (29.90 Ca$^{2+}$/mol). The fact that [Mg$^{2+}$] was not in excess over [Ca$^{2+}$] in these overlay conditions may have resulted in a greater degree of binding by calcium to non-specific sites. This is supported
Figure 5.4 Timecourse of Expression of the α-Actinin EF-hand Fusion Proteins in E. coli

Overnight Cultures (each 5ml) of cells transformed with EF-brain (lanes A to E) or EF-smooth (lanes F to J) were diluted 1/10 in fresh medium (final volume 10ml). The cells were grown to mid-log phase at 37°C induced by adding IPTG to a final concentration of 1mM. Samples (500μl) were taken at: 0 minutes (lanes A and F); 30 minutes (lanes B and G); 60 minutes (lanes C and H); 200 minutes (lanes D and I); and 240 minutes (lanes E and J) following induction with IPTG. Samples were pelleted for 1 minute in a high speed bench centrifuge. The supernatants were removed and replaced with an equal volume (500μl) of 1xSDS-PAGE sample buffer. The samples were boiled for 1 minute, and 50μl of each sample was subjected to 12% SDS-PAGE. The gel was then stained with Coomassie. The position and sizes (kDa) of the molecular weight markers are shown along the left hand side of the figure.
by the fact that the negative control protein cytochrome-C also appeared to bind Ca\(^{2+}\) in this experiment (7.07 Ca\(^{2+}/\text{mol}\)).

5.2.ii. Stopped-Flow Spectrophotometry.

Fusion proteins containing the EF-hands of chick brain or smooth muscle \(\alpha\)-actinin were expressed in \textit{E. coli} using the following strategy. The synthetic oligonucleotides EF-1 and EF-2 (see Section 2.15) were used to amplify cDNA fragments from either chick brain or chick smooth muscle \(\alpha\)-actinin cDNA templates. The amplified cDNA fragments were made to extend from the region encoding the end of the fourth repeat to the stop codon of each isoform (see Section 2.15), and therefore contained the region encoding both EF-hands. The amplified products (approx. 580bp) were digested with Bam HI (see Section 2.15), and ligated into Bluescript. It has been estimated, that during the PCR-amplification of DNA approximately 1 nucleotide in 9000 will be mis-incorporated, and approximately 1 in 41,000 nucleotides will be mis-incorporated and result in a frame-shift (Anglian Biotec.). The chick brain and chick amplified cDNA fragments were therefore sequenced on both strands in Bluescript, to check for mis-matches introduced by Taq polymerase. However, these fragments were found to be identical in sequence with respect to their parent cDNA sequences. The chick brain and smooth muscle inserts were liberated from Bluescript, using the restriction enzyme Bam HI, and cloned into the prokaryotic expression vector pGEX-1 (Smith and Johnson, 1988). The correct orientation and reading frame of the inserts in pGEX-1 was verified by sequencing across the insertion site using the synthetic oligonucleotide 5'GAGGCTCGGAACTCATT3' (which primes within each of the cDNA inserts). The pGEX-1 constructs containing the EF-hand encoding fragments of either chick brain or chick smooth muscle \(\alpha\)-actinins were referred to as EF-brain or EF-smooth respectively.

Cultures of \textit{E. coli} transformed with EF-brain or EF-smooth (or pGEX-1 plasmid alone) were grown to mid-log phase and induced with IPTG. Whole cell extracts made from the induced cultures were found to contain relatively large quantities of a 45-kDa protein (Figure 5.2), which correlated approximately with the predicted molecular weight of the fusion proteins (42-kDa). The 45-kDa fusion proteins were found to be stained specifically in western blots using antibodies against chick smooth muscle \(\alpha\)-actinin, but not when using pre-immune serum (Figure 5.3). Fusion protein was not detected in extracts of either non-transformed \textit{E. coli}, or \textit{E. coli} transformed with the pGEX-1 vector alone (Figure 5.3). A time-course of expression of the \(\alpha\)-actinin fusion proteins \textit{E. coli} is shown in Figure 5.4. The quantity of fusion protein was found to increase steadily over a period of 4hrs following induction with IPTG, with no visible
sign of degradation. The fusion proteins were purified to homogeneity (Figure 5.2) by affinity chromatography using glutathione/agarose as described in Section 2.15. The yield of protein obtained using this method was approximately 2mg per 250ml of bacterial culture. A large proportion of the fusion protein was also associated with the insoluble fraction of *E. coli* (Figure 5.2). The insoluble fusion protein may have been contained within cellular inclusion bodies (reviewed by Hartley and Kane, 1988).

The calcium-binding properties of the fusion proteins were measured using stopped-flow spectrophotometry (Figures 5.5 and 5.6). This work was done in collaboration with Drs A. Walmsley and C. Bagshaw (Department of Biochemistry, University of Leicester) using the apparatus described by Jackson and Bagshaw (1988). Chick brain α-actinin contains a cysteine residue within its N-terminal EF-hand (Chapter 4). In order to examine the possible effect of the formation of disulfide bonds between this cysteine and other cysteines present in the fusion protein, the first stopped-flow experiment was performed in the presence of 0.5mM DTT (Figure 5.5), and the second in the absence of DTT (Figure 5.6).

In the presence of DTT, gelsolin was found to be associated with calcium, and induced a strong increase in Quin-2 fluorescence (Figures 5.5). The data in Figure 5.5 (B) was fitted to a single exponential using the programme supplied with the Applied Photophysics stopped-flow apparatus. The amplitude of the fluorescence change was found to represent 35% of the total reaction amplitude. The single phase corresponded to a dissociation rate of 2.35s⁻¹. Gelsolin has been shown to contain two calcium-binding sites, and each binds a single ion with an affinity of 0.5-0.8μM (Way *et al.*, 1989). Despite the strong calcium-binding signals detected for gelsolin, both the chicken smooth muscle and chick brain fusion proteins of α-actinin were found not to bind calcium, and there was no increase in Quin 2 fluorescence (Figure 5.5). The traces given by these proteins were almost identical with those obtained with assay buffer alone (Figure 5.5). Similar results were obtained when the experiment was performed in the absence of DTT (Figure 5.6). Under these conditions gelsolin was found to give a strong calcium-binding signal. However, a significant change in fluorescence could not be detected for either the chick brain or the chick smooth muscle α-actinin fusion proteins. The traces given by these proteins were again almost identical with those obtained with assay buffer or expressed glutathione-S-transferase alone.

5.3 Discussion.

The calcium-binding activity chicken brain α-actinin was first measured using a dot-blot ⁴⁵Ca²⁺-overlay procedure. This technique is rapid and allows a large number of samples to be analysed simultaneously. Furthermore, it requires relatively
Figure 5.5 Analysis of the α-Actinin Fusion Proteins by Stopped-flow Spectrophotometry using the Fluorescent Reagent Quin-2 (in the Presence of DTT).

The α-actinin EF-hand fusion proteins were analysed by stopped-flow spectro­photometry using the fluorescent calcium chelator Quin-2, as described in Chapter 2.9. This experiment was performed in the presence of 0.5mM DTT. The proteins were expressed in E. coli and purified as described in Chapter 2, except for human plasma gelsolin which was a kind gift from Alan Weeds (MRC Laboratory of Molecular Biology, Cambridge). Calcium dissociation was measured over a time range of 1 second (A, C, E and G) or 4 seconds (B, D, F and H). The temperature was 20ºC. Quin-2 was used at a concentration of 200μM. A and B show the results obtained for gelsolin (200μg/ml). The data in B was fitted to a single exponential using the programme supplied with the Applied Photophysics stopped-flow apparatus. The amplitude of the fluorescence change was found to represent 35% of the total reaction amplitude. The single phase corresponded to a dissociation rate of 2.35s⁻¹. C and D show the results obtained for the chick brain α-actinin/glutathione-S-transferase fusion protein at a concentration of 300μg/ml. E and F show the results obtained for chick smooth muscle α-actinin/glutathione-S-transferase fusion protein at a concentration of 300μg/ml. G and H show the results obtained for reaction buffer alone (see Chapter 2). Each division on the vertical axis represents an increase in Quin-2 fluorescence of 4.6%.
small quantities of protein (50-200pmols spots) when compared with other techniques, such as equilibrium dialysis (Bennett et al., 1984) or continuous flow dialysis (Minowa and Yagi, 1984), which require several millilitres of protein at micromolar concentrations (a 2mg/ml solution of α-actinin). The latter point was obviously a major consideration in our choice of assay system, because the yield of brain α-actinin was fairly low (0.2-0.3mg/50g of wet tissue). Using this assay system reasonably accurate calcium-binding stoichiometries were obtained with the positive control protein gelsolin, and very little binding was detected for the negative control protein cytochrome C. However chicken brain α-actinin (like the chicken skeletal and smooth isoforms of the protein) was found to bind calcium at a level comparable with the negative control (cytochrome C). Chicken brain α-actinin may not have bound 45Ca2+ in this experiment simply because the assay system was unsuitable for analysing this particular protein. For example, brain α-actinin may not retain its native functional configuration when adsorbed onto nitrocellulose. In support of this hypothesis, rabbit macrophage α-actinin was shown not to bind 45Ca2+ when analysed by a similar 'dot-blot' assay (Pacaud and Hurricane, 1993) and also by SDS-PAGE/gel overlay (Pacaud and Molla, 1987). However, both calmodulin (Krinks et al., 1988) and spectrin (Wallis et al., 1992) have been shown to bind 45Ca2+ under these conditions, and these proteins also contain EF-hands. It is also possible that some aspects of the protocol used to purify chicken brain α-actinin might have caused it to become non-functional with respect to binding calcium. However, Duhammadan and Bamburg (1984) have shown that chicken brain α-actinin (purified using a very similar protocol to this study) is a calcium-sensitive protein by falling-ball viscometry. This suggests that chick brain α-actinin does retain the ability to interact with calcium following its purification from tissue.

In an effort to verify the findings of the dot-blot calcium-binding assay, chick brain and smooth muscle EF-hand fusion proteins were analysed by stopped-flow spectrophotometry, using the fluorescent calcium chelating compound Quin-2 as indicator (Tsien et al., 1982). Fusion proteins containing the EF-hands of either chick brain or chick smooth muscle α-actinin fused to glutathione-S-transferase were expressed in E. coli using the prokaryotic expression vector pGEX-1 (Smith and Johnson, 1988). When the stopped-flow experiments were performed no calcium-binding activity could be detected for either of the α-actinin fusion proteins, (Figure 5.5 and 5.6). In this situation, one could argue that calcium-binding was not detected because the EF-hands were not in their native configuration when attached to glutathione-S-transferase. Furthermore, the EF-hands of α-actinin may require additional interactions, absent from the fusion protein but present in the intact α-actinin dimer, so that they can function properly. α-Actinin forms an antiparallel homodimer,
Figure 5.6 Analysis of the α-Actinin Fusion Proteins by Stopped-flow Spectrophotometry using the Fluorescent Reagent Quin-2 (in the Absence of DTT).

The α-actinin EF-hand fusion proteins were analysed by stopped-flow spectrophotometry using the fluorescent calcium chelator Quin-2, as described in Chapter 2.9. The experiments were performed in the absence of DTT. The proteins were expressed in E.coli and purified as described in Chapter 2, except for human plasma gelsolin which was a kind gift from Alan Weeds (MRC Laboratory of Molecular Biology, Cambridge). The experiment was performed at 20°C. Quin-2 was used at a concentration of 400μM. The calcium dissociation curves shown are: (A) gelsolin at 0.2 mg/ml; (B) chick brain α–actinin/glutathione-S-transferase fusion protein at a concentration of 300μg/ml; (C) chick smooth muscle α–actinin/glutathione-S-transferase fusion protein at a concentration of 300μg/ml; (D) glutathione-S-transferase at 400μg/ml; and (E) reaction buffer alone. Note that the vertical scale varies from diagram to diagram, and each division on the vertical axis represents an increase in Quin-2 fluorescence of: 5% (A); 0.5% (B); 1% (C); 1% (D); and 0.5% (E). Therefore, the increase in Quin-2 fluorescence observed in curve A (gelsolin) is far more significant than the decrease in Quin-2 fluorescence observed in curves B to E. The reason behind the slight decrease in Quin-2 fluorescence observed in curves B to E is not known, but is likely to be artefactual since the effect was observed with reaction buffer alone (curve E). This effect may have been caused by partial decomposition of the Quin-2 reagent. This artefactual decrease in fluorescence may be masked when the protein being assayed releases a relatively large amount of calcium to Quin-2, such is the case with gelsolin (curve A).
and it is therefore conceivable that some residues within the ABD of the molecule may also participate in the co-ordination of the calcium ion.

Chick brain α-actinin is only predicted to contain one functional EF-hand, the N-terminal EF-hand. However, there is evidence that a functional N-terminal EF-hand may only bind calcium with high affinity when coupled with a functional C-terminal EF-hand. Schleicher et al. (1991) introduced point mutations at critical sites within the EF-hands of Dictyostelium α-actinin and looked at the calcium-sensitivity of the mutant proteins. Mutation of the N-terminal EF-hand alone was found to completely abolish calcium-sensitivity. Mutation of the C-terminal EF-hand alone did not abolish calcium-sensitivity, but 500 times more calcium was required to bring about inhibition. This suggests that the calcium-binding affinity of the N-terminal EF-hand is reduced considerably following the mutation of the C-terminal EF-hand (Schleicher et al., 1991). Furthermore, this data suggests that calcium may bind to α-actinin in a co-operative manner (Schleicher et al., 1991). When calcium binds to the C-terminal EF-hand of native Dictyostelium α-actinin, the molecule may change its conformation, and this change may enable the N-terminal EF-hand to bind calcium with high affinity. The binding of calcium to the N-terminal EF-hand may then induce a second change in the conformation of the molecule, which inhibits actin-binding. If the C-terminal EF-hand of chick brain α-actinin is non-functional then the N-terminal EF-hand of this protein may only bind calcium with very low affinity, and this may provide a further reason for the lack of detectable calcium-binding by chicken brain α-actinin in this analysis. The assay systems used in this study may have been too insensitive to detect the binding of Ca^{2+} to chick brain α-actinin.
Chapter 6. General Discussion
In chick brain there are distinct variants of non-muscle α-actinin which display a high level of sequence identity with their counterparts in chick smooth muscle and chick skeletal muscle tissues. For example, the sequence of the complete chick brain α-actinin cDNA 7a/9a is near identical with the sequence of a complete chick smooth muscle α-actinin cDNA, C17 (Baron et al., 1987b). These sequences are divergent only across a small stretch of sequence encoding the latter half of the N-terminal EF-hand and the EF-hand linker region. The large areas of total sequence identity and the localised region of divergence between these isoforms strongly suggests that they are encoded by the same gene. This has been confirmed by the sequence of several chick genomic clones derived from the 3' end of the smooth muscle/non-muscle α-actinin gene (Waites et al., 1992). The region of divergence is encoded on two separate exons which are alternatively spliced to give distinct mRNAs. Outside of this region, both isoforms are encoded by the same exons of the gene. Mutually-exclusive splicing of two adjacent exons is an uncommon event in non-viral genes, and the exceptional cases are usually confined to alternative muscle-specific splicing patterns (Waites et al., 1992). The majority of these cases are specifically modulated in skeletal muscle, such as troponin-T (Medford et al., 1984) and myosin light chain 1/3 (Periasamy et al., 1984). The α-actinin system is only the second example of a smooth muscle-specific modulation, the first case being exon 2 of rat α-tropomyosin (Wieczorek et al., 1988). It will be interesting to determine if the α-actinin and tropomyosin splicing systems are regulated by common smooth muscle-specific regulators of splicing.

Chick brain cells also express a second variant of non-muscle α-actinin, which displays a high degree of amino acid identity with the chick skeletal muscle isoform identified by Arimura et al. (1988). A partial cDNA encoding this variant (8W) was found to be identical with chicken skeletal muscle α-actinin, except in the region encoding the N-terminal EF-hand and the EF-hand linker peptide (Parr et al., 1992). The gene encoding chick skeletal muscle α-actinin has also been isolated and sequenced, and it is a distinct gene from the one encoding the chick smooth muscle/non-muscle isoforms (Parr et al., 1992). The chick skeletal muscle gene also contains two alternative exons in the region encoding the latter half of the N-terminal EF-hand and EF-hand linker peptides. These exons undergo alternative splicing, giving rise to the divergent EF-hand regions in the chick skeletal muscle isoform and the chick brain variant encoded by 8W (Parr et al., 1992). Therefore, in chick there are at least two genes for α-actinin, and both produce transcripts which are alternatively spliced. In human, three α-actinin genes have been identified to date, and each is located on a different chromosome (Youssoufian et al., 1990; Beggs et al., 1992). Firstly, there is the human non-muscle α-actinin gene on chromosome 14q22-24 (Youssoufian et al., 1990), which is presumably the human homologue of the chick gene encoding smooth muscle/non-
muscle α-actinin (Waites et al., 1992). Secondly, there is the gene encoding the human skeletal muscle α-actinin variant HuActSk1, which has been localised to chromosome 1q42-q43 (Beggs et al., 1992). HuActSk1 (104-kDa) is the human homologue of the chick skeletal muscle α-actinin isolated by Arimura et al. (1988). Thirdly, there is the gene encoding a second variant of human skeletal muscle α-actinin (HuActSk2), which is located on chromosome 11q13-q14 (Beggs et al., 1992). HuActSk2 (103-kDa) is a novel variant of skeletal muscle α-actinin, which has so far only been detected in human (see Chapter 1 and Figure 4.11). The extent to which these human genes undergo alternative splicing has yet to be determined. HuActSk1 and HuActSk2 are as distinct from one another as they are from the chick brain or human placental isoforms, displaying approximately 80% identity (Beggs et al., 1992). The high degree of amino acid sequence conservation between these respective proteins implies that the duplication events that gave rise to the various isoforms occurred before the divergence of the two species. Furthermore, the homologous genes created during this process have been highly conserved because of the functional constraints imposed on each isoform. Chick brain and human placental α-actinin only differ at 24 amino acid positions spread throughout the molecule, suggesting that every portion of the protein performs an important structural or functional role in vertebrate cells. For example, the highly conserved N-terminal region has been shown to interact with F-actin (Mimura and Asano, 1986; Hemmings et al., 1992), and also with the adhesion plaque protein zyxin (Crawford et al., 1992). The central region, containing 4 spectrin-like repeats, is important for the formation of the antiparallel dimer (Imamura et al., 1988), but also interacts with the transmembrane receptor, integrin (Pavalko and Burridge, 1991) and with long-chain fatty acids (Kahana and Gratzer, 1991). There is also some sequence in the C-terminal domain, flanking the EF-hand motifs, which is of unknown function. This sequence may have a structural role or may interact with some of the ligands that have already been described, or ligands that have not yet been characterized. This may explain why the high degree of sequence conservation between these vertebrate α-actinins extends throughout the whole length of the molecule. A similar argument may also be applied to the adhesion plaque protein vinculin, which is also highly conserved between human and chick (>95% amino acid identity) (Weller et al., 1990).

At this stage, it is not clear why chick brain cells express both smooth muscle α-actinin- and skeletal muscle α-actinin-based variants of the non-muscle isoform. These variants may contain distinct ligand binding sites and function in distinct areas of the cell. If both variants are expressed simultaneously within the same brain cell then there is the potential for the formation of heterodimers. However, this may be prevented by separating the translation of each variant, either spatially or temporally. It is also possible that the skeletal muscle α-actinin-based variant of the non-muscle isoform is
only expressed at a basal level in adult chick brains. This is supported by the N-terminal amino acid sequence of several thermolytic cleavage polypeptides of tissue-purified chick brain α-actinin (Chapter 3). These polypeptides exactly matched the sequence of chick smooth muscle α-actinin in the region of the ABD and the repeats. The amino acid sequence trace showed no indication of sequence derived from the skeletal muscle α-actinin-based variant encoded by 8W, suggesting that it is expressed at a very low level in adult chick brains. Complementary DNAs encoding the smooth muscle α-actinin-based variant of non-muscle α-actinin have been isolated from several non-muscle cDNA libraries, which include: chick fibroblast (Baron et al., 1987a); chick brain (this study, Waites et al., 1992); human placenta (this study, Millake et al., 1989); human endothelia (Yousoffian et al., 1990); and human hepatoma (Nishiyama et al., 1990). However, cDNAs encoding the skeletal muscle α-actinin-based variant of non-muscle α-actinin have so far only been isolated from one cDNA library (see Chapter 4 and Parr et al., 1992). This variant may only be expressed at specific stages during development and in specific tissues. Some support for this hypothesis has come from a quantitative assay involving PCR methodology (Parr et al., 1992). In adult chick non-muscle tissues (such as brain and liver) the mRNA corresponding to 8W is transcribed at a far lower level than the non-muscle variant encoded by 7a/9a and also the smooth muscle isoform. The skeletal muscle mRNA is also transcribed at higher levels (~3x) than its alternative spliceomer in adult non-muscle tissues. However, in chick embryo cDNA derived from whole embryo or fibroblast the situation is reversed, with the 8W-type mRNA transcribed at higher levels than the skeletal muscle isoform (Parr et al., 1992). A larger 'embryonic' variant of skeletal muscle α-actinin has been observed in extracts of chick skeletal muscle cells (Kobayashi et al., 1989). The embryonic variant is present in extracts of skeletal muscle tissues derived from chick embryos up to 21 days old, but is replaced with the 'adult' isoform of skeletal muscle α-actinin (Kobayashi et al., 1989). At present the relationship between the chick brain variant encoded by 8W and the embryonic variant of chick skeletal muscle α-actinin is not known, but conceivably they could be the same protein.

The non-muscle α-actinins derived from chick brain, chick fibroblast, human placental, human endothelia and human hepatoma are each predicted to bind a single calcium ion per subunit (Arimura et al., 1988; Millake et al., 1989; Yousoffian et al., 1990; Nishiyama et al., 1990; Waites et al., 1992). In contrast, those derived from smooth and skeletal muscle tissues are each predicted not to bind calcium (Baron et al., 1987b; Arimura et al., 1988; Waites et al., 1992; Beggs et al., 1992). This would appear to explain why sensitivity to calcium is restricted to the non-muscle isoform of α-actinin. However, when the calcium-binding activity of chick brain α-actinin was analysed by two independent assay procedures no binding activity could be detected,
suggesting that either the protein does not bind calcium or else the assay systems used in this analysis were unsuitable for this protein. For instance, the EF-hands of chick brain α-actinin may bind calcium with very low affinity \( (80-100\, \mu\text{M}) \) and this level of binding may have been beyond the sensitivity of the assay systems used in this study. However, this level of binding may be sufficient to induce the observed calcium sensitivity of chicken brain α-actinin (Duhaiman and Bamburg, 1984). Another possibility is that the chicken brain isoform may bind calcium with higher affinity when associated with F-actin, and F-actin might actually provide some of the calcium co-ordination points. In this situation, α-actins with low calcium binding affinities (when assayed in the absence of F-actin) might clearly display calcium sensitivity when associated with F-actin. Initially, this hypothesis could be investigated by performing viscometry or high speed sedimentation experiments with F-actin (in low and high \( [\text{Ca}^{2+}] \)) using the chick brain α-actinin purified by the method described in Chapter 3. A demonstration of calcium sensitivity would provide some support for the hypothesis, but would need to be backed up with unambiguous calcium binding data demonstrating a low affinity for the interaction. If on the other hand calcium sensitivity is not observed then an obvious conclusion is that the chicken brain α-actinin purified in this analysis is non-functional with respect to the equivalent material utilized by Duhaiman and Bamburg (1984). Non-functionality may have stemmed from partial denaturation or slight proteolysis of the protein during purification and subsequent analysis. However, there was no obvious indication of this having occurred to the chick brain protein during this study. In the case of the EF-hand fusion proteins described in Chapter 5, the EF-hands of chick brain α-actinin may not have retained their native calcium-free conformation when attached to glutathione-S-transferase. This problem might be overcome in future experiments by designing constructs encoding α-actinin C-terminal domains with varied length EF-hand flanking sequence. Alternatively, the EF-hand segments might be expressed using different pGEX vectors, such as pGEX-2 or pGEX-3 (Smith and Johnson, 1988). These vectors encode cut sites for the proteases factor-X or thrombin at the junction of the fusion protein, enabling the glutathione-S-transferase to be cleaved away from the protein, and then removed by affinity chromatography using glutathione/agarose. If the EF-hand peptides prove to be stable and soluble on their own, then it may be possible to monitor their change in conformation on going from the calcium-free to the calcium-saturated state, using circular dichroism (CD) or nuclear magnetic resonance (NMR). NMR spectroscopy can be useful in measuring subtle changes in the microenvironment of specific residues during the binding of calcium, such as those determined for calmodulin (Ikura et al., 1983). If the calcium binding/dissociation process is very rapid then the possibility remains that the process may have occurred during the dead-time of the stopped flow instrument (~1ms). Assays based on conformational change, such as
CD, NMR or tyrosine/tryptophan fluorescence (see below) will not be subject to this limitation.

Chicken brain was chosen as the source of non-muscle α-actinin in this study, largely because this isoform has been well characterized as a calcium sensitive non-muscle α-actinin. However, many problems were encountered whilst trying to obtain a suitable and adequate supply of chicken heads for this analysis. Considering the low yield of chick brain α-actinin described in Chapter 3 (and also the possibility that the limited amount of protein obtained may have been rendered non-functional at some stage during the preparation) it may be necessary in future experiments to choose an alternative protocol/source for the non-muscle isoform. Several non-muscle preparations have been published including: chicken liver (Kuo et al., 1982); bovine brain coated vesicle fraction (Schook et al., 1978); and rabbit macrophage (Bennett et al., 1984; Pacaud and Harricane 1993). Proteins obtained from these sources might initially be assayed using the methods described in Chapter 5. However, if we can obtain non-muscle α-actinin with greater yield from these sources, then we would be in a position to consider using alternative assay procedures, such as equilibrium dialysis (Bennett et al., 1984) or tyrosine/tryptophan fluorescence (Pacaud and Harricane, 1993). Using equilibrium dialysis, Bennett et al. (1984) observed that rabbit macrophage α-actinin binds 4 mol of Ca\(^{2+}\)/mol of protein, with a dissociation constant of 4.0x10\(^{-6}\)M in the presence of 2mM MgCl\(_2\) and 100mM KCl. In the absence of these ions Pacaud and Harricane (1993) found that [Ca\(^{2+}\)] ranging from 10-100\(\mu\)M induced significant perturbations in the environment of Tyr and Trp residues of rabbit macrophage α-actinin. However, Mg\(^{2+}\) also bound to this protein giving rise to the same spectral perturbations, suggesting that this bivalent cation might act by a similar mechanism to Ca\(^{2+}\). Indeed, Mg\(^{2+}\) and Ca\(^{2+}\) compete for identical sites on calmodulin (Crouch and Klee, 1980; Haeich et al., 1981). The spectral changes induced by Ca\(^{2+}\) and Mg\(^{2+}\) were not detectable in the presence of 140mM KCl, suggesting that monovalent cations might also bind to α-actinin (Pacaud and Harricane, 1993). The K\(^{+}\) ions may interact with negative charges on the protein thereby decreasing its Ca\(^{2+}\)/Mg\(^{2+}\) binding affinity, without interacting directly with the EF-hands of the protein. Pacaud and Harricane (1993) conclude that under physiological ionic conditions the apparent affinity of α-actinin for Ca\(^{2+}\) may be low (-80-100\(\mu\)M). However, it is noteworthy that all of the viscometry/sedimentation experiments demonstrating the calcium sensitivity of non-muscle α-actinin were performed in the presence of 1-4mM Mg\(^{2+}\) and 40-100mM KCl (Burridge and Feramisco, 1981; Duhaiman and Bamburg, 1984; Bennett et al., 1984; Landon et al., 1985), conditions which would appear to inhibit the binding of Ca\(^{2+}\) to α-actinin. In this analysis, the affects of K\(^{+}\) and Mg\(^{2+}\) on the calcium binding activity of chick brain α-actinin were not investigated, but such considerations will obviously need
to be made in all future experiments. The equilibrium dialysis experiments of Bennett et al. (1984) were performed in the presence of 50μM EGTA. Metal binding proteins such as calmodulin and parvalbumin bind EGTA with high affinity (Haiech et al., 1979, 1980). α-Actinin may also interact with EGTA and this may cause it to bind Ca²⁺ with anomalously high binding stoichiometry/affinity. Pacaud and Harriance (1993) did not include EGTA in their assay conditions (nor was it included in this study) and this might explain why their α-actinin bound to Ca²⁺ with >20 fold lower affinity than that of Bennett et al. (1984).

The C-terminal EF-hand of chick brain α-actinin is predicted to be non-functional and recent experiments suggest that this might reduce the binding affinity of the predicted functional N-terminal EF-hand. For example, Schleicher et al. (1991) introduced point mutations into the EF-hands of Dictyostelium α-actinin, and looked at the ability of the mutant proteins to cross-link F-actin in the presence of Ca²⁺. The single mutation of the N-terminal EF-hand completely abolished the calcium-sensitivity of Dictyostelium α-actinin. The single mutation of the C-terminal EF-hand did not remove calcium-sensitivity completely but 500-fold more calcium was required to observe the affect produced by the N-terminal EF-hand. This strongly suggests that the calcium-binding affinity of the N-terminal EF-hand is reduced considerably following the mutation of the C-terminal EF-hand (Schleicher et al., 1991). Similarly, the predicted functional N-terminal EF-hand of chick brain α-actinin may not bind calcium with high affinity when associated with the non-functional C-terminal EF-hand. It will be interesting to investigate this possibility by making site-directed mutants of chick brain α-actinin. For example, the C-terminal EF-hand could be engineered into a predicted functional calcium-binding site. If the hypothesis described above is correct then the mutant protein should bind calcium with much higher affinity. The function of the extended EF-hand linker peptide of chick brain α-actinin could also be investigated by making a series of site-directed or deletion mutants in this region, and studying the affects of these changes on the calcium-sensitivity of the protein by falling-ball viscometry. If chick brain α-actinin does bind calcium with very low affinity then the physiological relevance of this event must also be explored. Similar experiments have been performed in-vivo using a severely disrupted double mutant of Dictyostelium, which lacks the proteins α-actinin and ABP-120 (see Chapter 1 and Witke et al., 1992). The disturbed phenotype could be rescued by inserting a construct expressing a mutant of Dictyostelium α-actinin with low calcium-binding affinity (containing a functional N-terminal EF-hand and non-functional C-terminal EF-hand). However, it could not be rescued with a mutant of Dictyostelium α-actinin lacking the ability to bind calcium (containing a non-functional N-terminal EF-hand and a functional C-terminal EF-hand). This strongly suggests that the calcium-sensitivity of non-muscle α-actinin is required
for normal development in Dictyostelium, and that non-muscle α-actinins with very low
calcium-binding affinities can function in-vivo. The molecular mechanism underlying the
calcium-sensitivity of non-muscle α-actinin will only become fully resolved when the
crystal structures of this protein (in the calcium-free and calcium-saturated states) have
been determined. Now that we have complete cDNAs encoding both chick and human
non-muscle α-actinins we are in a position to express large quantities of the proteins in
prokaryotic (or eukaryotic) systems for crystallisation studies.

Baron et al. (1987b) were the first to suggest that the non-muscle and smooth
muscle isoforms of α-actinin may be expressed simultaneously in non-muscle cells,
performing unique functions at distinct sites within the cell. Indeed, using a quantitative
assay based on the polymerase chain reaction it has been shown that MRC-5 fibroblasts
and HeLa cells do express mRNAs encoding both isoforms (Waites et al., 1992). The
actin filaments in MRC-5 fibroblasts are more tightly organised and 'muscle-like', than
those of HeLa cells (Waites et al., 1992). However, the adhesion plaques of HeLa cells
are far more prominent than those found in MRC-5 cells (Waites et al., 1992). This
observation may be a reflection of the proportion of the smooth muscle isoform of α-
actinin in MRC-5 cells (25%) when compared with HeLa cells (3%) which contain more
of the non-muscle isoform (97%) (Waites et al., 1992). These results appear to suggest
that the calcium-insensitive smooth muscle isoform might be required for the formation
of stress fibres, which contain a muscle-like sarcomeric arrangement of cytoskeletal
proteins (Langanger et al., 1985; Drenckhahn and Wagner, 1986). In contrast, the
calcium-sensitive non-muscle isoform might be localized at the cell-matrix junctions
(Baron et al., 1987b). Indeed, Namalwa lymphoblastoid cells which lack actin stress
fibres, only express the non-muscle mRNA (Waites et al., 1992). To investigate this
hypothesis complete cDNAs encoding chick brain or chick smooth muscle α-actinin
were expressed in monkey COS cells and their intracellular localizations were probed
using an antibody specific for chicken α-actinin. Surprisingly, both isoforms were
found to target to both the stress fibres and cell-matrix junctions. Chick skeletal muscle
α-actinin was also found incorporated into these sites in transfected mouse fibroblasts
(Tokkue et al., 1992). Similar results were also obtained when α-actinins from
Dictyostelium, calf thymus, chicken smooth muscle or chicken skeletal muscle were
microinjected into non-muscle cells (Schulze et al., 1989; Meigs and Wang 1986;
McKenna et al., 1985; Sanger et al., 1986). Taken together these results strongly
suggest that a differential localisation of muscle and non-muscle isoforms does not occur
in non-muscle cells. However, it remains possible that the high levels of intracellular α-
actinin achieved by microinjection or transient expression leads to aberrant localization of
the protein. So far, a differential localisation of distinct α-actinin isoforms within the
same cell has only been clearly observed in fused myoblasts (Endo and Masaki, 1984).
In these cells, antibodies rendered specific for the skeletal muscle isoform (by cross-absorption against smooth muscle \(\alpha\)-actinin) were found to stain the Z-discs specifically, whereas antibodies specific for the smooth muscle isoform only detected membrane-bound structures. Indeed, when complete cDNAs encoding chick smooth muscle \(\alpha\)-actinin were transfected into skeletal muscle myotubes, the expressed protein could only be detected at membranous sites (Personal communication: Dr. D. Critchley, Department of Biochemistry, University of Leicester). It will be interesting to determine the localisation of the two variants of chick brain \(\alpha\)-actinin in fused myoblasts. The smooth muscle \(\alpha\)-actinin-based variant of non-muscle \(\alpha\)-actinin is very similar in sequence to the smooth muscle isoform (97% identity) and is therefore likely to target to the membranous sites. Similarly, the skeletal muscle \(\alpha\)-actinin-based variant of non-muscle \(\alpha\)-actinin may associate with the Z-discs of fused myoblasts by virtue of its homology with the chick skeletal muscle isoform.
References
265, 9236-9240.
266, 12989-12993.
100, 237-243.
140, 253-260.
Brink, M., Gerisch, G., Isenberg, G., Noegel, A., Segall, J. E., Walraff, E., and
314, 469-472.
46, 587-597.
Byers, T. J., Husain-Chishti, A., Dubreuil, R. R., Branton, D., and Goldstein, L.S.B.
28, 8506-8514.
39, 313-320.


Gerlin, J. B., Yamin, R., Egan, S., Stewart, M., Stossel, T. P., Kwiatkowski, D. J.,
345, 132-135.
12, 579-584.
272, 149-151.
18, 2752-2758.
105, 18-23.
Acta. 1061, 121-131.
116, 1369-1380.
Herrenknecht, K., Ozawa, M., Eckerskorn, C., Lottspeerh, F., Lenter, M., and
Sci. USA 83, 6470-6474.
14, 271-287.
Jones, P., Jackson, P., Price, G. J., Patel, B., Ohanion, V., Lear, A. L., and
101, 2223-2232.


Nucleic Acids Res. 13, 1347-1368.

Cell Motil. Cytoskel 17, 276-290.

Medford, R. M., Nguyen, H. T., Destree, A. T., Summers, E., and Nadal-Ginard, B.


Research 17, 6725.


Commun. 171, 1217-1223.


FEBS Letters 224, 156-160.


Nishiyama, M., Ozturk, M., Frohlich, M., Mafune, K., Steele, G., and Wands, J. R.
Winkelmann, J. C., Leto, T. L., Watkins, P. C., Eddy, R., Shows, T. B.,
Linnenbach, A. J., Sahr, K. E., Kathuria, N., Marchesi, V., and Forget, B. G.,
Current Topics in Membranes 38, 207-215.
47, 62-72.
581, 365-370.
Zubrzycka-Gaarn, E. E., Bulman, D. E., Karpati, G., Burghes, A. H. M., Belfall, B.,
Nature 333, 466-469.