Identification and Characterisation of Proteins Associating with the Collagen Homology Domains of p66Shc.

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester.

By

Mark John Wright B.Sc. (Hons) ARCS.

July 2002
UMI Number: U161433

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.

UMI U161433
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author. Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346
Identification and Characterisation of Proteins Associating with the Collagen Homology Domains of p66Shc.

Abstract

Mark John Wright

The Src-homology collagen protein (Shc) is expressed as three isoforms of 46, 52 and 66 kDa. The 66 kDa isoform of Shc (p66Shc) has been reported to inhibit the MAPK pathway and to sensitize cells to oxidative stress. To investigate the unique characteristics of the p66Shc isoform, it was attempted to identify proteins that associate with the second collagen homology domain (CH2). Using the yeast two-hybrid system, the receptor tyrosine phosphatase Lar and the colonic and hepatic tumour over expressed gene Ch-Tog were identified as binding partners for the CH2 domain of p66Shc. These observations were verified through co-immunoprecipitation and GST fusion protein precipitations. A common motif of SSKXXQ was identified in both Lar and Ch-Tog. Mutational analysis of this motif in Lar demonstrates that lysine 1285 of human Lar is essential for its association with p66Shc, as demonstrated with the yeast two-hybrid system, co-immunoprecipitation and GST fusion protein precipitation experiments. Although p66Shc is constitutively associated with the Lar tyrosine phosphatase, it is not, however a substrate of Lar. Ch-Tog is a component of the spindle checkpoint. Activators of the spindle checkpoint such as the anticancer drug Taxol induce a substantial serine phosphorylation of p66Shc. p66Shc is required for the efficient apoptosis of mouse embryo fibroblast cells as cells lacking p66Shc show considerable resistance to Taxol even at high doses (1μM). Taxol induced serine phosphorylation of p66Shc is an M phase specific event that is independent of microtubule condition. Jnk, p38 and Erk activation are not involved in the resistance of p66Shc knockout cells to Taxol. The Cyclin dependent kinase inhibitor Roscovitine completely inhibits the serine phosphorylation of p66Shc after Taxol treatment. In gel kinase assays have identified five kinases capable of phosphorylating the CH2 domain of p66Shc, but their identity is not yet known. Jnk, p38, Cdk1, Erk1 and Erk2 do not phosphorylate the CH2 domain of p66Shc in vitro.
Acknowledgements.

I would like to thank Dr. Sally Prigent for all her support and guidance throughout my time in the laboratory. I am grateful to all the members of the laboratory for making my time in laboratory and in Leicester a pleasure.

I am grateful to my work colleagues for their cooperation and valuable advice. I would especially like to express my gratitude to Dr. R. Patel for his help with the kinase assays and reagents. Many thanks also to Dr. A. Hyman for providing antibodies to Ch-Tog.

Finally, I would like to thank my wife, Shona, for her help with the preparation of my thesis and her support the whole time of my research.
CONTENTS

Title........................................................................................................................... I
Abstract....................................................................................................................... II
Acknowledgements .................................................................................................... III
Contents..................................................................................................................... IV
List of Figures........................................................................................................... X
List of Tables............................................................................................................ XIII
Abbreviations............................................................................................................ XIV

Chapter 1. Introduction ........................................................................................... 1
1.1 Overview of She signalling ................................................................................. 1
  1.1.1. Intracellular signal Transduction ............................................................. 1
  1.1.2. The She family of adaptor proteins have a conserved domain structure 2
  1.1.3. She binds phosphorylated tyrosine motifs in the intracellular domains of activated cell surface receptors ........................................................... 4
1.2 Signal transduction pathways involving She activation ............................... 6
  1.2.1. She is involved in the activation of the extracellular regulated kinase (ERK) signalling pathway ................................................................. 6
  1.2.2. She associates with the SH2 domain containing inositol phosphatase SHIP ................................................................. 8
  1.2.3. She is involved in cell motility and cytoskeletal organisation ............... 10
  1.2.4. The She tyrosine phosphorylation site 239/240 and 317 are differentially regulated and may be functionally distinct ....................... 12
1.3 She expression is essential for cardiac and vascular tissue development during embryogenesis ................................................................. 15
1.4 The She family members Sck and N-Shc show distinct tissue localisation and function ................................................................. 17
  1.4.1 N-Shc .......................................................................................................... 17
  1.4.2 Sck ............................................................................................................... 18
  1.4.3 N-Shc and Sck have some overlapping and some unique functions ....... 18
1.5 p66She.................................................................................................................. 20
1.5.1 The p66Shc isoforms acts as a negative regulator of the MAPK pathway................................................................. 20
1.5.2 p66Shc is serine phosphorylated at residues within the CH2 domain...... 22
1.5.3 p66Shc is phosphorylated at serine residue 36 in response to oxidative stress............................................................... 24

1.6 Microtubule disrupting drugs induce the serine / threonine phosphorylation of p66Shc.................................................. 28
1.7. Microtubule drugs activate the spindle checkpoint and arrest the cell cycle at mitosis.............................................................. 28

Chapter 2. Materials and Methods............................................................... 34
2.1 Materials............................................................... 34
2.1.1 General chemicals and reagents......................................................... 34
2.1.2 Bacterial Strains and growth media..................................................... 34
2.1.3 Yeast strains and growth media........................................................... 34
2.1.4 Antibodies......................................................................................... 34
2.1.5 Mammalian cell culture................................................................. 35
2.1.6 Cell culture media and supplements.................................................. 35
2.1.7 Drugs and inducers of signal transduction......................................... 35

2.2 Methods................................................................. 36
2.2.1 Yeast................................................................. 36
   2.2.1.1 Yeast Media................................................................................. 36
   2.2.1.2 Preparation of transformation competent L40................................ 37
   2.2.1.3 Yeast two-hybrid growth assay..................................................... 37
   2.2.1.4 Yeast two-hybrid β-galactosidase assays...................................... 38
2.2.2: Tissue culture................................................................................ 39
   2.2.2.1 Maintenance of cell lines................................................................. 39
   2.2.2.2 Storage of cells............................................................................... 39
   2.2.2.3 Recovery of frozen cells................................................................. 40
   2.2.2.4 Transfection of Hek293 cells......................................................... 40
   2.2.2.5 Cell cycle analysis......................................................................... 40
   2.2.2.6 Determination of apoptosis............................................................ 40
2.2.5.6 Electroporation of recovered plasmids into electrocompetent HB101............................................................................................... 56
2.2.5.7 Selective isolation of library plasmids............................................. 56

Chapter 3. Identification of proteins interacting with the second collagen homology domain of Shc using the yeast two-hybrid system................. 57

3.1 Introduction.......................................................................................... 57
3.2 Results................................................................................................. 60

3.2.1 Characterisation of proteins interacting with the CH1 domain of Shc.................................................................................................. 60
3.2.2 Characterising the CH2 domain construct for use in the screening of cDNA libraries........................................................................ 63
3.2.3 Selection of suitable cDNA libraries for use in yeast two-hybrid library screening........................................................................... 64
3.2.4 Results of first yeast two-hybrid library screen................................. 65
3.2.5 Further characterisation of clone 23.2.............................................. 67
3.2.6 Optimisation of the yeast two-hybrid screen.......................................... 71
3.2.7 Results of the second library screen................................................... 72
3.2.8 p66Shc interacts with the juxtamembrane region of Lar...................... 73
3.2.9 The microtubule associated protein Ch-Tog interacts with the CH2 domain of p66Shc................................................................. 75
3.2.10 A common sequence motif is present in the clones identified in the yeast two hybrid library screens....................................................... 76

3.3 Discussion............................................................................................ 78

Chapter 4. Further characterisation of the interaction of Ch-tog and Lar with the CH2 domain of p66Shc................................................................. 86

4.1 Introduction.......................................................................................... 86
4.2 Results................................................................................................. 87

4.2.1 Preparation of a GST / CH2 domain fusion protein.............................. 87
4.2.2 Lar associates with p66Shc in Lar transfected Hek293 cells............... 88
4.2.3 Mutational analysis of the CH2 domain-binding site in Lar.................. 90
4.2.4 The SSKXXQ motif of human Lar is required for its association with p66Shc .......................................................... 91

4.2.5 Tyrosine phosphorylated p66Shc is not a substrate for Lar ............................................................ 95

4.2.6 The microtubule binding protein Ch-Tog interacts with p66Shc .......................................................... 96

4.2.7 Co-localisation studies between Lar, Ch-Tog and p66Shc .......................................................... 98

4.3 Discussion ............................................................................................................................................. 101

Chapter 5. Microtubule disrupting drugs induce serine / threonine phosphorylation of the CH2 domain of p66Shc .......................................................... 103

5.1 Introduction ....................................................................................................................................... 103

5.2 Results ............................................................................................................................................... 107

5.2.1 Taxol induces p66Shc serine / threonine phosphorylation ........................................................... 107

5.2.2 Taxol induced p66Shc phosphorylation is specific to M phase .................................................. 108

5.2.3 Microtubule disruption alone does not induce p66Shc phosphorylation ........................................ 110

5.2.4 The cyclin dependent kinase inhibitor Roscovitine inhibits p66Shc phosphorylation after Taxol treatment ...................................................................................................................... 112

5.2.5 The CH2 domain of p66Shc is a substrate for up to five unidentified protein kinases in an in gel kinase assay .......................................................................................................................... 116

5.2.6 The CH2 domain of p66Shc is not phosphorylated by p38, Jnk, Erk1, Erk2 or Cdk1 in vitro ............... 120

5.3 Discussion ....................................................................................................................................... 121

Chapter 6. p66Shc expression is required for efficient induction of apoptosis by Taxol and other microtubule disrupting drugs .......................................................... 125

6.1 Introduction ....................................................................................................................................... 125

6.1.1 Taxol efficiently induces apoptosis in rapidly proliferating cells ..................................................... 125

6.1.2 Taxol mediated apoptosis is independent of the tumour suppressor p53 ......................................................................................................................................................... 126

6.2 Results ............................................................................................................................................... 129

6.2.1 Taxol efficiently induces apoptosis in mouse embryo fibroblast cells .................................................. 129

6.2.2 p66Shc -/- MEF cells show resistance to Taxol induced apoptosis .................................................. 131

6.2.3 p66Shc -/- cells are resistant to high concentrations of Taxol ............................................................. 134
6.2.4 Blocking entry to mitosis prevents apoptosis in MEF cells...................... 135
6.2.5 The cyclin dependent kinase inhibitor Roscovitine protects MEF
cells against Taxol mediated apoptosis ...................................................... 137

6.3 Discussion .......................................................................................................... 139

Chapter 7. General discussion ................................................................................ 142

References ................................................................................................................. 149
List of figures.

Chapter 1.

1.1 The domain structure of the Shc protein family .................................................... 3
1.2 Genomic organisation of the Murine Shc locus and the assembly of Shc transcripts ............................................................................................................... 4
1.3 Direct interactions with the individual domains of Shc ......................................... 12
1.7 Shc signalling cascades ........................................................................................ 16
1.5 The primary sequence of the CH2 domain of p66Shc ........................................... 25
1.6 Pathways to ensure the fidelity and timing of mitosis.......................................... 31
1.7 Two branches of the spindle checkpoint ................................................................ 32

Chapter 3.

3.1 The yeast two hybrid system ................................................................................... 58
3.2 The SH2 domains of Gads, Grb2 and Shc interact with the CH1 domain of Shc ........................................................................................................................ 61
3.3 The SH2 domain of Sck interacts strongly with Y317 in the CH1 domain of Shc ...................................................................................................................... 62
3.4 p66Shc expression is widespread throughout the adult and embryonic tissues ................................................................................................................................. 64
3.5 Clone 23.2 specifically associates with the CH2 domain of p66Shc .................... 66
3.6 Clone 23.2 is expressed during embryogenesis ..................................................... 67
3.7 A PCR approach to obtain a larger fragment of clone 23.2 .................................. 68
3.8 PCR amplification of 23.2 sequence generates multiple bands .............................. 69
3.9 The original clone 23.2 DNA sequence is present in the cloned material ............ 70
3.10 The PCR cloning technique generates a 750bp fragment containing clone 23.2 sequence and novel 3’sequence ................................................................. 71
3.11 The domain structure of Lar ................................................................................. 74
3.12 The shortened construct containing only the juxtamembrane sequence retains the ability to interact with the CH2 domain of p66Shc ............................... 75
3.13 Ch-Tog also interacts with the CH2 domain of p66Shc in the Yeast Two-Hybrid assay ................................................................. 76

X
Chapter 4.

4.1 GST/CH2 fusion protein is partially degraded during purification of the GST/CH2 fusion protein to yield three forms of the fusion protein ................................................. 87
4.2 Lar binds to a GST fusion protein of the CH2 domain of p66Shc and co-immunoprecipitates with Shc from Hek 293 cells ................................................................. 89
4.3 Individual point mutations of the SSKXXQ motif reduce the affinity of the CH2 domain for LAR in the yeast two-hybrid system ......................................................... 91
4.4 The lysine 1285 mutant of human LAR is post translationally processed and expressed at similar levels to WT LAR ................................................................. 93
4.5 Lysine 1285 is essential for the interaction of LAR with the CH2 domain p66Shc ..................................................................................................................... 94
4.6 p66Shc is not a substrate of LAR ........................................................................... 96
4.7 Ch-Tog interacts specifically with the CH2 domain of p66Shc in Hek293 cells .......................................................................................................................... 97
4.8 Expression of a full-length ds red protein tagged human p66Shc construct in Hek293 cells ............................................................................................................ 99
4.9 Production of polyclonal antibodies against the CH2 domain of p66Shc ............. 100

Chapter 5.

5.1 Simplified scheme of Jnk and p38 signal transduction pathways ....................... 105
5.2 p66Shc is phosphorylated after Taxol treatment .................................................. 108
5.3 Taxol induced p66Shc phosphorylation is specific to M phase ......................... 109
5.4 Phosphorylation of p66Shc is M phase specific and occurs independently of microtubule injury ................................................................................................. 111
5.5 The cyclin dependent kinase inhibitor Roscovitine inhibits Taxol induced p66Shc phosphorylation ......................................................................................... 113
5.6 Roscovitine inhibits p66Shc phosphorylation at concentrations greater than 40μM ................................................................................................. 114
5.7 Cell cycle analysis confirms that Roscovitine treatment will disrupt entry to mitosis ..................................................................................................................... 115
5.8 The CH2 domain of p66Shc is phosphorylated by several kinases enzymes following hydrogen peroxide and Taxol treatment ....................................................... 118
5.9 The CH2 domain of p66Shc is not phosphorylated by Jnk, p38, Cdk1, Erk1 or Erk2 ................................................................................................ 120

Chapter 6.

6.1 Apoptotic pathways induced by low doses of Taxol ............................................. 126
6.2 Cellular responses to high doses of Taxol .............................................................. 127
6.3 MEF cells undergo distinct apoptotic nuclear changes after Taxol treatment ....... 130
6.4 FACS analysis confirms G2/M phase cell cycle arrest leading to apoptosis in Taxol treated MEF cells .......................................................................................... 131
6.5 Shc knockout MEF cells show an increased resistance to Taxol-induced apoptosis .............................................................................................................. 132
6.6 Puromycin does not affect apoptosis levels in the p66Shc +/- MEF cell line ...... 132
6.7 Shc expression in Wt MEF cells, Shc +/- and p66Shc +/- MEF cells ..................... 134
6.8 p66Shc +/- MEF cells are resistant to high doses of Taxol ................................... 134
6.9 p66Shc MEF cells do not show increased resistance to microtubule damage induced apoptosis ......................................................................................... 135
6.10 High doses of Taxol induce Jnk and p38 activation in MEF cells ...................... 136
6.11 Roscovitine inhibits Taxol mediated apoptosis in MEF cells .......................... 137

Chapter 7.

7.1 p66Shc acts to recruit Lar to its substrates ......................................................... 144
7.2 p66Shc phosphorylation is involved in the apoptotic process induced by mitotic arrest ................................................................................................. 147
List of tables.

Chapter 3.

3.1 The yeast two-hybrid library screen identifies a potentially novel protein sequence that interacts with the CH2 domain of p66Shc ........................................  65

3.2 The yeast two-hybrid library screen identifies two possible associating proteins, LAR and Ch-Tog .............................................................................................  73

3.3 The yeast two-hybrid library screen selects serine rich basic sequences ..........  77
**Abbreviations.**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-AT</td>
<td>3-amino-1,2,4-triazole.</td>
</tr>
<tr>
<td>A</td>
<td>Absorbance.</td>
</tr>
<tr>
<td>AD</td>
<td>Activation domain.</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase promoting complex/cyclosome.</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate.</td>
</tr>
<tr>
<td>ATF2</td>
<td>Activating transcription factor 2.</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate.</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma / leukaemia 2.</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pairs.</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin.</td>
</tr>
<tr>
<td>BUB</td>
<td>Budding uninhibited by benzimidazole.</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase.</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA.</td>
</tr>
<tr>
<td>CH1</td>
<td>Collagen homology domain 1.</td>
</tr>
<tr>
<td>CH2</td>
<td>Collagen homology domain 2.</td>
</tr>
<tr>
<td>Ch-Tog</td>
<td>Colonic and hepatic tumour over expressed gene.</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled water.</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide.</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid.</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease.</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxy nucleotide triphosphate.</td>
</tr>
<tr>
<td>DOM</td>
<td>Drop out mix.</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded.</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol.</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence.</td>
</tr>
<tr>
<td>EDTA</td>
<td>Diaminoethanetetra -acetic acid (Disodium salt).</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor.</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor.</td>
</tr>
<tr>
<td>Esp1</td>
<td>Extra spindle poles 1.</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag.</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase.</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting.</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase.</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum.</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor.</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor.</td>
</tr>
<tr>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Gap 1 cell cycle phase.</td>
</tr>
<tr>
<td>G&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Gap 2 cell cycle phase.</td>
</tr>
<tr>
<td>GAB</td>
<td>Grb2 associated binder.</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein.</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanine diphosphate.</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor.</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein.</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor bound protein 2.</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3.</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione –s- transferase.</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate.</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney.</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase.</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation.</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl ß-D-thiogalactoside.</td>
</tr>
<tr>
<td>Jnk</td>
<td>c-jun N terminal kinase.</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases.</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton.</td>
</tr>
<tr>
<td>Lar</td>
<td>Leukocyte common antigen related protein.</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani.</td>
</tr>
<tr>
<td>M Phase</td>
<td>Mitosis cell cycle phase.</td>
</tr>
<tr>
<td>MAD</td>
<td>Mitotic arrest deficient.</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase.</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein.</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase.</td>
</tr>
<tr>
<td>Msps</td>
<td>Mini spindles</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor.</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell.</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase.</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline.</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor.</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor.</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology.</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide.</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol –3-kinase.</td>
</tr>
</tbody>
</table>
PKA  Protein kinase A.
PKC  Protein kinase C.
PLCγ Phospholipase Cγ.
PMSF Phenylmethylsulphonylfluoride.
PTB Phosphotyrosine binding domain.
PY  Phosphotyrosine.
RNA Ribonucleic acid.
RNase Ribonuclease.
Rpm  Revolutions per minute.
RPTK Receptor protein tyrosine kinase.
RPTP Receptor protein tyrosine phosphatase.
S Phase DNA synthesis phase of the cell cycle.
SAPK Stress activated protein kinase.
SCC1p Sister chromatid cohesion.
SDS Sodium dodecyl sulphate.
SDS PAGE SDS polyacrylamide gel electrophoresis.
SH2 Src homology 2 domain.
SH3 Src homology 3 domain.
SHIP SH2 domain containing inositol phosphatase.
SOS Son of sevenless.
TBST Tris buffered saline-Tween 20.
TCA Trichloroacetic acid.
TCF Ternary complex factor.
TE Tris/EDTA buffer.
TEMED N,N,N',N'- Tetramethylenediamine.
TNFα Tumour necrosis factor α.
UV Ultraviolet.
V Volts.
+ve Positive.
-ve Negative.
Wt Wild type.
X-gal 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside.
Chapter 1. Introduction.

1.1 Overview of She structure and function.

1.1.1 Intracellular signal transduction.

Cells of a multicellular organism are exposed to multiple extracellular stimuli to co-ordinate and control cellular function. These signals, from various sources must be passed from the exterior of the cell through the plasma membrane to achieve the desired response. To achieve this, most signalling molecules bind to cell surface receptors, which act to transduce the signal across the plasma membrane. Examples include the receptor tyrosine kinases, which dimerise and autophosphorylate on ligand binding or the G protein couple receptors, a large family of seven transmembrane pass receptors, which utilise heterotrimeric G proteins. Once across the cell membrane the signal can be propagated cell wide through the use of second messenger molecules such as cAMP and Ca^{2+} or more precisely though specific protein – protein interactions. Protein phosphorylation also plays a key role in signal transduction, by either creating binding sites for other proteins to associate or by inducing a conformational change in the proteins structure, which regulates its activity. Protein phosphorylation is tightly regulated by the action of kinase and phosphatase enzymes, which add or remove phosphate, allowing positive and negative regulation of protein activity in a particular signal transduction pathway.

Distinct protein domains have evolved which are closely associated with signal transduction. These domains are usually separate from any catalytic domain, if present, and often mediate transient and highly specific interactions. The classic example is the Src protein, a cytoplasmic tyrosine kinase originally identified from the transforming Rous Sarcoma Virus. Src contains two distinct signalling domains termed SH2 and SH3 (Src Homology). These domains allow the Src protein to associate with other proteins in a regulated manner. SH2 and SH3 domains are seen in many signalling molecules. Although all SH2 and SH3 domains recognise a related sequence, differences in the individual SH2 and SH3 domains means that each individual domain recognises a particular binding sequence, allowing specificity of binding. Many other
domains have been identified which mediate protein – protein interactions as well as domains which mediate protein – lipid interactions.

The Shc family of proteins act to couple cell surface receptors to downstream targets. Shc has many of the characteristics of a signal transduction molecule, as it includes domains that allow it to interact with activated receptors and also other proteins and some lipids. It has no detectable enzymatic activity, which suggest that it functions as an adaptor or scaffolding protein by acting as an intermediate in the binding of other proteins to the receptor. This has the effect of localising large complexes of proteins to the membrane, which allows the signal to be propagated further. To date, Shc has been implicated with a variety of different processes, including cellular growth and differentiation, cell motility, amplification of signals, apoptosis and resistance to apoptosis and as a convergence point for many signalling pathways.

1.1.2 The Shc family of adaptor proteins have a conserved domain structure.

The Shc family of adaptor proteins are involved in a variety of different signal transduction pathways where they act to couple activated cell surface receptors to downstream targets (Pelicci et al., 1992). The Shc protein family consists of three loci ShcA (Shc), ShcB (Sck, Sli) and ShcC (N-Shc, Rai) (figure 1), which encode at least six Shc like proteins of which Shc is the most widely expressed and best characterised family member (Luzi et al., 2000).

The ShcA loci codes for three overlapping proteins with relative molecular masses of 46, 52 and 66 kDa. The three Shc isoforms are identical except for the length of their N-terminal extensions, which are created through differential use of translation initiation sites (p46 and p52Shc) or alternative splicing (p66Shc) (Migliaccio et al., 1997). All members of the Shc family have a characteristic domain structure of an N-terminal phosphotyrosine binding domain (PTB), a central collagen homology (CH1) domain and a C-terminal Src homology 2 domain (SH2). The domain structure of the Shc family members is shown in figure 1.1.
p66Shc has an additional collagen homology domain (CH2) at the N terminus (Luzi et al., 2000; Pelicci et al., 1996). Both ShcB and ShcC isoforms are reported to have CH2 domain like N-terminal extensions. Sequencing of the p66Shc cDNA has given insight to the Shc locus (figure 1.2). The Shc locus contains 13 exons and 3 ATG start codons. The ATG start codons at positions 83 and 218 code for 473 and 428 amino acid proteins of 46.8 and 51.7 kDa respectively. Exon 1 is largely non-coding but contains the first 75 nucleotides of the p46 and p52 isoforms. Exon 2 contains the sequence for the CH2 domain of p66Shc. This includes the 521 base pairs of the CH2 domains including the start codon and 5’ untranslated sequence. This region is spliced to form the p66Shc transcript. The remaining 3’ 168 base pairs of exon 2 are found in all isoforms, as are exons 3 – 13 (Migliaccio et al., 1997). Expression of the p46 and p52 isoforms gives a constant ratio of the two isoforms. p66Shc expression however, varies dependent on cell type and can also be altered under certain conditions (Jackson et al., 2000; Xie et al., 1996; Leslie et al., 1998). Shc expression is widespread throughout
the body but is notably absent in the neural tissue, where N-Shc and Sck are predominantly expressed (Nakamura et al., 1998).

Figure 1.2: Genomic organisation of the Murine Shc locus and the assembly of Shc transcripts.
(A) The Shc locus consists of 13 exons, which are differentially spliced to give the p46/52Shc transcript and the p66Shc transcript. (B) The ATG start codons for the Shc isoforms are located in exon 2 and 2a, ATG 1 = p66Shc, ATG 2 = p52Shc and ATG 3 = p46Shc.

1.1.3 Shc binds phosphorylated tyrosine motifs in the intracellular domains of activated cell surface receptors.

Shc binds phosphotyrosine residues on activated receptor tyrosine kinases including epidermal growth factor receptor (EGFR) (Pelicci et al., 1992), platelet derived growth factor receptor (PDGFR) (Ward et al., 1996), fibroblast growth factor receptor (FGFR) (Vainikka et al., 1994), nerve growth factor receptor (NGFR) (Stephens et al., 1994), hepatocyte growth factor receptor (HGFR) (Pelicci et al., 1995) and insulin receptor (Pronk et al., 1993). Binding to activated receptors is mediated through either its PTB
domain, and/or its SH2 domain (Bonfim et al., 1996). Shc was identified in a screen for novel SH2 domain containing proteins using primers based on the c-fes SH2 domain (Pelicci et al., 1992). Using GST constructs of Shc it was shown that Shc could bind phosphotyrosine motifs independently of its SH2 domain, leading to the discovery of the PTB domain (Kavanaugh et al., 1994; Blaikie et al., 1994). Shc appears to preferentially use the PTB domain for binding to activated receptors, as mutation of the PTB domain has most inhibitory effect on Shc receptor binding. Although the PTB domain and SH2 domain have a similar function, in that they bind phosphorylated tyrosine motifs, they have little sequence or structural homology (Cohen et al., 1995). The specificity of the two domains is also different as the PTB domain recognises the residues N-terminal to the phosphotyrosine residue whereas the SH2 domain recognise residues C-terminal to phosphotyrosine motif. The PTB domain of Shc recognises a specific NPXpY motif where X is any amino acid and pY is phosphorylated tyrosine (Prigent et al., 1995; Farooq et al., 1999). This has been subsequently refined to just NXXpY (Laminet et al., 1996). This sequence can be found on many receptor kinases and adaptor proteins allowing Shc to complex with them. The affinity of the interaction is in the region of 50nM. The SH2 domain of Shc is less well characterised, as its binding motif is more degenerate and the function of the SH2 domain is not as clear as that of the PTB domain. The Shc SH2 domain recognises a consensus sequence of pY(I/E/Y/L)X(I/L/M) with an affinity in the region of 50 – 130μM (Zhou et al., 1996). The PTB domain of Shc can also function as a pleckstrin homology domain (PH domain). The PH domain allows Shc to associate with phosphoinositols (Rameh et al., 1997). The SH2 domain of Shc has been widely used as an inhibitor of Shc signalling as expression of the Shc SH2 domain alone in cells inhibits EGF signalling, inhibiting MAPK activation (Gotoh et al., 1995). The SH2 domain of N-Shc also retains this inhibitory ability (O'Bryan et al., 1998). The N-Shc SH2 inhibition did not appear to act by blocking binding of the endogenous Shc to the activated EGF receptor so the actual mechanism of the inhibitory action remains unclear. This could suggest that there is some wider function for the SH2 domain of Shc. Recently PAL (Protein expressed in Activated Lymphocytes) was shown to associate with the SH2 domain of Shc in a phosphotyrosine independent manner unlike other previously described Shc SH2 domain associating proteins (Schmandt et al., 1999). Expression of PAL is limited to
tissues with actively dividing cells and its expression is up regulated by growth factor treatment. These observations, and its association with Shc would suggest that PAL participates in a mitotic pathway. Expression of the SH2 domain of Shc alone would disrupt the Shc / PAL complex and could explain the ability of the SH2 domain of Shc to inhibit cellular proliferation.

1.2 Signal transduction pathways involving Shc activation.

1.2.1 Shc is involved in the activation of the extracellular regulated kinase (ERK) signalling pathway.

Shc recruitment to phosphorylated receptors has been demonstrated to be an early step in growth factor induced ERK activation. The ERK pathway is the best characterised of the mitogen activated protein kinase (MAPK) pathways (Robinson et al., 1997). Immunofluorescence and GFP tagged Shc constructs have shown Shc proteins to be located cytoplasmically, and bound to the rough endoplasmic reticulum (RER) membrane in unstimulated cells (Lotti et al., 1996). The distribution of Shc on the RER is polarized, with only the most central perinuclear cisternae showing Shc association. The outer nuclear membrane shows no Shc association, despite being continuous and functionally similar to the RER. Growth factor stimulation causes Shc proteins to leave the cytoplasm and the RER membrane and to migrate to the plasma membrane to activated receptors, endocytotic structures and the actin cytoskeleton (Lotti et al., 1996; Sato et al., 2000). Upon recruitment to an active tyrosine kinase receptor, Shc is tyrosine phosphorylated at residues Y239, Y240 and Y317 in its first collagen homology domain (Salcini et al., 1994), creating a binding site for SH2 domain containing proteins such as Growth factor Receptor Bound protein (Grb2) (Rozakis-Adcock et al., 1992; Ogura et al., 1999). Shc is also phosphorylated by receptors with no intrinsic kinase activity after ligand binding, such as the IL-2, Erythropoietin and B and T cell receptors through the recruitment of cytoplasmic tyrosine kinases. Cells expressing active cytoplasmic tyrosine kinases such as Src, Lck, Fps or Sea show extensive tyrosine phosphorylation of Shc (McGlade et al., 1992; Crowe et al., 1994; Dilworth et al., 1994; Baldari et al., 1995). Shc phosphorylation is also observed upon activation of G-protein coupled receptors such as the thrombin receptor and the angiotensin II receptor (Biesen et al., 1995; Collins et al., 1997;
Sadoshima and Izumo, 1996). Signalling by second messengers such as Ca\(^{2+}\)
stimulates Shc phosphorylation (Daulhac et al., 1997), as does protein kinase C
activation (Dabrowski et al., 1996; Daulhac et al., 1997; Miranti et al., 1999; Ohmori
et al., 2000). Viral oncogenes such as the polyoma virus middle T and large T antigens
signal through Shc (Dilworth, et al., 1994; Gottfredi et al., 1999; Webster et al., 1998).

Grb2 (Sem5 in C.elegans or Drk in Drosophila) is a 25kDa adaptor protein with a
SH3-SH2-SH3 domain structure and is associated with SOS (Son of Sevenless) through
its SH3 domain (Lowenstein et al., 1992; Egan et al., 1993). SOS controls the activity
of the Ras family of proteins through a complex with the active receptor, Shc, and
Grb2 (Buday et al., 1993). Ras family proteins are active in the guanine triphosphate
(GTP) bound state and inactive in the guanine diphosphate (GDP) bound state. All
members of the Ras family possess a slow intrinsic ability to hydrolyse GTP to GDP
(GTPase), as well as an ability to exchange GDP for GTP at a slow rate. The rate of
these changes is controlled by nucleotide exchange factors (GEF) that control the
exchange of GDP for GTP, and GTPase activating proteins, which enhance the rate of
GTP hydrolysis (Avruch et al., 1994). SOS is a 170kDa guanine nucleotide exchange
factor for the small G protein Ras that promotes Ras to enter the active GTP bound
form. Shc functions to recruit the Grb2 / SOS complex to the activated receptor at the
plasma membrane, which is essential for the activation of the membrane bound Ras
(Buday et al., 1993).

Active Ras associates with the serine / threonine kinase Raf. Ras functions to anchor
Raf to the plasma membrane, which is important to its activation (Daum et al., 1994).
Further stimuli are required for Raf activation. Raf requires phosphorylation on serine
and threonine residues and tyrosine residues 340 and 341 for full activity (Marais et
al., 1995). Raf is also phosphorylated by protein kinase C (PKC) and by non-receptor
protein kinases such as Src, suggesting that Raf activation is relatively complex.
MEK1 and MEK2 have been shown to be phosphorylated on serine and threonine
residues by Raf (Dent et al., 1992). MEK1 and MEK2 are dual specificity threonine /
tyrosine kinases that phosphorylate ERK1 and ERK2. ERK1 and ERK2 translocate
into the nucleus upon activation, where they phosphorylate the ELK1/TCF
transcription factor, which activates the serum response element activating genes such
as c-fos (Karin and Hunter 1995).
Growth-factor induced MAPK activation usually leads to cellular proliferation or differentiation. Over expression of Shc leads to strong activation and an increase in MAPK activity, which has the effect of transforming NIH-3T3 cells (Pelicci et al., 1992). The ability of Shc over expression to trigger transformation in this cell type is dependent on the tyrosine 317-phosphorylation site being present (Salcini et al., 1994). Similar experiments in PC12 cells demonstrate that Shc over expression leads to terminal differentiation, an event that can be blocked by expression of a dominant negative Ras construct (Thomas and Bradshaw, 1997). These observations show that Shc plays a role in transducing the growth and differentiation signals via Ras utilising Grb2 as an intermediate. This hypothesis is supported by the observation that constitutive Shc phosphorylation is seen in a range of cancers and particularly in those expressing mutated oncogenic receptor tyrosine kinases (McGlade et al., 1992; Pelicci et al., 1995; Xie et al., 1995).

1.2.2 Shc associates with the SH2 domain containing inositol phosphatase SHIP.

SHIP, a 145kDa protein associates with Shc following growth factor stimulation (Lioubin et al., 1996). The protein was cloned and was shown to contain one potential SH2 domain, two PTB domains, an ATP/GTP binding site and several potential SH3 domain-binding sites. Homology with other inositol phosphatases was demonstrated and SHIP can convert phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) to phosphatidylinositol 3,4 bisphosphate (PtdIns(3,4)P2). SHIP was found to be restricted to myeloid tissues, but a related protein SHIP2 has been identified which shows wider expression (reviewed in Emeux et al., 1998).

SHIP is thought to act as an antagonist to PI3 kinase as it catalyses the conversion of the PI3 kinase product (PtdIns(3,4,5)P3) (Amen et al., 1998; Wymann and Pirola, 1998). Protein kinase B or Akt is a downstream target of PI3 kinase that is dependent on specific serine and threonine phosphorylation and specific binding of phosphoinositides to the Akt PH domain for its activity. SHIP inhibits activation of Akt by conversion of the (PtdIns(3,4,5)P3) required by Akt for activity. PI3 kinase activation in immune cells triggers an influx of calcium through the activation of (PtdIns(3,4,5)P3) leading to the activation of Bruton's tyrosine kinase and PLCγ
(Katan, 1998; Okada et al., 1997; Gupta et al., 1999; Tridandipani, et al., 1999). SHIP deletion prolongs calcium influx into mast cells leading to sustained degranulation (Huber et al., 1998). Shc signalling is actively involved with lymphocyte antigen receptor activation (Plyte et al., 2000; Baldari and Telford, 1999) and NK cell activation (Galandrini et al., 2001).

SHIP expression appears to be tightly regulated and is present as multiple extensively spliced isoforms. The isoforms are generated through C-terminal truncations (Damen et al., 1998), or through internal deletions (Lucas and Rohrschneider, 1999). 145, 135, 125 and 110kDa isoforms are generated through C-terminal truncation through proteolytic cleavage by calpain. Only the 145kDa isoform precipitates Shc suggesting different roles for the other isoforms. A SHIP isoform expressed during myeloid development shows an internal deletion of 61 amino acids removing a potential PI3 kinase binding site (Lucas and Rohrschneider, 1999).

SHIP has been shown to be tyrosine phosphorylated upon growth factor treatment and this promotes its association with Shc. In vitro studies show that the SH2 domain of SHIP binds phosphorylated tyrosine residue 317 with a $K_D$ of 290nM (Lui et al., 1996). The PTB domain of Shc was also shown to bind SHIP (Lamkin et al., 1997). There have been contradictory observations on the exact configuration of the Shc/SHIP complex. Erythropoietin stimulates the formation of a Gab1, Shc, SHIP, SHP2, PI3-kinase complex (Lecog-Lafon et al., 1999). Some groups have shown Grb2 to be present (Lucas and Rohrschneider, 1999; Lecog-Lafon et al., 1999), or absent (Tridandipani et al., 1997) from the SHIP complex. The absence of Grb2 from the complex has been suggested as a reason for the growth inhibitory nature of SHIP. The role of Grb2 in the complex may be important for the stability of the complex (Harmer and DeFranco, 1999). Depletion of Grb2 gave a decrease in the amount of Shc co-immunoprecipitated with SHIP. Cell lines deficient in Grb2 showed roughly 20-fold reduction in the amount of Shc associated with SHIP. Addition of Grb2 corrected this. Mutation of the 239 and 317 tyrosine phosphorylation sites of Shc has been reported to disrupt the interaction between Shc and SHIP. Grb2 is thought to bind to the Shc tyrosine phosphorylation sites with a 10 fold higher affinity than SHIP, suggesting that Grb2 is likely to be the physiological binding partner for Shc. A model was therefore proposed where the PTB domain of Shc associates with tyrosine phosphorylated
motifs on SHIP and that tyrosine phosphorylated Shc recruits Grb2 which further stabilises the interaction with SHIP, possibly through its SH3 domains. The SH2 domains of SHIP and Shc would then be free to interact with other effectors.

SHIP2 is less well characterised. It was originally cloned using degenerate primers to other related inositol phosphatases (Pesesse et al., 1997; Wisniewski et al., 1999; Ishihara et al., 1999). Initial characterisation indicates that it is functionally similar to SHIP, in that it becomes tyrosine phosphorylated after growth factor stimulation, has an inhibitory effect on growth and Akt activity and associates with Shc (Habib, et al., 1998). Differences in substrate specificity have been reported, suggesting different signalling roles for SHIP and SHIP2. The proline rich regions of SHIP and SHIP2 have been investigated for any potential differences. SHIP2 has been reported to associate with the SH2 domain of Abl, which SHIP does not. SHIP2 does not associate with the SH3 domain of Grb2, so the earlier proposed stability model for SHIP may not be applicable to SHIP2 (Wisniewski et al., 1999). Association of p52Shc and p66Shc with SHIP2 was observed after EGF and PDGF stimulation of SH-SY5Y cells. Only p52Shc association with SHIP2 was observed after NGF treatment of PC12 cells, despite the ability of NGF to stimulate tyrosine phosphorylation of p52shc and p66Shc (Habib et al., 1998).

1.2.3 Shc is involved in cell motility and cytoskeletal organisation.

At present, the best-characterised role for Shc within the cell is its role in signalling to Ras and the MAPK pathway. It is also clear that Shc can be associated with a variety of other signalling complexes. Shc associates with signalling complexes and structures involved with cell migration and cytoskeletal rearrangement (Collins et al., 1999; Mauro et al., 1999). Shc has also been shown to associate with PTEN (Phosphatase and Tensin homologue), a tumour suppressor shown to inhibit some aspects of cell motility (Gu et al., 1999). PTEN is a dual specificity phosphatase, dephosphorylating both phosphorylated Serine / Threonine residues as well as Tyrosine residues. Unusually it also has a phosphoinositide 3 phosphatase activity, allowing it to inhibit PI3-kinase signalling pathways. PTEN has been shown to directly associate with all three isoforms of Shc and dephosphorylate both the 239 / 240 and 317 sites. Cells over expressing Shc showed increased numbers of focal adhesions, disorganisation of the
actin cytoskeleton and increased random migration (Gu et al., 1999). Addition of PTEN or Shc dominant negatives inhibited these effects. Shc is also involved with transducing mitogenic signals derived from the extracellular matrix via the integrins (Wary et al., 1996; Mainiero et al., 1997). A dominant negative Shc construct blocks MAPK activation and prevents cell cycle progression after integrin ligation. The association of Shc is only found in a subset of the integrin family and may be indirect, through caveolin.

Using Glutathione S transferase / Shc fusions, it was shown that Shc binds to actin via its N terminus and that neither Shc nor actin needs to be phosphorylated (Thomas et al., 1995). These observations suggest that Shc may play some role in the regulation of the actin cytoskeleton. Disassembly of actin filaments by compounds such as Botulinum C2 toxin inhibits the association of Grb2 with Shc but not Shc phosphorylation, suggesting that interaction between Shc and the actin may be important in controlling Shc signalling pathways (Tsakiridis et al., 1998). Shc has also been shown to associate with the adaptor proteins adaptins (Okabayashi et al., 1996). This interaction is mediated via amino acids 346 - 355 of Shc (RDLFDMKPF). The adaptins have been shown to be involved with clathrin coated vesicle dynamics, suggesting a link for Shc with endocytosis and vesicle formation. The adaptins have also been demonstrated to be involved with the recycling of cell surface receptors, such as the EGF receptor. These observations suggest potentially wider signalling roles for Shc than just participation in the Ras / MAPK pathway. Proteins interacting with Shc are summarised in figure 1.3.
1.2.4 The She tyrosine phosphorylation site 239/240 and 317 are differentially regulated and may be functionally distinct.

Some aspects of the regulation of the tyrosine phosphorylation of She remain unclear. The 317 (YVNI) site was the first characterised and was thought to be the major Grb2 binding site as mutation of this site removed the transforming potential of over expressed She in NIH 3T3 cells (Salcini et al., 1994). Subsequently a second tyrosine phosphorylation site was identified at Y239/240 (YYND) (Gotoh et al., 1996). The consensus-binding site for the SH2 domain of Grb2 is pYXNX and Grb2 association has been demonstrated (Thomas and Bradshaw 1997). Insight into the potential functions of the two sites comes from the analysis of dShc (Luschnig et al., 2000). The Drosophila She protein (dShc) was identified using antibodies directed against human She, which detect a ubiquitously expressed 45 kDa protein that is present throughout the life cycle of Drosophila. dShc has around 46% homology to human She, with highest homology seen in the PTB and SH2 domains. Interestingly, only the 239/240 site is present, suggesting that the acquisition of the 317 site is a relatively recent event in the evolution of She. dShc is recruited to activated receptors and tyrosine phosphorylated in a manner consistent with its mammalian homologues. Analysis of
dShc has shown that the recruitment of Grb2 to Shc and consequently the activation of Ras may also be a relatively new evolutionary acquisition, as Drk, the Drosophila homologue of Grb2 does not associate with tyrosine-phosphorylated dShc. This suggests that dShc may participate in a signalling pathway separate from the Ras/MAPK pathway and a more fundamental role for Shc has possibly been overlooked (Luzi et al., 2000).

It has been reported that Interleukin-3 induced c-myc expression is dependent on the 239/240 tyrosine phosphorylation site of Shc (Gotoh et al., 1996). c-myc controls a variety of cellular activities including control of the cell cycle, differentiation and apoptosis (Grandori and Eisenman 1997; Schmidt, 1996). Abnormal c-myc activity has also been reported in a range of neoplasms. As the IL-3 receptor has no intrinsic kinase activity, it is thought that a cytoplasmic tyrosine kinase would be responsible for inducing the tyrosine phosphorylation of Shc. EGF induced c-myc expression is also blocked by the 239/240 dominant negative Shc, but MAPK activation remains largely unaffected. (Gotoh et al., 1997). Src has been implicated in the induction of c-myc from signalling from the PDGF receptor and dominant negative experiments have suggested that this occurs in a Ras independent manner (Barone and Courtneidge, 1995). Shc has been shown to be an efficient substrate for the Src family and it has been reported that the 239/240-tyrosine phosphorylation site is preferentially phosphorylated when the PTB domain of Shc is acting as a PH domain by binding phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 3,4,5-trisphosphate. Intermediates in this signalling pathway have yet to be identified (Sato et al., 1997; Sato et al., 1998).

Using mutant Shc constructs that have either or both tyrosine phosphorylation sites mutated to a phenylalanine residue, it is possible to distinguish functionally between the two sites (Gotoh et al., 1996; Thomas and Bradshaw 1997). Differences between the two sites have been reported in function and Grb2 recruitment. As Y317 was the first identified tyrosine phosphorylation site, many groups have mutated this site to attempt a dominant negative Shc construct. This approach does inhibit signalling from a variety of receptors (Ishihara et al., 1998; Salcini et al., 1994; Stevenson et al., 1999; Wary et al., 1996) and blocks transformation of 3T3 fibroblast by Shc over-expression, which confirms the importance of this site in Grb2 recruitment. It as also been reported
that mutation of either site causes a greater than 50% decrease in Grb2 association with Shc in B cells, where Shc is efficiently phosphorylated at both sites by the tyrosine kinase Syk (Harmer and DeFranco 1997). This could suggest that Grb2 binds Shc in a co-operative manner, where two Grb2 molecules are required for high affinity binding. This could have the effect of blocking significant Grb2 association with Shc when just one site is deleted, explaining the ability of the single Y317 mutant to attenuate activation of Ras. A truncated Shc construct retaining only the Y239/240 phosphorylation site but removing the SH2 domain and Y317 phosphorylation site inhibits the transforming ability of constitutively active neu. No association with Grb2 was reported (Li et al., 1999).

Other groups have reported Y239/240 as being the major Grb2 binding site. Nerve growth factor stimulated PC12 cells show little Grb2 binding at the Y317 site when compared to Grb2 binding at Y239/240 (Thomas and Bradshaw 1997). In these cells the Y239/240 site is much more efficiently phosphorylated, which means that cell type specific differences will determine the preferential Grb2 binding site. Investigation of the signalling pathways involving the middle T antigen have demonstrated that the Y239/240 and Y317 phosphorylation sites are not equal in the propagation of mitogenic signals. To investigate whether Shc association is required for signalling stimulated by the middle T antigen, the Shc binding site on the middle T antigen was replaced with the known Shc Grb2 binding site Y239/240 or Y317 (Nicholson et al., 2001). Using a rat fibroblast transformation assay it was demonstrated that only the Y239/240 Grb2 binding site of Shc could induce transformation by the mutant middle T antigen. Replacement of the Shc binding domain of middle T antigen with Y317 Grb2 binding site or the Grb2 binding sites of EGF receptor failed to transform the cells. Addition of a second Grb2 binding site from either Shc Y317 or a Grb2 binding motif from EGFR with Y239/240 induced transformation of the rat fibroblast as efficiently as wild type middle T antigen. Combinations of the Y317 site and Grb2 binding sites from EGFR in the Shc binding mutant of middle T antigen failed to induce transformation. This would imply that Grb2 bound to the Shc Y317 or to receptor Grb2 binding sites is not equivalent to Grb2 bound at the Y239/240 site. Interestingly, addition of double Y239/240 sites into middle T antigen transforms cells more efficiently than the wild type middle T antigen, showing a faster growth and a
more transformed phenotype. Y239 and Y240 are required, but mutation of the second site has less effect.

Y239/240 may also be differently regulated to Y317. Using a substrate trapping phosphatase inactive form of the T cell protein tyrosine phosphatase (TCPTP) it was shown that TCPTP associates specifically with phosphorylated Y239 (Tiganis et al., 1998). This identifies the Y239 site as being a substrate for TCPTP. This inactive form of TCPTP does not bind Y317, suggesting that it is not regulated by this phosphatase. This could mean that the two phosphorylation sites of Shc can be individually regulated by the action of specific kinase and phosphatase enzymes and therefore carry out separate functions.

1.3 Shc expression is essential for cardiac and vascular tissue development during embryogenesis.

The Shc gene has also recently been knocked out in mice, which gives an embryonic lethal phenotype with death at day 11.5 (Lai et al., 2000). Defects in cardiac development, and blood vessel maturation were visible in the embryonic knockout mice. Cardiac and vascular tissue from the knockout mice showed a decreased ability to remodel the cellular cytoskeleton in response to extracellular matrix derived signals and reduced sensitivity to growth factor signals and consequently reduced MAPK activation. These defects prevent spreading of cells in the vascular tissue and slow proliferation, leading to failings in cardiac development and vasculogenesis. Mouse embryo fibroblast isolated from the knockout mice are viable, indicating that Shc is not an absolute requirement for the transmission of, but is important for the fine control and co-ordination of signals required for complex functions such as tissue formation. It has been reported that Grb2 can bind to activated tyrosine kinases such as the EGF receptor independently of Shc, which may explain why Shc is not essential for Ras activation (Ward et al., 1996). It would therefore appear that Shc gives an extra level of complexity to growth factor signalling to Ras allowing a further level of regulation over Ras signalling. Further differences in Shc function are apparent between the individual isoforms of Shc, which will be discussed in more detail later. The basic Shc signalling pathways are summarised in figure 1.4.
Figure 1.4: Shc signalling cascades.

Simplified scheme of Shc signalling showing the well-characterised role of Shc in activating the MAPK pathway and the possible signalling pathway from Shc to c-myc.
1.4 The She family members Sck and N-Shc show distinct tissue localisation and function.

1.4.1 N-Shc.

Human and mouse clones of N-Shc were cloned at similar times using two different methodologies. The human form was cloned from a brain cDNA library, and the clone identified was termed N-Shc (Nakamura et al., 1996). The mouse form was cloned using a low stringency primer generated against the Shc SH2 domain sequence, identifying a clone termed ShcC (O’Bryan et al., 1995). N-Shc has a 1782 base pair open reading frame, containing two AUG start codons and a single TGA stop codon. The two start codons allow for a 594 residue protein and 474 residue protein, with molecular mass of 64kDa and 52kDa. N-Shc shows an overall homology of 54.3% with Shc. Highest homology is found in the PTB and SH2 domains, showing 73% and 69% homology to Shc. Lowest homology is seen in the CH1 domain at just 34% homology to Shc. N-Shc retains some of the proline rich nature of the Shc CH1 domain, but shows little homology with collagen. Early characterisation of N-Shc shows it to have a functional SH2 and PTB domain as GST fusions of these domains could bind to activated EGF and Trk receptors (O’Bryan et al., 1998). Tyrosine phosphorylation of N-Shc could be detected after stimulation of PC12 cells with NGF, as could association with Grb2 (Nakamura et al., 1996). These observations suggest that N-Shc probably functions in a manner analogous to Shc in that it is recruited to activated receptors and associates with Grb2. The conserved nature of the phosphotyrosine binding domains suggests that this function is probably functionally similar to Shc, although differences with specificity have been reported for the SH2 domain. The SH2 domain of N-Shc shows similar selectivity at the pY+1 and +3 positions to the SH2 domain of Shc, but a preference for hydrophobic residues at the pY+2 position. The N-Shc PTB domain recognises a NPXpY motif (O’Bryan et al., 1995). The lower homology seen in the CH1 domain could indicate that N-Shc is bound by other factors, possibly specific to neuronal cell types. Association with Grb2 is thought to be a major function of Shc and this is retained.
1.4.2 Sck.

Sck (Shc like) was discovered through database searches using the PTB domain of Shc as a template (Kavanaugh et al., 1994). A partial clone was identified from this search, which allowed the majority of the Sck gene to be cloned from a brain cDNA library. The N terminal sequence of Sck has only been more recently cloned showing that Sck can have an N terminal extension of around 120 amino acids before the PTB domain. This sequence includes a potential start codon and two PXPXXP sequences that may allow binding of SH3 domain containing proteins (Sakai et al., 2000). It has been reported that Sck is expressed as a 68 kDa protein (Kojima et al., 2001). Sck shows lower homology to Shc as the Sck PTB domain shows 67% homology with Shc and the Sck SH2 domain shows 65% homology to Shc. Lowest homology is seen in the CH1 domain, which shows 36% to Shc. Sck signalling pathways are relatively uncharacterised, although it appears that unlike Shc and N-Shc, Sck is poorly phosphorylated by Src. It also associates with an unidentified tyrosine phosphorylated protein with a mass of 135kDa (Nakamura et al., 1998). A candidate for this protein could be SHIP1 or SHIP2, which has been shown to associate with Shc and is tyrosine phosphorylated in growth factor treated cells and has isoforms in that mass range. Sck has been shown to associate with the active EGF receptor and to associate with Grb2, suggesting that Sck too, can activate Ras like the other Shc family members (Nakamura et al., 1998). Sck also interacts with the KDR receptor via its SH2 domain, suggesting a possible role for Sck in angiogenic signalling (Warner et al., 2000).

1.4.3 N-Shc and Sck have some overlapping and some unique functions.

The expression of N-Shc and Sck show considerable overlap, as they are both widely expressed throughout the adult nervous system (Nakamura et al., 1998). There is some overlap between Shc expression and Sck but no significant overlap between Shc and N-Shc expression. Expression of Shc in the brain is only seen in the olfactory epithelium, where neuronal cell renewal occurs throughout life (Conti et al., 1997). Shc expression is seen in the developing brain but is only seen in damaged neurones in adults (Tanabe et al., 1998). In damaged neurones, Shc expression is up regulated and N-Shc expression is down regulated. In the developing brain Shc expression is detectable only in regions of growth. Sck and Grb2 expression remains constant.
throughout life (Conti et al., 1997). This suggests a role for Shc in development of the nervous system, with selective expression of different Shc family members acting as a switch between growth and differentiation. Sck and N-Shc expression instead of Shc, could allow different factors to associate with receptors due to their differences in the CH1 domain, while maintaining the basics of Shc function such as receptor binding and Grb2 recruitment. This would allow a receptor to trigger different signalling cascades after differentiation, and shut off those associated with growth as Shc expression is replaced with N-Shc. Due to the differences in Sck phosphorylation and the overlap in Sck expression with Shc and N-Shc expression, it is possible that Sck is carrying out some other function required throughout the life span of the organism.

Recently, both Sck and N-Shc knockout mice have been generated (Sakai et al., 2000). These mice are viable and are born with the expected Mendelian ratio. To analyse where there is a redundancy in function between Sck and N-Shc, a combined Sck / N-Shc knockout was also bred, which are also viable and are born with the expected Mendelian ratio. Analysis of the knockouts showed no detectable defects physically and no obvious defects in behaviour, but under microscopic analysis, changes were detected in neurone number. Sck deficient mice show a decrease in peptidergenic and nonpeptidergenic nociceptive sensory neurones. Analysis of neurone types expressing high levels of N-Shc show no abnormalities in the N-Shc knockout mice. The double knockout mice show a loss of neurones in the superior cervical ganglion that is not seen in either mutant alone. This would suggest that Sck and N-Shc possess some overlapping functions and some unique.
1.5 p66Shc.

1.5.1 The p66Shc isoforms acts as a negative regulator of the MAPK pathway.

p66Shc differs from the p46Shc and p52Shc isoforms in structure, function and expression. Expression of p66Shc varies, unlike the invariant expression of p46Shc and p52Shc, but remains widely expressed throughout development and into the adult organism. p66Shc is notably absent from the haematopoietic cell lines and lymphocytes (Pelicci et al., 1992). The control of Shc expression is driven through two alternative promoters, inducing the transcription of a p46/p52Shc mRNA and a p66Shc mRNA. The p66Shc promoter is silenced through epigenetic modifications in cell lines that do not express p66Shc. Histone deacetylase inhibitors and demethylating agents can induce the expression of p66Shc in cell lines that do not normally express p66Shc. There is an inverse correlation between p66Shc expression and promoter methylation density. These observations would implicate histone deacetylation and cytosine methylation in the regulation of the p66Shc promoter (Ventura et al., 2002). This could mean that there is a specific function for p66Shc that is required in some cell types. This would probably involve the unique CH2 domain, which could allow proteins to be recruited to activated receptors through a CH2 domain interaction.

A key difference between p66Shc and p46/52 Shc is that over expression of p66Shc does not transform 3T3 fibroblast, whereas p46Shc and p52Shc over expression does (Migliaccio et al., 1997). This indicates that p66Shc may function differently from the p52Shc and p46Shc isoforms and may function as a negative regulator of cell growth. This could mean that growth and differentiation signals are controlled by an antagonistic relationship between the Shc isoforms, which either act to promote or repress growth. p66Shc is efficiently recruited to activated receptors where it is tyrosine phosphorylated and binds Grb2 (Migliaccio et al., 1997). In this respect it is indistinguishable from p52Shc and p46Shc, which would suggest that the failure to promote cellular growth observed with p66Shc over expression is not occurring by blocking recruitment of Grb2 to the active receptor.

As p66Shc does not appear to inhibit the recruitment of Grb2 to the activated receptor, the effect of p66Shc expression on MAP kinase was investigated (Migliaccio et al.,
Using a tagged Map kinase and the myelin basic protein kinase assay it was shown that cells over expressing p66Shc were inefficient in stimulating MAP kinase after EGF stimulation. Downstream targets of the MAPK pathway were also investigated. The relationship between p66Shc and the Fos promoter was investigated. p66Shc over expression was shown to have an inhibitory effect on Fos expression suggesting that p66Shc over expression inhibits the MAPK pathway and some of the associated changes in gene expression brought about by MAPK activation.

These observations could explain the inability of p66Shc over expression to transform NIH 3T3 cells. It is also interesting that p66Shc inhibits EGF signalling downstream from the receptor, at the level of the MAPK pathway, as Shc activation is an early step in the signal transduction cascade. Expression of the CH2 domain alone could inhibit MAPK activation (Migliaccio et al., 1997). This suggests that p66Shc may have functions downstream of the receptor, as this fragment alone has not been shown to be able to interact with an activated receptor. As many tyrosine kinases signal through the Shc isoforms it is possible that p66Shc is a negative regulator of many pathways.

Recently it has been reported that p66Shc forms a complex with p120RasGAP and p190RhoGAP during gastrulation in *Xenopus* (Dupont and Blancq 1999). Exact contacts in the complex are not identified, but it does appear that p120RasGAP forms a complex with Shc that is dependent on the p66Shc isoform being tyrosine phosphorylated. Interestingly in this system Grb2 only co-immunoprecipitates with Shc when p66Shc is tyrosine phosphorylated even when other Shc isoforms are strongly tyrosine phosphorylated. The significance of these observations is unclear, as it difficult to see how Grb2 could distinguish between the individual isoforms of Shc. The identification of p120RasGAP within a complex with p66Shc is significant, as p120RasGAP is a negative regulator of the MAPK pathway. p120RasGAP functions by enhancing the rate at which Ras hydrolyses GTP to GDP, a step which is sufficient to deactivate Ras and to block signalling downstream to the MAPK pathway. This would be an ideal candidate for the inhibition of MAPK activation seen with p66Shc over expression.

The ability of p66Shc to activate the MAPK pathway remains contentious and some contradictory results have been reported. Cripto, a member of the EGF family was
shown to induce tyrosine phosphorylation of the p46, p52 and p66 isoforms of Shc and trigger MAPK activation (Kannan et al., 1997). The inhibitory effect on MAPK activation by p66Shc was not observed, although time course differences in the experiment could account for this. It has also been reported that cells over expressing p66Shc can activate ERK activity and at longer time courses p66Shc accelerates the inactivation of ERK (Okada et al., 1997). This suggests that p66Shc regulation of MAPK pathway activation is more complex than purely inhibiting MAPK activation, but is more likely to be part of a negative feed back loop acting through the MAPK pathway. This also means that there is likely to be further levels of regulation controlling p66Shc function. As the CH2 domain alone retains the inhibitory effect, it is unlikely that an association with p120RasGAP is responsible solely for the negative regulation of MAPK signalling seen with p66Shc.

Investigation of the role of p66Shc in the differentiation of bronchial epithelial cells showed that growth on collagen gels prevented high levels of p66Shc tyrosine phosphorylation and Grb2 association despite high levels of EGFR phosphorylation (Moghal and Neel 1998). p52Shc phosphorylation and Grb2 association was unaffected, but this failed to effectively stimulate MAPK activation. Unidentified 120 and 130 kDa tyrosyl phosphorylated proteins were present during growth on collagen gels and potentially could be part of an unidentified signalling pathway acting on p66Shc under these conditions. This could mean that p66Shc is involved in complex regulation of growth factor signalling. These contradictory observations could be due to differences in the cell types and protocols used, so the action of p66Shc on MAPK activation remains inconclusive.

1.5.2 p66Shc is serine phosphorylated at residues within the CH2 domain.

Several groups observed an electrophoretic mobility shift in p66 after stimulation with EGF, insulin, fibroblast growth factor (FGF) and platelet derived growth factor (PDGF)(Okada et al., 1997; Kao et al., 1997; El-Shemerly et al., 1997). Treatment of the p66Shc with phosphatases returned the mobility shift of p66Shc back to normal, suggesting that serine / threonine phosphorylation was occurring. Low levels of serine and threonine phosphorylation of Shc family members have been detected, but this is unchanged by stimulation suggesting that Shc is stably serine and threonine phosphorylated.
phosphorylated under normal conditions (Kao et al., 1997). As the levels of tyrosine phosphorylation are much greater after stimulation, serine and threonine phosphorylation was thought not to be so important in Shc regulation and function (Pelicci et al., 1992). The electrophoretic mobility shift seen in p66Shc following growth factor treatment, could mean that serine and threonine phosphorylation of p66Shc is important to its activity.

12- O-tetradecanoylphorbol- 13-acetate (TPA) is a diacylglycerol analogue; a potent activator of protein kinase C. Treatment of cells with TPA induces the mobility shift in p66Shc (El-Shemerly et al., 1997), suggesting that protein kinase C activation could be important. Phorbol ester stimulation also triggered the association of PEST tyrosine phosphatase with p52 and p66Shc, and the mobility shift was observed in p66Shc again (Habib et al., 1994). G protein coupled receptors capable of activating protein kinase C acted in a similar manner to the phorbol ester treatment, suggesting the observation was physiologically relevant. PEST tyrosine phosphatase could potentially disrupt the phosphorylation dependent interactions between the receptor and Shc or between Shc and Grb2. This is unlikely as it has been demonstrated that only the serine / threonine phosphorylated forms of p66Shc are tyrosine phosphorylated and associated with Grb2, but this complex is not receptor bound (Okada et al., 1997). This accounts for about 50% of the p66Shc present. The other 50% is receptor bound, and is not phosphorylated on tyrosine, serine or threonine residues. This implies that there is no decrease in phosphotyrosine levels in either serine / threonine phosphorylated p66Shc or the receptor, but only a decrease in serine / threonine phosphorylated p66Shc affinity for the active receptor. This suggests that p66Shc serine / threonine phosphorylation impairs its ability to complex with the receptor; making it unlikely that PEST tyrosine phosphatase is deactivating either p66Shc or the receptor through dephosphorylation.

This evidence fits well with a model for p66Shc acting as a step in a negative feedback loop. The pathways leading to Ras activation are tightly regulated. There are similarities between Sos regulation and p66Shc regulation (Zhao et al., 1998). Sos is part of a negative feedback loop, which is regulated by Ras activation. After prolonged Ras activation, Sos becomes phosphorylated on serine and threonine residues that decrease its ability to associate with Grb2, having the effect of attenuating further Ras
activation. A similar model could explain the action of p66Shc. It has been shown that
p66Shc serine/threonine phosphorylation occurs after tyrosine phosphorylation and
that inhibitors of the MAPK pathway partially inhibit the serine/threonine
phosphorylation of p66Shc. These observations could mean that Shc activation leading
to Ras activation triggers a downstream kinase that can serine/threonine
phosphorylate p66Shc leading to its inactivation, through eliminating its ability to bind
to active receptor. Co-immunoprecipitation suggests that there is a limiting amount of
Grb2 and that the Shc isoforms compete for this (Okada et al., 1997). This could
indicate that p66Shc acts to sequester Grb2 by forming non-membrane localised,
unproductive signalling complexes, therefore inhibiting further activation of the
MAPK pathway.

1.5.3 p66Shc is phosphorylated at serine residue 36 in response to oxidative stress.

A breakthrough in the understanding p66Shc action came with the development of the
p66Shc knock out mouse (Migliaccio et al., 1999). This knock out involved a targeted
mutation in exon 2 coding for the CH2 domain, which does not affect the p52Shc and
p46Shc coding sequences. The resulting mice appear to be physically healthy and
phenotypically indistinct from wild type mice (full details of the knockout are yet to be
published). The effect of this mutation only became apparent as the mice aged. Wild
type mice had an average life span of 850 days. The knock out mice had an average
life span of over 1,100 days. Stressing cells with hydrogen peroxide and UV radiation
triggered p66Shc to be phosphorylated at serine residue 36. Mutating this residue to
Alanine (S36A) increased cellular resistance to stress by protecting against apoptosis
induced by those agents. Transfecting this S36A into p66Shc knockout cells did not
restore the normal stress response to the cell. p66Shc knockout cells or cells expressing
the S36A mutant showed increased levels of p53 expression following stress but
reduced levels of cyclin dependent kinase inhibitor p21, suggesting that p66Shc
regulated a p53 independent pathway. The p66Shc knock out mice showed increased
resistance to paraquat compared to wild type mice demonstrating \textit{in vivo} the role of
Shc in stress resistance. The primary sequence of p66Shc is shown in figure 1.5.
Figure 1.5: The primary sequence of the CH2 domain of p66Shc.
The CH2 domain of p66Shc contains several potential SH3 binding sites and several potential serine / threonine phosphorylation sites. (CK2 = casein kinase 2).

Cellular stress has been implicated as a major factor in ageing suggesting p66Shc may regulate stress responses. Although molecular oxygen is an essential element for life, its incomplete reduction during normal aerobic respiration generates reactive oxygen species (ROS). ROS such as singlet oxygen, hydrogen peroxide, hydroxyl radical, superoxide anion and nitric oxide are part and parcel of aerobic life and as a consequence many biochemical and enzymatic methods for detoxification have evolved. Environmental changes can cause the cellular redox balance to shift, through the production of excess ROS, a state known as oxidative stress. Molecular oxygen is relatively inert despite being a free biradical due to the parallel spins of its unpaired electrons on each oxygen atom. Environmental factors such as UV irradiation can activate oxygen by altering the spin state of oxygen-unpaired electrons to an antiparallel spin state by elevating one electron to a higher energy level. Under certain conditions the activated oxygen will then produce singlet oxygen (Reviewed in Dalton et al., 1999). Most reactive oxygen is produced as the relatively unreactive superoxide
anion, which is a product of incomplete reduction by organic electron donors. Superoxide dismutase (SOD) converts the superoxide anion to hydrogen peroxide and oxygen. Hydrogen peroxide can be converted to oxygen and water by catalase or in the presence of reduced transition metals such as ferrous or cuprous ions to the highly reactive hydroxyl radical. Excess ROS are damaging as they react and modify all major classes of biological macromolecules causing cytotoxic and mutagenic damage. Oxidative damage activates stress activated signalling pathways, up regulating oxidant defences such as free radical scavenging enzymes or leading to apoptosis with severe damage (Adler et al., 1999). Disruption of the cellular redox balance has been implicated in the pathogenesis of many human diseases.

All multicellular organisms face a progressive and irreversible decline in the efficiency of a variety of physiological processes after reproductive age is achieved. At the cellular level it has been shown that cells have a finite ability to replicate. After a number of divisions, which varies from species to species, the cell enters a terminally non-dividing state known as senescence (Smith and Pereira-Smith 1996). This is different from terminal differentiation, which can be induced by specific stimuli. Cells can rarely escape senescence to become immortalized as cancer cells. Many investigations have established a link between the proliferation potential of cells in culture and aging and more recently in in vivo models (Jazwinski et al., 1996). It can be postulated from this that damaged cells cannot be replaced indefinitely, leading eventually to organ failure and death. Therefore factors that damage and kill cells will accelerate the aging process and shorten the organism's life span. The best characterised method for extending life is known as Calorie Restriction (Sohal and Weindruch 1996). A link had been noticed between metabolic rate and life span. Organisms with a high metabolic rate tend to have short life spans. Reducing metabolic rate though calorie restriction reduced physical activity and induced hibernation all increased life span in test animals. Reducing metabolic activity can dramatically reduce the production of ROS and decreases the proportion of cellular macromolecules showing ROS induced damage. This discovery suggests that under steady state condition cellular antioxidative defences are not fully efficient and that cells are under constant oxidative stress. Support for this hypothesis includes the observation that over expression of antioxidant enzymes such as SOD and Catalase extend life in Drosophila and C.elegans (Lithgow and Kirkwood 1996) The age-1
mutant identified in *C. elegans* extends average life span by 65% and maximum life span by 110%. This mutant showed greatly increased resistance to a variety of stresses, including heat shock, UV irradiation, paraquat treatment and hydrogen peroxide treatment. The age-1 mutant has been demonstrated to have elevated activities of catalase and superoxide dismutase, and shows less signs of oxidative damage. The mutation responsible is thought to be in a regulatory gene, as many changes are apparent. The life-extending mutant Sir2 has recently been demonstrated as being a NAD-dependent histone deacetylase, suggesting that many genes could be involved in the extension of life (Imai *et al*., 2000).

Increased life span comes with a penalty. Calorie restriction can extend the life span of mice by 50% or more, but at the cost of reduced fertility and impaired physical development. Mice undergoing calorie restriction tend to be 30% smaller than control animals (Guarante *et al*., 1999). Large reductions in cellular ROS levels may also be harmful. It is now known that ROS are involved in cellular signalling. The discovery that the deletion of p66Shc can extend life is a great breakthrough, as it appears to have little cost to the organism. Evolutionary theory suggests that aging results from the absence of selection in the germ line against mutations that cause a post-reproductive decline. It is possible that p66Shc may be beneficial in development up to reproductive age but then may act negatively in later life. This suggests that there maybe defects in the knockout mice that are not immediately obvious. Even if this were the case, p66Shc would be a good target for therapies to prevent cellular aging and damage that could be given later in life. Oxidative stress has been reported to be a major determinant in ventricular dysfunction and failure in cardiomyopathy and p66Shc has been reported to be involved in this process (Cesselli *et al*., 2001). Any potential therapy to significantly slow the progress of ageing would have profound social and economic implications (Roush, 1996).
1.6 Microtubule disrupting drugs induce the serine/threonine phosphorylation of p66Shc.

A shift in the electrophoretic mobility of p66Shc has also been reported to occur after treatment with microtubule disrupting drugs (Yang and Horwitz 2000). Microtubule disrupting drugs have been used with some success in the treatment of cancer and function by blocking normal chromosome segregation during mitosis (Schiff and Horowitz, 1980). Examples of microtubule disrupting drugs include Colchicine, Podophyllotoxin, Paclitaxel (Taxol), Nocodazole, Vinblastine and Vincristine. The shift in the electrophoretic mobility of p66Shc after treatment with agents such as Taxol occurs after around 4 hours and peaks at 16 hours. This is in contrast to growth factor and oxidative stress-induced phosphorylation, which can be detected as little as 5 minutes after treatment. The shift in mobility induced by Taxol is also insensitive to the MEK inhibitor PD90859. Cycloheximide treatment blocks Taxol induced phosphorylation of p66Shc suggesting additional protein synthesis is required for the phosphorylation event. These observations would suggest that the phosphorylation event induced by Taxol and other microtubule disrupting drugs maybe mechanistically different to that observed after growth factor treatment and oxidative stress. Taxol also stimulates tyrosine phosphorylation of Shc and its association with Grb2. The significance of this in the cellular response to Taxol is not clear (Wolfson et al., 1997).

1.7. Microtubule drugs activate the spindle checkpoint and arrest the cell cycle at mitosis.

The cell cycle in eukaryotes comprises of four distinct phases, gap 1 (G₁), DNA synthesis (S), gap 2 (G₂) and mitosis (M). The G₁ phase is considered the start of the cell cycle. During this phase, the cell makes the decision to proceed or exit the cell cycle, dependent on mitogenic and inhibitory signals (Van den Heuvel, 1993). During this phase, preparations are made for replication of the genome. G₁ phase leads to S phase, where the cell replicates its DNA. After DNA replication the cell enters G₂ phase, which is the preparation for cell division. The cell is tetraploid at this time. M (Mitosis) phase is the last phase of the cell cycle. During mitosis, sister chromatids are segregated to either pole of the cell before the cell divides through a process known as cytokinesis. A scaffold of microtubules mediates the movement of chromosomes.
through the cell during mitosis. The minus end of the microtubule associates with the
mitotic spindle pole and the plus end associates with the kinetochore, a structure on the
centromeric chromosomal DNA. Cells that are non-dividing are said to be quiescent
(G0) (Reviewed in Sherr 1996).

The cell cycle is tightly regulated and contains a series of checkpoints to ensure the
fidelity of mitosis (Johnson and Walker 1999). A family of protein kinases known as
the cyclin dependent kinases (Cdks) have been identified, which are important in
regulating the cell cycle. These enzymes require a bound cyclin for activity as well as
phosphorylation for activity (reviewed in Morgan 1995). D type cyclins are important
for progression through G1 phase into S phase and associate with Cdk 4 and 6. The
expression of the D type cyclins is regulated by extracellular mitotic signals. The
cyclin D / Cdk complex phosphorylates the retinoblastoma (Rb) protein.
Phosphorylated Rb up regulates the activity of the transcription factor E2F, activating
genes requires for DNA synthesis and cell cycle progression. Cyclin E is also
expressed at this time forming complexes with Cdk2 and functions to prolong the
phosphorylation of Rb. Cyclins A and B are expressed later in the cell cycle and form
complexes with Cdk1. Regulation of the cdks is achieved through phosphorylation and
peptide inhibitors. The cyclin D / cdk complexes are inhibited by p16\(^{ink4a}\), p15\(^{ink4c}\) and
p19\(^{ink4d}\). Cyclin A, D and E dependent kinases are inhibited by p21\(^{cip1}\), p27\(^{kip1}\) and
p57\(^{kip2}\) (reviewed in Johnson and Walker 1999). Tight regulation of the cell cycle is
required to prevent inappropriate cell division. Tumour cells often acquire mutations
deactivating cell cycle regulation and checkpoint genes, which may lead to
uncontrolled cell division seen in tumour cells (Molinari, 2000).

Several checkpoints exist to monitor the accuracy of cell cycle transitions and to
prevent them from occurring inappropriately. Cells can be arrested throughout the cell
cycle depending on the damage they sustain. Two checkpoints control the entry to
mitosis and mitosis itself (Reviewed in Molinari, 2000). The G2/M checkpoint
controls entry to mitosis and is activated by cellular damage, particularly to DNA.
Cells with damaged DNA are prevented from proceeding through the G2/M
checkpoint into mitosis. Second is the spindle checkpoint, which is active during the
process of mitosis itself. The spindle checkpoints function is to detect unattached
kinetochores in the cell or failures in spindle function. On detection of unattached
kinetochores or spindle function failure, the spindle checkpoint functions to prevent exit from mitosis by arresting the cell cycle at the metaphase – anaphase transition. This allows the cell to repair any damaged system or induce apoptosis if the damage is too great to repair. The regulation of the spindle checkpoint is complex, requiring cell cycle controlling protein kinases such as the cyclin dependent kinases, the anaphase-promoting complex or cyclosome (APC/C) as well as spindle checkpoint-specific proteins such as the Mad and Bub family. Proteins such as Mad and Bub sense damage or incomplete attachment of kinetochores and relay this information to regulate the functions of the cell cycle components such as the cdks and APC/C. This allows for proper co-ordination of the individual steps in mitosis and ensures correct separation of chromosomes to prevent aneuploid or polyploid offspring. This step is vital for normal mitosis to occur, and errors in this checkpoint lead to cell death or tumourigenesis.

Much of the work on the spindle checkpoint has been done in Yeast, but because of the vital nature of the spindle checkpoint, mammalian homologues are very closely conserved (Wassmann and Benezra, 2001). Under a normal mitotic division, progression through mitosis is controlled by the destruction of inhibitory proteins by ubiquitination and destruction by the 26S proteosome. The key step controlled by the spindle checkpoint is the destruction of Pds1 in *S.cerevisiae* or securin in mammalian cells. Destruction of Pds1 leads to the liberation of Separin/Esp1, a protease that cleaves Scc1 triggering the loss of sister chromatid cohesion. The destruction of Pds1 is triggered by ubiquitination by the APC/C which is in complex with Cdc20 (Zachariae et al., 1999). APC/C<sup>Cde20</sup> also targets Clb5, Clb2 as well as Pds1. These proteins inhibit the activity of a complex of the APC/C and Hct1 (also known as Cdh1). The APC/C<sup>Hct1</sup> complex is required for a second stage that sees the destruction of Clb2. Hct1 is activated by its dephosphorylation by the phosphatase Cdc14. Cdc14 is a part of the mitotic exit network (MEN). The MEN consists of several proteins, Tem1, Lte1, cdc15, cdc5, Dbf2, Dbf20, Mob1, Net1 and Cdc14. The MEN is controlled by the GTPase activity of Bub2. An increase in the levels of GTP bound Tem1 triggers the release of Cdc14 from the nucleolus. This dephosphorylates Swi5, a transcription factor that increases the levels of the CDK inhibitor Sic1.
Cdc14 also stabilises Sic1 by dephosphorylating it, which prevents its destruction. Cdc14 also activates Hct1, which in complex with the APC/C triggers the destruction of Clb2. The increase in Sic1 and the destruction of Clb2 acts to reduce the activity of the Cdc28/B cyclin complex allowing cytokinesis to occur. To ensure correct timing of these events, Pds1 and possibly Esp1 inhibit activation of the MEN (Cerutti and Simanis, 2000).

The spindle checkpoint consists of two separate branches that inhibit either the APC/C:\textsuperscript{Cdc20} or the MEN (Gardner and Burke, 2000). The kinetochore pathway of the spindle checkpoint has been shown to require Mad 1, 2, 3 and Bub1 and 3. These genes were identified as mutations that increase the frequency of errors in chromosome segregation after low doses of Nocodazole. These proteins localise to unattached kinetochores and it is thought that these checkpoint proteins monitor the physical condition of the kinetochores. Upon activation of the spindle checkpoint, Mad2 associates with Cdc20, which prevent activation of the APC/C complex ubiquitin ligase activity. By maintaining the APC/C is an inactive state, Pds1 destruction does not occur, preventing chromatid separation and MEN activation. Upon full attachment of kinetochores, the spindle checkpoint ceases to signal, preventing the repression of Cdc20 by Mad1, 2 and 3. This allows activation of the APC/C, allowing continuation through mitosis through the destruction of Pds1 and activation of the MEN.

Figure 1.6: Pathways to ensure the fidelity and timing of mitosis.
The second branch of the spindle checkpoint controls activation of the MEN (figure 1.6). Bub2 mutants were observed to behave differently from the Mad mutants or Bub1 and 3 mutants. Bub2 mutants do arrest normally at the M – A transition after treatment with low doses of Nocodazole. Single Bub2 and Mad2 mutants can arrest mitosis but the double mutant of Bub2 and Mad2 cannot. This would suggest that the loss of both genes has an additive effect and that Bub2 and Mad2 are probably not in the same linear pathway. Bub2 functions as a GTPase for Tem1, hydrolysing Tem1 bound GTP to GDP. The GDP bound Tem1 is inactive. This prevents activation of the MEN. Activation of the Bub2 branch of the spindle checkpoint therefore does not inhibit the M – A transition but prevents the exit from mitosis by maintaining high levels of activity of the Cdk1/Cyclin B complex (Burke, 2000).

![Diagram of spindle checkpoint](image)

**Figure 1.7: Two branches of the spindle checkpoint.**

Both pathways are of interest to understanding the effects of the microtubule disrupting drugs. Mutants of Bub2 and Mad2 show that microtubule-disrupting drugs activate both branches of the spindle checkpoint. The role of the Bub2 pathway is...
unclear as the Bub/Mad pathway is sufficient to arrest the cell cycle after Nocodazole treatment. The Bub2 pathway has been proposed to monitor the end of the M –A transition or to monitor the separation of the spindle poles to the daughter cells as it located at the spindle pole and inhibits the late steps of mitosis.

The observation that p66Shc is phosphorylated in response to microtubule disruption and / or mitotic arrest induced by agents such as Taxol could link p66Shc into the apoptotic process induced by these compounds. It will therefore be of interest to see whether any microtubule localised proteins are identified in the yeast two-hybrid library screens. Microtubule disrupting agents have been reported to activate many of the same stress signalling pathways as oxidative stress. It could therefore be predicted that p66Shc phosphorylation is part of a generalised stress response induced by many different stimuli.

It would appear that p66Shc is a multi-functional protein involved with sensing oxidative damage, negative regulation of growth factor signalling and possibly mitotic arrest and microtubule disruption. In order to gain insight into the mechanisms by which p66Shc may modulate proliferative signalling pathways and also regulate the cells response to oxidative stress, we sought to identify novel binding partners for the distinct functional CH2 domain. Using a yeast two-hybrid library screening approach it may be possible to identify proteins associating specifically with the CH2 domain which may link p66Shc to particular signalling pathways. The phosphorylation of p66Shc seems key to its activity. Using the yeast two-hybrid assay, it may be possible to identify the kinase(s) responsible for the phosphorylation events. Using \textit{in vitro} kinase assays it is possible to test individual kinase enzymes ability to phosphorylate the CH2 domain of p66Shc.
Chapter 2: Materials and Methods

2.1: Materials

2.1.1 General chemicals and reagents

All chemical reagents were of analytical grade and were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK), Fischer (Fischer Scientific Ltd., Loughborough, UK) or Fisons (Fisons Scientific Equipment, Loughborough, UK).

2.1.2 Bacterial Strains and growth media.

The following *Escherichia coli* strains were used: DH5α, HB101 and XL-1 Blue. All bacterial stocks were stored at -80°C as glycerol stocks, made by adding 0.2ml of glycerol to 0.8ml of overnight culture. DH5α were routinely grown in liquid LB with shaking at 250 rpm or on solid media plates at 37°C. Bacteria transformed with antibiotic resistance plasmids were grown in L Broth or solid LB media (Bacto-tryptone (10 g/L), Bacto-yeast extract (5 g/L) and NaCl (10 g/L) supplemented with 100μg/ml Ampicillin or Streptomycin or 30μg/ml Kanamycin. All bacterial culture reagents were obtained from Oxoid (Unipath, Basingstoke, Hampshire, UK).

2.1.3 Yeast strains and growth media.

Reagents for yeast cell culture were obtained from Oxoid (Oxoid Ltd., Basingstoke, UK), Becton Dickinson (Sparks, MD). The general yeast strain used was *Saccharomyces cerevisiae* LA0, genotype [MATα his3Δ200 trp1-901 leu2-3112 ade2 lys2::(4lexAop-HIS3) ura3::(8lexAop-lacZ)] gal 4 gal 180. This yeast strain can be purchased from Invitrogen (9704 CH Groningen, The Netherlands). All yeast manipulations were carried out under sterile conditions using sterile materials.

2.1.4 Antibodies.

Rabbit polyclonal antibodies recognising Shc and mouse monoclonal Lar antibodies were purchased from Transduction Labs (Lexington USA). Mouse monoclonal Shc
antibody sc-967, anti-phosphotyrosine (sc-7020); FLK-1 sc-6251, p34 cdc2 sc-954, Erk2 c-14, and Erk-1 c-16 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit polyclonal Ch-Tog antibody was a gift provided by Dr. A Hyman, EMBL, Heidelberg.

2.1.5 Mammalian cell culture.

The following cell lines used are listed below:

- Hela - Human epithelial carcinoma cell line.
- Hek293 - Human Embryonic Kidney cells.
- MEF - Mouse Embryo Fibroblast cells.

Wild type, Shc and p66Shc knockout MEF cells were provided by Dr Tony Pawson (University of Toronto, Canada). p66Shc -/- cells are derived from the Shc knockout, but have been stably transfected to express wild type levels of p46 and p52Shc.

2.1.6 Cell culture media and supplements.

Cell culture reagents were obtained from Gibco BRL (Life Technologies Ltd., Paisley, Scotland). Hela, Hek293 and MEF cells were routinely cultured in Dulbecco’s modified eagle’s medium (DMEM) with Glutamax-I (L-alanyl-L Glutamine) 4500mg/l glucose with pyridoxine and sodium pyruvate, supplemented with 10% (v/v) fetal calf serum (FCS), 100 μg/ml streptomycin and 100 units/ml penicillin. Puromycin was added to media to a final concentration of 10μg/ml for the culture of p66Shc -/- cells. All cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. All media and solutions for cell culture were prepared and handled under sterile conditions.

2.1.7 Drugs and inducers of signal transduction.

Taxol, Nocodazole, Roscovitine and Anisomycin were obtained from Sigma and were made up as 100μM, 500ng/ml, 10mM and 100μM stock solutions respectively in Dimethylsulphoxide (DMSO). PD98059 and SB20190 were obtained from
Calbiochem (Nottingham, UK). Hydroxyurea was purchased from Sigma and made up as 100 mM stock solutions in distilled water. Epidermal growth factor was made up to a 1 μM stock in phosphate buffered saline.

2.2: Methods.

2.2.1 Yeast.

2.2.1.1 Yeast media.

Untransformed yeast were routinely cultured in YPD media (Yeast Nitrogen Base without amino acids (10 g/L) and Bactopeptone (20 g/L) pH 5.8-6.0). Glucose was added to a final concentration of 100 mM after sterilization. For solid media, Bacto-agar (18 g/L) was added prior to autoclaving. Yeast were grown at 30°C with shaking at 250 rpm for liquid media. Three drop out mixes are used to screen and select in the yeast two-hybrid system. The basic composition of the drop out mix is shown below with the following modifications for DOM1, which lacks leucine, tryptophan and histidine, DOM2, which lacks leucine and tryptophan, DOM3, which lacks uracil and lysine and DOM4, which lacks tryptophan.

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>0.5 g</td>
<td>Leucine</td>
<td>4.0 g</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>2.0 g</td>
<td>Lysine</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>2.0 g</td>
<td>Methionine</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>2.0 g</td>
<td>p-Aminobenzoic acid</td>
<td>0.2 g</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2.0 g</td>
<td>Phenylalanine</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>2.0 g</td>
<td>Proline</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.0 g</td>
<td>Serine</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2.0 g</td>
<td>Threonine</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0 g</td>
<td>Tryptophan</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>2.0 g</td>
<td>Tyrosine</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Inositol</td>
<td>2.0 g</td>
<td>Uracil</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.0 g</td>
<td>Valine</td>
<td>2.0 g</td>
<td></td>
</tr>
</tbody>
</table>
Drop out medium, (Yeast Nitrogen Base without amino acids (6.7 g/L) and drop-out mix (2 g/L) 100mM glucose, pH 5.8-6.0), was used for screening and selection of yeast transformants. Filter sterilised 1M 3-amino-1, 2, 4-triazole (3-AT), was added to cooled, autoclaved DOM1 media to the appropriate final concentration. For solid media, Bacto-agar (18 g/L) was added prior to autoclaving.

### 2.2.1.2 Preparation of transformation competent L40.

5 ml YPD was inoculated with a single colony of L40, and the culture grown at 30°C, with agitation at 250 rpm, overnight. The following day, 100 µl of this culture was diluted 1:500 into 50 ml YPD and grown overnight, (250 rpm, 30°C), until the OD$_{600}$ was between 0.8 - 1. Cells were pelleted at 2500 rpm, 4°C for 5 min, washed with 50 ml 0.1 M LiOAc in TE and repelleted. The cells were resuspended in 1 ml 0.1 M LiOAc in TE, shaken at 30°C, 250rpm for 30 minutes. 100 µl yeast was added to a eppendorf tube already containing 1 µg of each of the appropriate bait and prey plasmids. 400µl of 50% PEG 3350 in TE buffer containing 0.1M LiOAc was added and vigorously mixed. Transformations were incubated at 30°C for 30 min, after which 55µl of DMSO was added and mixed before heat-shock at 42°C for 6 min. The yeast was then pelleted by centrifugation for 30 sec at 13,000 g, and the supernatant aspirated. The pellet was washed with PBS before being re-pelleted and resuspended in100 µl PBS. The entire mixture was then plated out onto DOM2 plates and incubated for 3 days at 30°C.

### 2.2.1.3 Yeast two-hybrid growth assay.

Single transformants were spread onto DOM2 master plates and allowed to grow at 30°C for 3 days. The individual transformants were then replica plated onto DOM1 plates containing a range of 3-AT, and their growth was observed over the following week.
2.2.1.4 Yeast two-hybrid β-galactosidase assays.

(a) β-galactosidase filter assay.

Single transformants were patched onto DOM2 plates and allowed to grow at 30°C for 3 days. The individual transformants were then transferred onto grade 50 Whatman® filter papers (Whatman International Ltd, Maidstone, UK), (9 cm diameter), overlying solid DOM2 media. Yeast were allowed to grow on the surface of the filters at 30°C overnight. The filter was then submerged for 10 seconds in liquid nitrogen to permeabilise the cells. A Whatman grade 3 filter (9 cm diameter) was placed in a petri dish, and saturated with fresh X-gal substrate solution [0.27 % β-mercaptoethanol, 1.67 % X-gal stock solution in autoclaved Z-buffer (60 mM Na₂HPO₄.7H₂O, 40 mM NaH₂PO₄.H₂O, 10 mM KCl and 1 mM MgSO₄.7H₂O; pH 7.0)]. The frozen filter was then placed, colony side up, on top of this saturated filter, and incubated at 30°C until a blue colour develops.

(b) β-galactosidase solution assay.

1 ml DOM2 media was inoculated with a single colony of transformed L40, and the culture grown at 30°C, with agitation at 250 rpm, overnight. The following day, cells were pelleted at 5000 rpm for 5 min, resuspended in 500 μl Z buffer and repelleted. Cells were resuspended in 80 μl Z buffer by vortexing, and frozen in liquid nitrogen. Cells were then thawed at 30°C, and pelleted at 13000 rpm for 5 min. 15.4 μl supernatant was added to a well of two 96 well plates. The samples in one plate were used for a BCA protein assay. To the samples in the second plate, 106 μl Z buffer/0.27% β-ME was added. 24.6 μl ONPG (5mg/ml) was then added, and the time taken for the development of a yellow colour was recorded. Once yellow, 52.3 μl NaCO₃ was added to stop the reaction. 12 clones from each of three transformations was assayed for each construct. The mean ± S.D. of the results of one representative transformation are shown in the results chapters.
Absorbance at $\text{OD}_{405}$ was then read, and $\beta$-galactosidase activity calculated:

$$\text{Activity} = \text{nmol min}^{-1} \text{mg}^{-1}$$

$$= \frac{\text{OD}_{405} \times \text{total vol (ml)}}{0.0045 \times [\text{protein}] \text{ (mg/ml)} \times \text{extract vol (ml)} \times \text{time (min)}}$$

2.2.2: Tissue culture.

2.2.2.1 Maintenance of cell lines.

Hek293, Hela and Mouse Embryo Fibroblasts (MEFs) cells were routinely cultured as monolayers in medium sized plastic tissue culture flasks. Upon approaching confluence, the cells were passaged. Cells were washed once with PBS, 1 ml trypsin-EDTA (0.25% trypsin, 1 mM EDTA) was added, and the cells incubated at 37°C until they detach. 9 ml media was then added and the cells pelleted by centrifugation. Fresh media was added and the cells split as required. Cell number was determined by counting on a haemocytometer. New cell stocks were used after 20 – 25 passages.

2.2.2.2 Storage of cells.

Cell lines were frozen in liquid nitrogen for long-term storage. Cells approaching confluence in a medium sized flask, approximately $10^6$ exponentially growing cells, were washed once with PBS, and detached from the plate with 1 ml of trypsin-EDTA and resuspended in 1 ml FCS. An equal volume of 20% DMSO in serum free DMEM media, was added to the cells. Cells were aliquoted and frozen slowly by placing in an insulated box at -80°C overnight before transfer to a liquid nitrogen tank.

2.2.2.3 Recovery of frozen cells.

Cells were thawed quickly in a 37°C water bath, and added to 10 ml DMEM media in a medium sized flask. Once cells had adhered, media was replaced with fresh media so as to remove traces of DMSO and any dead cells.
2.2.2.4 Transfection of Hek293 cells.

Fugene reagent (Roche) was used to transfect Hek 293 cells with the pcDNA3 construct containing the full length Lar cDNA. 4µl of Fugene reagent was incubated in 96µl of serum free media for 10 minutes before being added to 1-2 µg of plasmid DNA. This mixture was then incubated at room temperature before being added to the cells to be transfected and incubated for 24 hours.

2.2.2.5 Cell cycle analysis.

For analysis of the cell cycle, 1x10⁶ cells were trypsinised and fixed in ice cold 70% ethanol. Cells were collected by centrifugation and incubated with PBS containing 0.1mg/ml RNAse and 5mg/ml propidium iodide. The DNA content was then analysed by flow cytometry (Becton Dickson FACScan) and the percentage of cells in each phase of the cell cycle analysed by Cell Quest software. Cyclin B expression was also used to analyse cell cycle location through western blot with an anti-cyclin B antibody.

2.2.2.6 Determination of apoptosis.

The number of apoptotic cells present in a population was estimated using Hoechst staining and counting using a fluorescence microscope. Cells were seeded onto covers slips and treated with the desired stimuli and analysed over a range of time points. Cells were then washed twice with PBS and fixed with ice-cold methanol for a minimum of 2 hours. The methanol was then washed off with PBS and the cells incubated with 1µM Hoechst stain in PBS for 5 minutes. The cell were then washed four times with PBS and mounted for microscope analysis. Three hundred cells were then counted per slide. Apoptotic cells are clearly visible as they show distinct nuclear changes. Experiments were repeated at least three times for each experimental condition in parallel with cells treated with DMSO alone. Background levels of apoptosis (DMSO alone) were subtracted. The mean percentage ± S.D. of cells undergoing apoptosis from three experiments are shown in the results chapters.
2.2.3: DNA Manipulations.

2.2.3.1 Further cloning of 23.2 clone.

Forward and reverse primers were synthesized from the 23.2 sequence cloned from the yeast two-hybrid screen.

23.2F
5'-CTCACC GCCCCTTTCTTGGTC-3'
23.2R
5'-AGGAAGGCATTGGTAGCTATA-3'

pGadF
5'-ACC ACTACAATGGATGATGTA-3'
pGadR
5'-AGAAATTGAGATGGTGCACGA-3'

Using the pGadF and pGadR primers that recognise vector sequence 5' and 3' of the library insert in combination with 23.2F and 23.2R it is possible to amplify 5' and 3' sequence to the 23.2 sequence. Sequencing of isolated products was achieved using either 23.2F or 23.2R depending on the direction of the fragment cloned.

2.2.3.2 Plasmids.

The pBTM116 vector was constructed by Paul Bartel and Stan Fields (Dept. of Microbiology, State University of New York, Stony Brook). The cDNA for p66Shc was provided by Dr. B. Margolis, (Howard Hughes Medical Institute, University of Michigan) and was used to amplified the CH2 domain of p66Shc by PCR using the following primers:

5'-CGATGGATCTCTGGATCTTCTACCCCA-3'; 5'-CGATGGATCCCGCCTCCACTCAG-3'
The PCR fragment was subcloned into the BamHI cloning site of pBTM116 for yeast two-hybrid library screening, and pGEX-3x (Pharmacia) for the production of GST-fusion protein (constructed by J.A. Brown, University of Leicester).

The construct encoding the juxtamembrane domain of LAR in the pVP16 vector (LAR\textsubscript{4190-4472}) was made by PCR amplification of LAR from the full-length human cDNA of Lar in pcDNA3 vector, kindly provided by Dr. T. S. Pillay, (University of Nottingham) using the following primers:

5'-CGATGGATCCTATTCAAGAGGAAGAACA-3';
5'-CGATGGATCCTTCGACATAGCGGTTCTTGGG-3'

The resulting PCR products were then ligated into the BamHI site of pVP16.

Sequences cloned into the pVP16 vector or identified in the yeast two-hybrid library screens were sequenced using the Sequenase 2.0 DNA sequencing kit (Amersham) with the following primers:

pVP16.F: 5'-GAGTTTGAGCAGATGTTTA-3'
pVP16.R: 5'-TGTAAAACGACGGCCAGT-3'

The construct encoding the full length p66Shc sequence in the pDsRed vector (Clonetech, Palo Alto, USA) was made by PCR amplification of the p66Shc cDNA provided by Dr. B. Margolis, (Howard Hughes Medical Institute, University of Michigan).

Sequences cloned into the pDsRed vector were sequenced using the following primers:

pDsRed.F: 5'-TAGGCGTGTACGGTGGGA-3'
pDsRed.R: 5'-GAAGCGCATGAACTCCTT-3'

This vector encodes a novel red fluorescent protein (RFP) that has been optimised for expression in mammalian cells. The p66Shc sequence cloned into this vector is expressed with RFP fused at the C-terminus. Fusions to the N-terminus of DsRed allow \textit{in vivo} localisation of the fusion protein (excitation maximum = 558nm,
emission maximum = 583nm). This vector contains a neomycin / kanamycin resistance marker.

2.2.3.3 Mutagenesis of LAR.

Mutagenesis of double-stranded DNA templates was performed using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene). For each specific mutagenesis reaction, this method requires that complementary primers encoding the desired mutation are synthesized for both sense and antisense strands. These primers are used to simultaneously initiate DNA synthesis in a PCR reaction, thereby producing double-stranded, mutated plasmids with staggered nicks. These PCR products are then digested with DpnI, which preferentially digests only the parental, methylated DNA, leaving the mutated strands intact. The digested products are then transformed into XL1-Blue Supercompetent Cells (Stratagene). These bacteria repair the nicks, generating closed circular, double-stranded, mutant plasmids. All reactions were conducted as described in the kit, using the reagents provided.

Point mutations were introduced into the LAR juxtamembrane construct (LAR\textsubscript{4190-4472}) using the following primers:

**LAR SS>AA:**

5' -GGACCCACTCTCCGCGCTAAGGATGAGCAG-3' and 5' -CTGCTCATCCTTAGCGGCCGGAGAGTGGGTCC-3'

**LAR Q>A:**

5' -CCTCTAAGGATGAGGCGTCGATCGGACTGAAG-3' and 5' -CTTCAGTCCGATCGACGCCTCATCTTAGAGG-3';

**LAR K>T:**

5' -CACTCTCCGTCCTCTACGGATGAGCAGTCCGT-3' and 5' -GATCGACTGCTCATCCGTAAGAGGACGAGTGG-3'.
The lysine 1285 mutation was introduced into the full-length human cDNA using the LAR K>T primer pair indicated above.

2.2.3.4 DNA purification.

DNA (100bp-10kb) was routinely purified from primers, nucleotides, enzymes and salts by the PCR Purification Kit using QIAquick spin columns (Qiagen Ltd., Crawley, West Sussex, UK). Gel purification of DNA from agarose gels was achieved using Qiagen gel extraction kit, which also utilises QIAquick spin columns.

2.2.3.5 Restriction digests.

Restriction endonucleases and their corresponding buffers were purchased from Roche (Roche Diagnostics Ltd., Lewes, East Sussex, UK). Digests were usually performed for 1-2 hours at 37°C, but longer incubations were required for some cleavages where the cut site is close to the end of the DNA sequence. In the case of double digests in which the buffers were not compatible, digests were performed sequentially, with the products from the first digest being purified before use in the second digest.

2.2.3.6 Alkaline phosphatase treatment.

1 unit of shrimp alkaline phosphatase (Roche Diagnostics Ltd., Lewes, East Sussex, UK) was added to digested, purified DNA, mixed with the appropriate amount of buffer, and incubated at 37°C for 1 h.

2.2.3.7 DNA ligations.

Ligation reactions contained an approximate insert to vector ratio of 3:1, or with an excess of insert over vector. Ligation reactions were carried out using 2 units of T4 DNA ligase, in 66mM Tris, 5mM MgCl₂, 1mM DTT, 1mM ATP, pH7.5 buffer overnight at 16°C. T4 DNA ligase was deactivated by incubating the reaction at 65°C before transformation.
2.2.3.8 Separation of DNA by agarose gel electrophoresis.

6 x gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was added to DNA samples prior to electrophoresis. The samples were electrophoresed in 1-2% agarose gels, containing ethidium bromide for visualization of DNA bands by UV light. Electrophoresis was performed at 100 V in 0.5 x TBE buffer.

2.2.3.9 Extraction of DNA from agarose gels.

DNA fragments (100bp-10kb) were routinely extracted from agarose gels using the Gel Extraction kit (Qiagen Ltd., Crawley, West Sussex, UK) as described in the kit.

2.2.3.10 Standard DNA minipreps.

5ml LB-Amp was inoculated with a single colony of DH5α, and the culture grown at 37°C, with agitation at 225 rpm, overnight. Cells were pelleted at 13,000 g, 4°C for 30 sec, and resuspended in 100μl ice cold, autoclaved, solution I by vortexing. 200 μl of fresh solution II was then added, the solutions mixed by inversion, and the samples placed on ice. 150 μl of ice-cold, autoclaved, solution III was added and the solutions mixed gently by inversion. The tubes were then incubated on ice for a further 5 min before centrifugation at 13,000 g, 4°C for 5 min. The supernatant was then subjected to phenol/chloroform extraction, and the DNA was isolated from the aqueous layer by ethanol precipitation.

2.2.3.11 Midi-DNA preps.

5ml LB-Amp was inoculated with a single colony of DH5α, and the culture grown at 37°C, with agitation at 225 rpm, overnight. The following day, 1 ml of this miniculture was used to seed 500 ml LB-Amp, and the culture grown at 37°C, with agitation at 225 rpm, overnight. Cells were pelleted at 8000 rpm for 15 min, and resuspended in 4ml cold, autoclaved, solution I (50 mM glucose, 25mM Tris/HCl, 10mM EDTA pH 8). 20 mg lysozyme was added, and the solution incubated at room temperature for 10 min. 10 ml fresh solution II (0.2M NaOH, 1% SDS) was then
added, and the solution transferred to ice for 10 min. 7.5 ml autoclaved solution III was then added (3.6 M potassium acetate in 11.5% acetic acid), and the solution was incubated on ice for a further 10 min. The mixture was centrifuged at 12,000 rpm for 10 min, after which the supernatant was divided into 2 x 10 ml aliquots. 6 ml isopropanol was added to each aliquot. The tubes were then incubated at room temperature for 5 min, centrifuged at 11,500 rpm for 10 min, and then washed with 4 ml 70% ethanol. Each pellet was resuspended in 1 ml TE and treated with 5 µl RNase A (10 mg/ml) for 30 min at 37°C. The solution was then divided into 500 µl aliquots and subjected to phenol/chloroform extraction. The aqueous layers were further divided into 250 µl aliquots, from which the DNA was isolated by ethanol precipitation. DNA was resuspended in 1 ml TE.

2.2.3.12 Synthesis of primers.

Oligonucleotide primers were synthesized, at the 40 nM scale, on an Applied Biosystems 394 synthesizer (Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, Leicester, UK). Oligonucleotides were purified by ethanol precipitation.

2.2.3.13 Amplification of DNA by the polymerase chain reaction.

PCR reactions were conducted in 0.5 ml, thin-walled, polypropylene microcentrifuge tubes. Each reaction contained 50-500 ng template, 1 mM primers, 200 mM of each dNTP (Pharmacia Biotech.) and 10 x reaction buffer (Stratagene), in a total volume of 100 µl. The PCR was performed in a GENIUS block (Techne). Unless otherwise stated, reactions were as follows: Template was denatured for 6 min at 94°C prior to the addition of 2 units of Taq (Stratagene). 19 cycles of denaturation (94°C, 30 sec), annealing (58°C, 50 sec), and elongation (72°C, 2 min) were finally followed by an extra 7 min of elongation (72°C) to allow completion of all initiated strands.

2.2.3.14 Preparation of transformation competent bacteria.

Chemically competent cells were prepared by inoculating 5ml of LB broth with a single colony of DH5α and grown overnight. 500 µl of this culture was diluted 1:100
into 50 ml LB and grown at 37°C, with agitation, until an OD<sub>600</sub> of 0.2 was reached. Cells were pelleted at 2000 rpm, 4°C, for 10 min. The cell pellet was resuspended in 5 ml ice cold, transformation buffer (10% w/v PEG 1500, 30 mM MgCl<sub>2</sub>, 5% fresh DMSO in LB) and chilled on ice for 20 min. Cells were then aliquoted into prechilled microcentrifuge tubes or snap-frozen in a dry ice/ethanol bath and stored at -70°C for later use.

2.2.3.15 Bacterial cell transformation.

An appropriate amount of plasmid DNA was added to the competent DH5α bacteria and mixed with the pipette tip. The mixture was then incubated on ice for 10 minutes, followed by incubation at room temperature for 10 min, and on ice for a further 10 min. 900 µl LB supplemented with 20mM glucose was added to the cells. To increase efficiency, a recovery step of 1h incubation at 37°C, with agitation at 225 rpm was included. 200µl of the culture was then plated out onto LB plates with appropriate selection and grown at 37°C overnight.

2.2.4: Protein Manipulations.

2.2.4.1 Quantitative protein assay.

Protein concentrations of samples were quantified with the BCA Protein Assay Kit (PIERCE, Rockford, IL) according to the manufacturer’s protocol. Unknown protein concentrations are calculated from a standard curve by plotting BSA concentration standards against A<sub>562</sub> readings.

2.2.4.2 Preparation of whole cell lysates.

50,000 cells were seeded per well of a 24 well plate and allowed to attach overnight. The following day, cells were washed once with PBS prior to the addition of 40µl, hot 1.5x SB. The lysates were then transferred into microcentrifuge tubes, 100 mM DTT was added, and the samples were boiled for 10 min before loading for SDS-PAGE.
2.2.4.3 Expression and purification of CH2 domain / glutathione S-transferase (GST) fusion protein.

0.5ml of an overnight culture of the pGEX or pGEX-CH2 vector in DH5α cells was used to inoculate desired final volume of LB-Amp media and grown at 37°C, with agitation at 225 rpm, until the OD₆₀₀ was 0.6. Expression of the fusion protein was then induced with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) and grown for a further 5 h. Cells were pelleted by centrifugation at 5000 rpm for 10 min at 4°C and then lysed on ice for 20 min in 5 ml lysis buffer (100 mM Tris (pH7.4), 100 mM NaCl, 1 mM EDTA) containing 5 mg lysozyme, 1 mM DTT and protease inhibitor cocktail. After 20 min, 4 mg deoxycholate (DOC; 40 mg/ml stock in lysis buffer) was added, and the mixture incubated at room temperature for 20 min. DNAse (10 mg/ml) and 500 μl 1M MgCl₂ were then added, and the mixture incubated for a further 20 min at room temperature. Insoluble debris was pelleted by centrifugation at 8000 rpm, 4°C for 10 min. The cell supernatant was then added to 0.5 ml glutathione-sepharose beads (Glutathione Sepharose R 4B; Pharmacia Biotech) in PBS, which had been washed in PBS to remove any alcohol preservative. The beads and supernatant were incubated with rotation overnight at 4°C. The following day, the beads were washed (1000rpm, 4°C, 1 min) twice with PBS/0.1% Triton/1 mM DTT, and twice with PBS/1 mM DTT. 1 volume of PBS was then added to the beads. The amount and purity of the fusion protein immobilized on the beads was estimated by comparison of varying volumes of beads with defined BSA standards. This was performed by subjecting the samples and the standards to SDS/PAGE, staining the gels with coomassie Brilliant Blue (45% methanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue R) for 30 mins, and then destaining (45% methanol, 10% acetic acid) as required. 20% glycerol was added to the bead-immobilized fusion proteins. These were then aliquoted and stored at -80°C.
2.2.4.4 Precipitation of Lar and Ch-tog from whole cell lysates using a CH2-GST fusion protein.

Cells were seeded at a density of 1 x 10^6 cells per 6 cm petri dish, and allowed to attach overnight. The following day, the medium was replaced with fresh media 1 h before beginning the experiment. Cells were stimulated with either 15 nM EGF for 5 minutes, 400 μM hydrogen peroxide for 5 minutes or with 100 nM Taxol for 16 hours. Following treatment, cells were washed twice with PBS and lysed for 10 min at 4°C in lysis buffer [1% Triton X-100, 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EGTA, 25 mM benzamidine] containing 100 mM NaF, 1 mM Na_3VO_4 and a mammalian protease inhibitor cocktail (Sigma). Cell debris was removed by centrifugation at 25,000g, 4°C for 5 min and protein precipitated from lysates with 5 mg beads by incubating with rotation at 4°C overnight. The following day, the beads were pelleted by centrifugation at 5000 rpm, 4°C for 2 min, and then washed four times with TBST (50 mM Tris/HCl, pH 7.4, 150 mM NaCl)/0.1% Triton X-100. Precipitated protein was eluted from beads by boiling for 5 minutes in 3 X SDS sample buffer, freshly supplemented with 100mM DTT. The preheated samples were analysed immediately by SDS-PAGE.

2.2.4.5 Immunoprecipitations.

Cells were seeded at a density of 1 x 10^6 per 6 cm petri dish, and allowed to attach overnight. 2.5 mg protein A-sepharose (for precipitations with the rabbit anti Shc antibody) or protein G-sepharose (for precipitations with the mouse anti Shc antibody), were washed once with PBS (5000 rpm, 2 min). 5 μg of antibody was then pre-bound to the beads by incubating at room temperature, with occasional gentle flicking, for 1-2 h. Cells were stimulated as described in individual figure legends. Following treatment, cells were washed twice with ice-cold PBS and lysed for 10 min at 4°C in lysis buffer [1% Triton X-100, 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EGTA, 25 mM benzamidine] containing 100 mM NaF, 1 mM Na_3VO_4 and a mammalian protease inhibitor cocktail (Sigma). Cell debris was removed by centrifugation at 25,000g, 4°C for 5 min and protein was precipitated from the supernatant by incubating with antibody, pre-bound to beads, with rotation at 4°C overnight. The following day, immunoprecipitates were pelleted by centrifugation at 4°C for 5 min and protein precipitated from lysates with 5 mg beads by incubating with rotation at 4°C overnight. The following day, the beads were pelleted by centrifugation at 5000 rpm, 4°C for 2 min, and then washed four times with TBST (50 mM Tris/HCl, pH 7.4, 150 mM NaCl)/0.1% Triton X-100. Precipitated protein was eluted from beads by boiling for 5 minutes in 3 X SDS sample buffer, freshly supplemented with 100mM DTT. The preheated samples were analysed immediately by SDS-PAGE.
5000 rpm, 4°C for 2 min, and then washed four times with TBST (50 mM Tris/HCl, pH 7.4, 150 mM NaCl)/0.1% Triton X-100. Associating proteins were eluted from beads by boiling for 5 min in 3 X SDS sample buffer, freshly supplemented with 100 mM DTT. The preheated samples were analysed immediately by SDS-PAGE.

2.2.4.6 Western Blot analysis.

(A) SDS polyacrylamide gel electrophoresis.
Proteins were separated by SDS-PAGE through 0.7mm thick, 7-10% gels, using a Biorad minigel apparatus and a tricine based buffer system:

Anode buffer: 0.2M Tris (pH 8.9)
Cathode buffer: 0.1 M Tris, 0.1 M tricine and 0.1% SDS; pH 8.25.

The choice of acrylamide concentration is dependent on the molecular mass of the proteins being studied. Lar (145 kDa) and Ch-Tog (200 kDa) were resolved on 7% gels. She proteins (46 - 66 kDa) were analysed on 10% gels. Products of the immune complex kinase assays (19 - 40 kDa) were resolved on 12% gels. The loaded proteins are initially stacked in a 4% gel before progressing into the 7-10% resolving gel. This is achieved by pouring the resolving gel first. A small volume of distilled water was added to ensure a level surface to the gel. Once the resolving gel is set the stacking gel is poured on top and the comb inserted. The acrylamide stock solution (Ultra Pure ProtoGel; National Diagnostics, Hull, UK or Prosieve gel) contained acrylamide and bis-acrylamide at a ratio of 37.5 : 1 respectively. For resolving gels, acrylamide was mixed with 33% gel buffer [3M Tris.HCl (pH 8.45), 0.3% SDS], 10.6% glycerol and water as required. Polymerisation was initiated by the addition of 0.05% fresh ammonium persulphate and 0.05% TEMED. For 4% stacking gels, acrylamide was mixed with 20.7% gel buffer and water. Polymerisation was then initiated by addition of 0.08% fresh ammonium persulphate and 0.08% TEMED. Samples were boiled for 10 min in sample buffer (3 x SDS sample buffer: 100 mM Tris HCl (pH 6.8), 4% SDS, 0.01% bromophenol blue, 20% glycerol), freshly supplemented with 100 mM DTT, immediately before loading on the gel. 5 μl of Rainbow (range: 14.2 kDa-205 kDa) molecular weight markers (Sigma) were run alongside samples, so as to
facilitate the estimation of sample protein molecular weights. Gels were run at 50 V until the proteins had stacked. The voltage was then increased to 150 V.

(B) Transfer of proteins to nitrocellulose membranes.

Proteins separated by SDS PAGE were transferred to nitrocellulose membranes for immunodetection. Six gel-sized sheets of Whatman 3MM filter paper were soaked in transfer buffer (48 mM Tris base, 39 mM Glycine, 20% methanol, 0.037% SDS) and placed at the anode of a horizontal, semi-dry electrophoretic transfer apparatus (Biorad). On top of this was placed one sheet of pre-soaked (in transfer buffer) Hybond ECL nitrocellulose membrane (Amersham), followed by the gel, which had been soaked in transfer buffer for 20 minutes. This was completed with 6 more pre-soaked sheets of 3MM paper and the cathode was then placed in contact with this layer. The transfer was conducted at 21-23 V for 45-120 min, depending on the size of the protein to be transferred. Once transferred, the membrane was blocked at 4°C overnight in TBST (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20) containing 5% low fat dried milk powder, (or 5% BSA if membrane was to be analysed for the presence of phosphoproteins), prior to being used for the immunodetection of proteins.

(C) Immunodetection of proteins bound to nitrocellulose membrane.

The blocked membrane was incubated with an appropriate dilution of primary antibody in TBST supplemented with 5% low fat dried milk powder, (or 1% BSA), for 1 h whilst agitating at room temperature. The membrane was washed four times (10 min each) with TBST, and then incubated, by agitating for 1 h at room temperature, with a 1:1000 dilution of horseradish peroxidase-coupled secondary antibody in TBST. The membrane was washed four times (10 min each) with TBST. The membrane was then covered with a 1:1 mix of ECL chemiluminescent detection reagents (Western Blot Chemiluminescence Reagent Plus; NEN Life Science Products) for 2 min and exposed to chemiluminescence-sensitive film. After exposure, if the blot needed to be re-probed, the membrane was incubated at 60°C for 50 min in blot-strip buffer (62.5 mM Tris.Cl (pH 6.7), 2% SDS), containing 0.8%
fresh β-mercaptoethanol. The membrane was then washed several times with de-ionised H₂O, four times with TBST, and then blocked at 4°C overnight.

2.2.4.7 Detection of kinase activity after SDS-PAGE.

Semi-confluent 10cm dishes of Hela cells were stimulated with the appropriate stimuli before being washed in PBS and lysed in 1ml of 1 X SDS-PAGE sample buffer. 0.4ml of the lysates were then separated by SDS-PAGE on a 10% gel containing between 0.1 – 0.5mg/ml GST-CH2 fusion protein immobilized in the resolving gel. Gels were run at 4°C to prevent overheating of the samples. On completion of the SDS-PAGE, the resolving gel is incubated in 100ml of 20% isopropanol in 50mM Tris-HCl (pH 8) with shaking for 30 minutes, with a change of buffer followed by a further 30 minutes incubation as before to remove SDS from the gel. The gel was then incubated with 250ml of Buffer A (50mM Tris-HCl / 5mM 2-mercaptoethanol (pH 8)) for 1h with shaking. Then gel was then denatured with two 30-minute incubations in 100ml 6M Guanidine/HCl in buffer A with gently shaking. The proteins are then renatured by incubating in Buffer A containing 0.04% Tween 40 at 4°C for 16h with frequent buffer changes. The gel was then incubated in Buffer B (40mM Hepes (pH 8), 2mM DTT, 0.1mM EGTA, 5mM MgCl₂) for 30 minutes at room temperature. The gel was then incubated at Buffer B containing 50μM ATP and 5μl of 10μCi/μl γ³²P ATP for 1 hour at room temperature with gentle agitation. The gel was then washed with 5% Trichloroacetic acid / 1% Sodium pyrophosphate buffer until radioactivity of solution become negligible. The gel is then dried on Whatman 3M paper and exposed to film at -70°C with an intensifying screen.

2.2.4.8 Immune-complex kinase assays.

Cells seeded at 1x10⁶ were allowed to attach overnight before being stimulation with 200nM PMA for 10 minutes (ERK1 and 2 assay), Nocodazole for 16 hours (CDK1 assay) or 100nM Anisomycin for 30 minutes (p38 and Jnk assays). Cell lysates were prepared as described in immunoprecipitation protocol. Lysates were cleared with a 1-hour incubation at 4°C with 0.5mg of protein A or protein G sepharose. The supernatant was then removed and incubated with protein A or protein G sepharose
Chapter 2

with 5μg of bound antibody to Cdk1, ERK1 and 2 (Protein A) or Jnk and p38 (protein G) overnight at 4°C. The precipitates were washed twice with Triton lysis buffer and once with either Cdk1 assay buffer, (50mM Hepes (pH 7.4), 1mM DTT, 15mM EGTA and 20mM MgCl$_2$), Jnk and p38 assay buffer (20mM Hepes (pH 7.2), 20mM β-glycerophosphate, 1mM DTT, 50μM sodium orthovanadate and 10mM MgCl$_2$) or ERK 1and 2 assay buffer (30mM Tris-HCl (pH 8), 20mM MgCl$_2$ and 2mM MnCl$_2$). The assays were then resuspended in 30μl of the appropriate kinase buffer containing 5μg of substrate; MBP for ERK1 and 2, Histone H1 for Cdk1, GST-jun for Jnk and GST-ATF2 for p38 in addition to 50μM ATP containing 2μCi of [$^{32}$P] ATP. Reactions were incubated at 30°C for 30 minutes and the reactions stopped by the addition of SDS PAGE sample buffer. The samples were then resolved on 10% SDS PAGE gels. $^{32}$P incorporation was detected by exposure of the dried gels to X-ray film.

2.2.4.9 Generation of polyclonal p66Shc antibodies.

GST / CH2 fusion protein was prepared as described in section 2.2.4.3. The GST / CH2 fusion protein was then eluted from the beads using a sterile solution of 10mM glutathione in PBS. The eluted protein was then quantified on coomassie stained gels. Two RB/NZW rabbits were immunised with 0.4 mg of GST / CH2 domain fusion protein in Freunds complete adjuvant. Two booster doses of 0.2 mg GST / CH2 domain fusion protein in incomplete adjuvant were given at monthly intervals, with titre checks 7 day after each booster. The sera was then analysed for ability to detect p66Shc in whole cell lysates by Western blot.
2.2.5 Yeast two-hybrid library screening.

2.2.5.1 cDNA libraries and vectors.

The pVP16 mouse embryo library was constructed by Stan Hollenberg, (Fred Hutchinson Cancer Research Center, Seattle, Washington). The pBTM116 vector was constructed by Paul Bartel and Stan Fields (Dept. of Microbiology, State University of New York, Stony Brook).

Sequencing primers
pVP16.F: 5'-GAGTTT GAGC AG ATGTTT A-3'

2.2.5.2 Media.

(A) Soc and SOB media.

Bacto-tryptone (20 g/L), Bacto-yeast extract (5 g/L) and NaCl (0.5 g/L) were dissolved in de-ionised water. 2.5 mM KCl was then added, the pH adjusted to 7.0, and the media autoclaved. For SOC media, autoclaved SOB was cooled to 60°C prior to the addition of 20 mM filter-sterile glucose. 10 mM autoclaved MgCl₂ was then added just before use.

(B) M9/agar.

410 mM Na₂HPO₄, 220 mM KH₂PO₄, 86 mM NaCl and 187 mM NH₄Cl were dissolved in de-ionised H₂O, the pH adjusted to 7.2-7.6, and the media autoclaved. Autoclaved media was cooled to 60°C prior to the addition of 4 mg/L thiamine (2 mg/ml stock). 1 x M9 was then mixed with bactoagar (20 g/L), and the media was re-autoclaved. When cooled to approximately 60°C, 0.2% filter sterile glucose, 100 mM autoclaved CaCl₂, 1mM autoclaved MgSO₄, and 2mg thiamine (2mg/ml) were added, the plates poured and allowed to set.
2.2.5.3 Screening yeast two hybrid cDNA libraries.

The method used for a library screen transformation was a scaled up version of the method used for a small-scale transformation, with the transformed yeast being plated onto plates lacking histidine as well as tryptophan and leucine. A 500 ml yeast culture was routinely used for library screens. The amounts of yeast, DNA and reagents used were doubled in comparison to those used for small-scale transformations. Transformations were plated onto 15 cm diameter DOM1 plates containing 3-AT at the appropriate concentration. The efficiency of transformation was estimated by plating 20 μl of one transformation mixture onto a 9 cm diameter DOM 2 plate.

2.2.5.4 Isolation of plasmid DNA from positive transformants.

Yeast colonies testing positive for both reporters were scraped into microcentrifuge tubes containing 100 μl yeast lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris.Cl (pH 8.0), 1 mM EDTA] and 200 μl phenol:chloroform:isoamyl alcohol (25:24:1). A small spatula of 425-600 μM, acid-washed glass beads (Sigma) was added, the tube vigorously vortexed for 2 min, and then centrifuged for 5 min. Plasmid DNA was isolated from the cell lysates using spin columns (Qiagen).

2.2.5.5 Preparation of Leu- (HB101) electrocompetent cells.

50 ml SOB (100μg/ml Streptomycin) was inoculated with a single colony of HB101, and grown at 37°C, with agitation at 225 rpm, overnight. 0.5 ml of this culture was diluted 1:1000 into 500 ml SOB (100μg/ml Streptomycin) and grown until the OD₅₅₀ reached 0.8. The cells were harvested by centrifugation at 5,000 rpm for 15 min. The pellet was washed three times with 500 ml ice-cold, autoclaved, wash buffer (10% redistilled glycerol) and then resuspended in 2 ml wash buffer. Unless used immediately, the cells were frozen in a dry ice/ethanol bath and stored at -80°C.
2.2.5.6 Electroporation of recovered plasmids into electrocompetent HB101.

1 μl DNA was electroporated into a 50μl aliquot of HB101. After a 1 hour incubation at 37°C with shaking, 100 μl of the electroporation mixtures were plated out onto LB-Amp (100 mg/ml) plates, which were then incubated at 37°C overnight.

2.2.5.7 Selective isolation of library plasmids.

Colonies resulting from the electroporation of positive plasmid DNA into HB101 cells were spotted onto M9 plates to select for bacteria containing library plasmids. Colonies growing on the M9 plates should contain library pVP16 vectors. Plasmid DNA was then isolated and mini prep DNA was used for sequencing.
Chapter 3. Identification of proteins interacting with the second collagen homology domain of Shc using the yeast two-hybrid system.

3.1 Introduction.

To characterise the signal transduction pathways responsible for the inhibitory effects of the p66Shc isoform, it is important to identify protein – protein interactions of potential signalling intermediates with the CH2 domain. Several methodologies exist for this purpose. Traditionally biochemical techniques have been used, such as co-immunoprecipitation, chemical cross-linking and co-fractionation. These methods have the considerable drawback of identifying the interacting protein. The yeast two-hybrid system goes someway to resolving this problem. The yeast two-hybrid system is a yeast based genetic approach for identifying and characterising protein – protein interactions (Fields and Song, 1989). The yeast two-hybrid system selects for proteins that interact with a target protein by activating the transcription of one or more reporter genes, which allows selection of yeast colonies showing positive interactions. The yeast two-hybrid system exploits the modular nature of transcription factors. Transcription factors require a DNA binding domain and a transcriptional activation domain to activate transcription. It has been shown that the DNA binding domain has to be in close contact with the transcriptional activation domain to activate transcription, although this does not necessarily have to be present in the same molecule. If one protein has a DNA binding domain and on another, a transcriptional activation domain and the proteins bring the two domains into close contact, they can activate transcription. This observation has been exploited in the yeast two-hybrid system. DNA sequences for the target proteins are cloned into vectors containing the transcriptional activation domain and the DNA binding domains to create an in frame protein fusion, fusing the target proteins to either DNA binding domain or the transcriptional activation domain. If the target proteins interact, the DNA binding domain and the transcriptional activation domain are localised together to form a complex capable of initiating the transcription of a reporter gene (Figure 3.1). This approach has been expanded by fusing cDNA library sequences to the transcriptional activation domain allowing whole cDNA libraries to be screened for proteins interacting with a target protein of interest. This makes the yeast two-hybrid system a powerful tool, as millions of clones can be screened and upon identification of a
positive interaction, the DNA sequence of the interacting protein can easily be retrieved from the yeast colony (Chien et al., 1991; Fields and Sternglanz, 1994).

Figure 3.1: The yeast two-hybrid system.
When transformed individually, neither the LexA/CH2 domain fusion (A) or the VP16/cDNA fusion (B) can initiate transcription. When co-transformed together, if the CH2 domain interacts with the protein encoded by the library cDNA, transcription is initiated (C). (UAS = Upstream activating sequence, BD = DNA binding domain.

Various derivatives of the yeast two-hybrid system have been developed (Keegan and Cooper, 1996; Lioubin et al., 1996) but all use this basic concept. False positives are a particular problem with the yeast two-hybrid, which is notorious for giving misleading results. Clones of DNA binding proteins or transcription factors may trigger activation of the reporter genes independently of any interaction with the bait protein. When a DNA is cloned into the yeast two-hybrid vectors to create a yeast two-hybrid cDNA library, it is possible for the cDNA to be cloned in either orientation or to be cloned out of frame. This creates artefactual peptides that do not exist in vivo, but may interact by
chance with the bait protein. The yeast two-hybrid system also allows for proteins that are not expressed in the same cell type or are physically separated in the cell by different sub cellular localisations to interact. These possibilities account for many of the false positives seen in a yeast two-hybrid library screen. Post translational modifications of the bait or prey may also be required for the interaction to occur. Some of these are possible to recreate in the yeast, for example it has been possible to demonstrate phosphorylation dependent interactions in yeast co-expressing active mammalian kinases. Despite its drawbacks, the yeast two-hybrid system remains a powerful tool in identifying interacting proteins (reviewed in Fields and Sternglanz, 1994).

Interactions within the CH1 domain of Shc have been well characterised, but potential CH2 domain interactions are fewer in number and poorly characterised. It has been reported that p120RasGAP interacts with tyrosine phosphorylated p66Shc during morphogenetic events of gastrulation in *Xenopus* (Dupont and Blancq, 1999). It has not been determined whether p120RasGAP associates directly or indirectly with p66Shc, but Dupont *et al* hypothesise that the SH3 domain of p120RasGAP binds the second collagen homology domain of p66Shc. As p66Shc tyrosine phosphorylation is requisite for this association, it is unlikely that this interaction is dependent on a possible p120RasGAP SH3 domain interaction with the CH2 domain of p66Shc. This could indicate that a second factor, possibly Grb2, is binding to phosphorylated tyrosine sequences in the CH1 domain of p66Shc and recruiting with it p120RasGAP. Unpublished reports have also suggested that p66Shc is in a complex containing p120RasGAP, first in 1996 (Bonfini *et al*., 1996) and repeated (Baldari and Telford, 1999), but it has yet to be shown that there is a direct interaction between p120RasGAP and p66Shc. The significance of these observations remains unclear. Recently it has been reported that p66Shc interacts with 14-3-3 (Foschi *et al*., 2001). 14-3-3 is a phosphoserine binding protein, and it is suggested that p66Shc serine phosphorylation is required. Agents shown to trigger serine phosphorylation of p66Shc appear to increase the association of p66Shc with 14-3-3. This is not exclusive to p66Shc as p52 Shc also associates with 14-3-3 and possibly p46Shc, which means that the interaction between p66Shc and 14-3-3 may not be responsible for any of the unique properties exhibited by this isoform.
Identifying other proteins associating with p66Shc could give further insight into its function and possibly aid in understanding the mechanisms by which it senses oxidative stress and inhibits MAPK activation. Using just the CH2 domain as bait, it will be possible to identify factors that are specific to p66Shc. Possible targets to identify are the kinase(s) and phosphatase(s) that regulate the serine phosphorylation of p66Shc. It has also been reported that this domain can inhibit EGF signalling, so it may associate with which constituents of EGF or other growth factor pathways. The interaction of p120RasGAP and the CH2 domain could be verified.

Constructs of the Wt and Y317F mutant CH1 domain of Shc in the pTBM116 Src vector were available. Earlier screens by J.A. Brown had identified Grb2 as a binding partner for the CH1 domain confirming the ability of the yeast two-hybrid system to detect binding partners for the CH1 domain. It would be of interest to determine whether other related SH2 domain containing proteins could interact with the CH1 domain. Candidate SH2 domains can be easily cloned into the pVP16 vector and tested in the yeast two-hybrid system without undertaking a labour intensive library screen.

3.2 Results.

3.2.1 Characterisation of proteins interacting with the CH1 domain of Shc.

Yeast two-hybrid screen have been carried out previously in the lab using the CH1 domain as bait. The CH1 domain of Shc was previously cloned into the pTBM116 plasmid to create an in frame fusion of the CH1 domain (amino acids 233-378) to LexA for expression in yeast by J. A. Brown. Several proteins have been identified that associate with the CH1 domain of Shc, of which Grb2 is probably the most significant. The CH1 domain is co-expressed in yeast with constitutively active Src, which allows phosphorylation dependent interactions to be studied. A construct with Y317 mutated to phenylalanine was also available to distinguish between the two main tyrosine phosphorylation sites. A previous screen had identified Grb2 as a binding partner, confirming the validity of this technique. As the CH1 domain interactions are relatively well characterised, it was decided that a yeast-two hybrid screen using the CH2 domain would be more productive. There are however Grb2 related proteins, which could be tested in the yeast two-hybrid system such as Gads (Liu and McGlade,
SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN
Interestingly, the Shc SH2 domain appears to interact weakly with the WT CH1 domain. This was unexpected as the ShcSH2 domain was included as a negative control. The interaction is specific, as the Shc SH2 domain only interacts with the WT CH1 domain and not the mutant CH1 domain or lamin. The SH2 domain of Sck has been reported to be used for high affinity receptor interactions, which could indicate that the SH2 domain of Sck is more functionally important than Shc. The Sck SH2 construct was also available and it was tested with the mutant and wild type CH1 domain constructs in parallel with the Shc SH2 domain and with Grb2 and Gads for a comparison of affinity compared to known associating proteins (figure 3.3).

![Figure 3.3: The SH2 domain of Sck interacts strongly with Y317 in the CH1 domain of Shc.](image)

The pVP16 construct encoding the SH2 domain of Sck was co-transformed with the WT and Y317F mutant CH1 domain constructs into L40 yeast. Gads, Grb2 and Shc SH2 domain interactions with the WT and Y317F mutant CH1 domain are shown for comparison. β-galactosidase activity was then assayed in a ONPG solution assay. Activity is expressed as nmol min⁻¹ mg⁻¹ protein. Error bars represent mean ± S.D.
The SH2 domain of Sck interacts strongly with the wild type CH1 domain, but only weakly with the Y317 mutant. This interaction compares closely in affinity to the Grb2 and Gads SH2 domain interactions, suggesting that this interaction maybe more relevant than the ShcSH2 domain interaction which is considerably weaker. This also indicates that the SH2 domain of Sck may recognise similar sequences to that of its family member Shc. This observation could have relevance in cells that express both Shc and Sck, possibly allowing a Shc / Sck dimer to form. Again, further experiments are required to see if this reaction can occur in vivo.

3.2.2 Characterising the CH2 domain construct for use in the screening of cDNA libraries.

The yeast two-hybrid system has proved effective in identifying and characterising CH1 domain interactions so would be hopefully a good choice of technique to screen for proteins interacting with the second collagen homology domain. The CH2 domain of p66Shc was previously cloned into the pBTM116 vector to create an in frame fusion of the CH2 domain (amino acids 1-110) to LexA for expression in yeast (kindly provided by J. A. Brown, University of Leicester.) Using 3 amino triazole (3-AT), an inhibitor of the His3 gene product, it is possible to quantify the level of background expression of the reporter gene activation triggered by the CH2/LexA fusion. Expressing this construct with empty pVP16 vector in yeast gives very low background activation of the reporter genes as concentrations of 3-AT as low as 1mM prevent any growth on media lacking histidine. Even without 3-AT, background growth is difficult to detect on media lacking histidine. To prevent background growth of yeast in the library screen, 3-AT can be added but it appeared that background transcriptional activation would not impair the results of the library screen, so 3-AT was not used for the first library screen. Alternatively, 3-AT can be added to screen for the strongest interactions. As there were no known interactions with the CH2 domain it is difficult to predict the affinity of interaction between the CH2 domain of p66Shc and a binding partner. To ensure that no interactions were missed from the screen it was decided that the first screen should have no 3-AT present, which would ensure that all positive clones would be identified, but possibly at the cost of increasing the number of false positives.
3.2.3 Selection of suitable cDNA libraries for use in yeast two-hybrid library screening.

Several cDNA libraries were available for use in the yeast two-hybrid library screen. An 8.5 – 9.5 day mouse embryo cDNA library was chosen, as p66Shc was originally cloned from such a library. This would suggest that p66Shc is active during embryogenesis and would hopefully indicate that any proteins associating with p66Shc would be present in a mouse embryo library.

![Image of protein expression](image_url)

**Figure 3.4: p66Shc expression is widespread throughout the adult and embryonic tissues.**

Lysates were prepared from homogenised mouse tissue for separation by SDS page. Mouse embryo extracts were provided by Dr C Prichard (University of Leicester). Shc protein was detected by immunoblotting with rabbit polyclonal anti-Shc antibodies.

Many biological processes are occurring during embryogenesis, giving a wide range of protein expression that is not normally seen in just one tissue, increasing the chances of finding an associating protein. The cDNA library was constructed by Dr S Hollenberg, in the pVP16 vector (Hollenberg *et al.*, 1995). The library contains short inserts of around 500 bp and contains around 2 million different clones. Despite the relatively small insert size this library is thought to be very comprehensive. This should give a good chance of identifying a positive interacting clone, although further cloning could be hampered if this was necessary. p66Shc may function differently in
adult tissues, so adult mouse tissues were screened for p66Shc expression by Western blot (figure 3.4).

3.2.4 Results of first yeast two-hybrid library screen.

250,000 transformants were generated in the first library screen. Both bait and prey plasmids were transformed simultaneously without giving a recovery step to ensure that there was no negative selection of yeast expressing strongly interacting clones. This method is not efficient enough to generate the numbers required for an adequate screen, so for a subsequent screen a more efficient method was required. This first screen did however generate over 250 potential positive interactions. Positive clones were checked with the more sensitive β-gal reporter gene to assay the affinity of the clones’ interactions and to confirm that both reporters were being activated. From these clones the fifty clones showing the highest β-gal activity were selected. Plasmid DNA was isolated from the positive yeast colonies and retransformed with an unrelated bait of lamin to test whether the prey could activate transcription independently of the bait. Of the clones that could be recovered only seven did not have the ability to activate transcription independently of the bait. (table 3.1).

<table>
<thead>
<tr>
<th>Clone</th>
<th>Cloned sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Antisense sequence coding for KIA0800.</td>
</tr>
<tr>
<td>4</td>
<td>Antisense sequence coding for L23 ribosomal protein.</td>
</tr>
<tr>
<td>9</td>
<td>Out of frame sequence coding for mph1.</td>
</tr>
<tr>
<td>11</td>
<td>Antisense sequence coding for KIAA1041.</td>
</tr>
<tr>
<td>23.2</td>
<td>Novel 350bp sequence.</td>
</tr>
<tr>
<td>23.3</td>
<td>Out of frame sequence coding for Fascin.</td>
</tr>
<tr>
<td>29</td>
<td>Out of frame sequence coding for eEF-Tu.</td>
</tr>
</tbody>
</table>

Table 3.1: The yeast two-hybrid library screen identifies a potentially novel protein sequence that interacts with the CH2 domain of p66Shc.

Over 250 potentially positive clones were identified from the screen. β-galactosidase activation was assayed for each clone and plasmid DNA was recovered from the 50 strongest interacting clones. Specificity of interaction was then assayed by retransformation with the unrelated bait of lamin. Clones that passed the retransformation step were sequenced and are listed above.
The large number of potentially positive clones generated in the screen suggested that it would also be a requirement to include some 3-AT in a future screen to reduce the number of the weaker clones, as screening clones is both labour and time intensive. Six of the seven clones are definitely artefacts of the yeast two-hybrid system as they are either cloned out of frame or in the antisense orientation. These frame shifted and antisense sequences code for peptides that do not occur in vivo. These artefactual peptides by chance bind weakly to the CH2 domain of p66Shc and therefore activate transcription of the reporter genes. These kinds of artefacts are hard to avoid, as they can be relatively specific for the CH2 domain, in that they associate significantly more strongly with the CH2 domain than with the negative control protein lamin, mimicking a real interaction. This allows them to pass the retransformation step with the negative control, which eliminates the majority of the non-specific interacting clones. They can only be avoided if their affinity for the CH2 domain was lower than real binding partners, as 3-AT can be added to screen out lower affinity interactions.

Of interest is clone 23.2 that had no match in the sequence database. This could mean that this is an as yet unidentified protein sequence. Sequence analysis of this clone fails to reveal any homology to any other known protein sequence.

Figure 3.5: Clone 23.2 specifically associates with the CH2 domain of p66Shc.
Plasmid DNA from yeast clone 23.2 was isolated and purified for DNA sequencing. The complete insert was sequenced using the Sequenase 2.0 DNA sequencing Kit (Amersham). (A) Complete DNA sequence of clone 23.2. (B) Translated sequence from the cloned open reading frame.
3.2.5 Further characterisation of clone 23.2.

Clone 23.2 contains relatively little sequence to give any clues to its identity or function. For any further characterisation more sequence information was required. A logical first step was to test whether this sequence was present in other similar cDNA libraries to confirm that it was not an artefact made during the construction of the library (Figure 3.6).

![Figure 3.6 Clone 23.2 is expressed during embryogenesis.](image)

Two mouse embryo libraries (A and B), one liver library (C) and one brain (D) library were screened for 23.2 using a PCR reaction with primers annealing with the 5' and 3' sequence of clone 23.2. If the 23.2 sequence is present, a 300bp DNA fragment is generated.

A PCR reaction with PCR primers directed to the 5' and 3' sequence of clone 23.2 confirmed that clone 23.2 was expressed in a second mouse embryo cDNA library. It cannot be detected in liver or brain cDNA library. The second mouse embryo library contains larger insert of up to 2 kb, which should allow further sequence of clone 23.2 to be cloned. To clone further sequence of clone 23.2, a biotinylated primer pull down procedure was attempted (Shepard and Rae, 1997). This method uses a series of biotinylated primers recognising the cloned 23.2 sequence. The primers anneal...
specifically to library plasmids containing the 23.2 sequence after denaturation and renaturation of the library DNA. Sequences that anneal with the primers are then recovered using streptavidin coated magnetic beads. The beads are then washed to remove non-specifically bound plasmids, before being transformed into bacteria. The plasmid DNA is harvested from the bacteria and the process repeated for three rounds to enrich for plasmids containing the desired 23.2 sequence. This procedure proved unsuccessful, so a second approach was used to clone more sequence.

The second approach used a PCR based method using four separate primers. Firstly, the forward and reverse primers used previously to detect 23.2 and primers annealing to sequence 5’ to the cloning site and sequence 3’ to the insert cloning site are required. These two primers recognising the vector sequence will allow the PCR reaction to proceed forwards into the insert from the 5’ end or reverse into the insert from the 3’ end. Using combinations of the forward and reverse primers for the vector and clone 23.2 it should be possible to identify sequence to the 5’ and 3’ of the sequence of clone 23.2.

![Diagram](image)

**Figure 3.7: A PCR approach to obtain a larger fragment of clone 23.2.**

Using combinations of PCR primers it should be possible to clone sequence to the 5’ (A) and 3’ (B) of the sequence identified in the 23.2 clone. This technique should amplify sequence from multiple different clones in the library, thereby generating multiple bands detectable on agarose gel electrophoresis.
Figure 3.8: PCR amplification of 23.2 sequence generates multiple bands.

PCR reactions were set up as described in figure 3.8 to clone 5' and 3' sequence of clone 23.2 DNA. The PCR reactions were then resolved by agarose gel electrophoresis and visualised under UV light.

The PCR cloning technique generates a ladder of bands representing cloned fragments of different length that potentially containing 23.2 sequence. Altering the annealing temperatures reduces the number of bands suggesting that some bands represent artefactual mispriming. To confirm whether the 23.3 sequence was present in any of the fragments, a second PCR reaction was attempted, using forward and reverse primer to 23.2. This should give a band of 350 bp if the cloned sequence is contained in the PCR cloned material (Figure 3.9).
The cloned DNA sequences were analysed for the presence of clone 23.2 by PCR using primers annealing to the 5’ and 3’ sequence of clone 23.2. A PCR product of 300 bp confirms the presence of clone 23.2 in the cloned fragments.

PCR analysis of the previously cloned material generates a 300 bp band, corresponding to the original sequenced cloned in the yeast two-hybrid library screen. This confirms the presence of 23.2 sequence in the cloned material. From this a 750 bp fragment of 3’ sequence was amplified through a further PCR reaction and directly sequenced. Sequence analysis of this fragment confirmed the presence of the original 23.2 sequence and allowed new 3’ sequence to be identified. Using this new sequence, a further database search was performed. No match was possible with the original sequence, but the newly identified sequence revealed homology to three EST clones present in the database. Comparison of the sequence contained in the EST clones and the sequence cloned for 23.2 showed that the 23.2 sequence cloned in the original yeast two-hybrid screen was not present in the EST clones.
Figure 3.10: The PCR cloning technique generates a 750bp fragment containing clone 23.2 sequence and novel 3' sequence.

A 750bp fragment was sequenced giving sequence to the 3' of the original 23.2 sequence. The new sequence was used to search the NCBI database and identified 3 EST clones. Sequence analysis of the EST sequence does not show the 23.2 sequence as being present. PCR analysis of the EST DNA also fails detect the presence of the 23.2 sequence.

This suggests that there could be a potential splice site between the EST clones sequence and the 23.2 sequence. This could indicate either that the 23.2 sequence is present as a less abundant splice variant of the protein or that the 23.2 sequence was spliced out as an intron. From the sequence data alone, it is hard to distinguish between these two possibilities. It is also still unclear what orientation and what frame the transcript is read in, as there is more than one reading frame that gives a long protein sequences. Because of these uncertainties over the possible validity of this result, it was decide to continue screening with a second more comprehensive library screen.

3.2.6 Optimisation of the yeast two-hybrid screen.

Several modifications were undertaken with the second library screen to optimise the procedure in terms of efficiency and accuracy. Firstly, the transformation efficiency of the first library screen was very low. To increase the yield of transformants a different transformation procedure was used (Hill et al., 1991). Three major differences were included. Transforming both the bait and prey plasmids simultaneously is relatively inefficient, so the yeast were transformed sequentially with the prey and bait plasmid alone. This requires modification of the procedure to include selection for the bait
plasmid prior to the library transformation. This step dramatically improves yields, as only the prey plasmid now has to be taken up during the library transformation. Secondly, DMSO treatment was included prior to the heat shock stage, which aids the permeabilisation of the yeast. This allows the single prey plasmid to be more easily taken up. Finally, the yeast were allowed to recover after the transformation, by not using immediate selection. This reduces the number of yeast that are killed through the stress of the transformation, but possibly may bias the transformant population against the strongest interacting clones as these clones will grow slower than weakly or non interacting clones in non selective media. To improve the fidelity of the screen, 3-AT was included in the library media. 5mM 3-AT was included in the library selection media as this was the minimum 3-AT concentration that inhibited the selection of the artefactual clones identified in the previous library screen.

3.2.7 Results of the second library screen.

The second screen again used the Dr S Hollenberg mouse embryo cDNA library described previously. The second library transformation generated 5.94 million transformants, which is large enough to ensure that library has been completely screened. This demonstrates the benefits gained from the modified transformation. Over two hundred potential positive clones were identified from the library plates. These were screened for the ability to activate both reporters, and then by strength of interaction by replica plating over a range of 10 – 50mM 3-AT containing plates. These steps narrowed the field to forty strongly interacting clones capable of activating both reporters. These clones were isolated and retransformed with the negative control lamin. The clones that passed the retransformation with the negative control were then sequenced.
Table 3.2: The yeast two-hybrid library screen identifies two possible associating proteins, LAR and Ch-Tog.

Clones were selected by their ability to strongly activate the β-gal reporter and their capability to grow in the presence of increasing 3-AT concentrations. Plasmid DNA was recovered from the strongest interacting clones and was retransformed with the unrelated lamin bait to assay the specificity of the interaction. Clones that passed the retransformation step were sequenced and are listed above.

The screen revealed multiple identical copies of two clones coding for Ch-Tog and Lar. An in frame clone of the methyl CpG binding protein was also identified as a binding partner for the CH2 domain of p66Shc. This clone induces activation of the reporter genes with both the CH2 domain and Lamin, but the interaction with the CH2 domain is of a higher affinity. Methyl CpG binding protein is a DNA binding protein. As She is known to be exclusively located in cytoplasm, an interaction in vivo is unlikely. The independent reporter gene activity observed with this clone and the different cell compartment localisation of these proteins make it likely that this observation is another artefact created by the yeast two-hybrid system.

3.2.8 p66Shc interacts with the juxtamembrane region of Lar.

Two positive identical clones were identified which contained a 372 bp sequence corresponding to residues 1197 – 1321 of the receptor tyrosine phosphatase Lar. Sequence analysis confirmed that the insert was cloned in the correct orientation and reading frame to express a fragment of Lar, suggesting that this was not an artefact of the yeast two-hybrid system. The cloned sequence included a small portion of the
extracellular domain, the transmembrane domain and the intracellular juxtamembrane
domain (The Lar domain structure is shown in figure 3.13). To identify the region
interacting with the CH2 domain, a new construct was designed containing just the
intracellular juxtamembrane region (residues 1273 – 1367), which was thought to be
the likely CH2 domain interaction site. The new construct containing just the
juxtamembrane region was introduced into the yeast two hybrid assay.

Figure 3.11: The domain structure of Lar.
The Leukocyte common antigen related protein is a transmembrane receptor tyrosine
phosphatase with a large extracellular domain consisting of immunoglobulin like and
fibronectin type III repeats, a short transmembrane region, with an intracellular tandem
phosphatase domain.
Figure 3.12: The shortened construct containing only the juxtamembrane sequence retains the ability to interact with the CH2 domain of p66Shc.

The construct encoding the juxtamembrane region of LAR in pVP16 was made by PCR amplification of LAR from the full-length human LAR cDNA and insertion into the BamH1 site of pVP16. This construct was co-transformed with the CH2 domain or lamin construct into L40 yeast and interaction assayed by ONPG solution assay. 23.2 co-transformed with the CH2 domain construct and lamin construct is shown for comparison. Activity is expressed as nmol min\(^{-1}\) mg\(^{-1}\) protein. Error bars represent mean ± S.D.

The shortened Lar juxtamembrane construct retains the ability to associate with the CH2 domain of p66Shc in the yeast two-hybrid system localising the interaction to this region. As p66Shc is a cytoplasmic protein, interaction with the transmembrane region or extracellular domain of Lar would be unlikely.

3.2.9 The microtubule associated protein Ch-Tog interacts with the CH2 domain of p66Shc.

The yeast two-hybrid screen also identified four identical clones encoding the Ch-Tog gene. DNA sequencing confirmed that the Ch-Tog insert was cloned in the correct orientation and reading frame. The clones identified in the yeast two-hybrid screen contained a 548 bp sequence encoding amino acid residues 1342 – 1523 of Ch-Tog, which are located towards the C terminus of the protein.
Figure 3.13: Ch-Tog also interacts with the CH2 domain of p66Shc in the Yeast Two-Hybrid assay.

The Ch-Tog / pVP16 construct isolated during the yeast two-hybrid library screen was used to co-transform L40 yeast with either the CH2 domain or lamin bait construct. The affinity of interaction between the CH2 domain and Ch-Tog was assayed semi quantitatively using an ONPG solution assay to measure β-galactosidase activity. Lar and 23.2 are shown for comparison. Activity is expressed as nmol min\(^{-1}\) mg\(^{-1}\) protein. Error bars represent mean ± S.D.

### 3.2.10 A common sequence motif is present in the clones identified in the yeast two hybrid library screens.

The first library screen did not generate any real interactions, although it does prove useful in that it has shown some patterns in the types of sequences that can associate with p66Shc. All of the clones that were sequenced bound specifically to the CH2 domain and not to the negative control bait lamin. This means that although the sequences that were identified did not exist as real proteins \textit{in vivo}, they did have some characteristics which allowed them to associate with p66Shc. Therefore the peptides generated could in some way mimic a real binding partner for the CH2 domain of p66Shc.

Comparison of the sequence isolated by the library screen revealed some similarities in terms of the frequency of individual amino acids residues present (Table 3.3). Firstly, only a very short sequence was required for association with the CH2 domain, suggesting that a large protein interaction domain such as a SH2 domain was not required for an interaction.
Table 3.3: The yeast two-hybrid library screen selects serine rich basic sequences.

Secondly, all of the sequences cloned were rich in Serine residues; usually having double or triple repeats of this residue (Shown in bold). Also present was a basic residue, usually Arginine but occasionally Lysine. Less common in the sequence are some hydrophobic groups, notably Proline and Leucine. As clone 23.2 has sequence similar to these, it was possible that the artefactual peptides generated in the screen were mimicking this protein, or one like it.

The second library screen repeated this trend, suggesting that this could be the case. Importantly Lar and Ch-Tog were identified. As these sequences were correctly cloned, it was possible that these proteins could be real binding proteins for the CH2 domain of p66Shc. Analysis of the sequence cloned in the yeast two-hybrid screen shows that a double Serine motif followed by a Lysine residue is present in both sequences. Two residues down from this sequence is a Glutamine residue that is also present in both. This gives a consensus sequence of SSKXXQ as a possible Shc binding site.
3.3 Discussion.

The interaction between the SH2 domain of Grb2 and the CH1 domain of Shc is well characterised and is probably one of the most significant functions of mammalian Shc proteins. The yeast two-hybrid system can be used to test for phosphorylation dependent interactions so is a useful method for studying potential SH2 domain interactions with the CH1 domain of Shc. This has been demonstrated, as it can be shown that Grb2 interacts with both the potential phosphorylation sites of the CH1 domain in a phosphorylation dependent manner. Grb2 interacts strongly with both sites but with higher affinity for the Y317 site as shown by a quantitative ONPG solution assay. The interaction of Gads with Shc was originally reported as occurring at both Y317 and Y239/240 (Lui and McGlade, 1998), but only an interaction at 317 is detectable as no activity is seen with the Y239 mutant. This contradictory result maybe due in part to different methodologies used. There is disagreement over the preferred Grb2 binding site on Shc. Although it is clear Grb2 can bind at both sites, evidence has been reported indicating both Y239/240 and Y317 as being the more important site in transducing signals through Shc (Li et al., 1999; Harmer and DeFranco, 1997; Gotoh et al., 1997). These contradictory results are probably due in part to the context in which they were studied, possibly indicating cell type and signal pathway specific differences. These results would indicate that Y317 is maybe the highest affinity Grb2 binding site on Shc, although the yeast-two hybrid system cannot account for other proteins factors that may compete for or regulate binding at these phosphorylation sites. These results also support the hypothesis that Grb2 binding is a relatively recent evolutionary event, as dShc lacks the Y317 site and cannot recruit Drk, which may mean that in vivo another protein out competes Grb2 for binding at Y239/240 as this site has a lower affinity for Grb2. Further studies in a more appropriate system that can account for more complex regulation of these sites would be required to confirm or disprove this hypothesis. This system would therefore be useful to test other Grb2 related proteins for the ability to bind the Shc CH1 domain. Gads has been reported to interact with the CH1 domain of Shc and that interaction can be seen in the yeast two-hybrid assay.

Using this system, the SH2 domain of Shc was also identified as a binding partner for the CH1 domain of Shc. The interaction was not detectable with the Y317 mutant,
suggesting binding was occurring at this site. Analysis of the sequence C terminal of Y317 shows a Valine residue at position +1 and an Isoleucine residue at position +3 (pYVNI) that matches the rather degenerate Shc SH2 domain binding motif of pY(I/E/Y/L)X(I/L/M). There is no consensus-binding site at Y239/240 (YYNSIP) explaining the absence of an interaction with the Y317 mutant construct. Harmer and DeFranco recently confirmed this observation using a peptide based approach (Harmer and DeFranco, 1999). It is also interesting whether other Shc family members could also use their SH2 domains in an analogous manner. Previous work has indicated that the SH2 domain of Sck maybe more important for Sck recruitment to EGF receptor than the SH2 domain of Shc as Shc tends to use its PTB domain preferentially (O'Bryan et al., 1995).

The low affinity of the Shc SH2 interaction compared to the comparable Grb2 SH2 domain interaction with the same site suggests that this interaction will not occur in vivo unless Grb2 levels are low, leaving phosphorylated Y317 unoccupied. It has been reported that Grb2 levels in the some cell types are limiting, and that there is competition for Grb2 binding (Okada et al., 1997). This could mean that this binding site is under some circumstances unoccupied, which would allow this interaction to occur. The physiological relevance of this observation remains unclear, but it could mean that Shc dimers are potentially possible or that the SH2 domain of Shc could bind to the Y317 site within the same molecule. The SH2 domain of Shc has also been used to inhibit signalling from the EGF receptor (Gotoh et al., 1995). This was originally proposed to act by blocking Shc binding motifs on the active receptor. Subsequently, it was shown that the Shc SH2 domain is relatively redundant when compared to the PTB domain, which mediates the majority of the interactions between Shc and activated receptors (Prigent et al., 1995). This could mean that the SH2 domain of Shc may function in a regulatory role as opposed to mediating receptor binding. This could explain why the Shc SH2 domain inhibits EGF signalling, although it is hard to see how this would be possible with such a low affinity interaction between the SH2 domain and the CH1 domain of Shc as predicted by the yeast two-hybrid assay. This interaction could require the involvement of other proteins to stabilise the interaction. Another possibility is that this interaction could regulate the association of mPAL with the Shc SH2 domain (Schmandt et al., 1999). This protein associates with the SH2 domain of Shc in a phosphotyrosine independent
manner, suggesting that this is not a traditional SH2 domain interaction. The mPAL interaction as it is an atypical interaction for a SH2 domain. This interaction could be much lower affinity than the traditional phosphotyrosine ligand making the possibility of an Shc SH2 domain inhibition more likely.

The SH2 domain of Sck also interacts with the CH1 domain of Shc. This interaction is significantly stronger, and is comparable to that seen with Grb2. This means that in cells expressing both Shc and Sck it is possible that Shc and Sck could interact to form a heterodimer, even in the presence of Grb2. As relatively little is know about Sck, it is hard to predict the significance of this interaction. It could possibly allow activated receptors to be clustered as both Sck and Shc still have their PTB domains free to interact with active receptors. The ability to bind the CH1 domain at Y317 seems to be conserved, which could mean that it has significance. It would be of interest to test whether N-Shc retains this ability. Further experiments are required to confirm these interactions in vivo.

After two screens of the mouse embryo library it has been possible to identify three potential binding partners. The apparent binding affinities for the interaction between p66Shc and Ch-Tog and p66Shc and Lar are very similar, which may suggests that this strength of interaction is what can be expected for an interaction with p66Shc. This could be useful for any further screens using the CH2 domain construct, as weaker artefactual interactions could be screened out using higher concentrations of 3-AT without missing relevant interactions.

The Leukocyte common Antigen Related protein (Lar) is a transmembrane tyrosine phosphatase, which has been shown to be important in the development of the nervous system and in the regulation of insulin and growth factor signalling (Streuli et al., 1988). Tyrosine phosphorylation regulates protein function and the degree of tyrosine phosphorylation is regulated by the actions of protein tyrosine kinases and protein tyrosine phosphatases. Lar has been shown to inhibit the downstream affects of several tyrosine kinases, including insulin receptor, Hepatocyte growth factor, EGF receptor and FGF receptor (Kulas et al., 1996; Mooney et al., 1997; Wang et al., 2000). Known substrates of Lar include p125FAK, p130Cas, insulin receptor, and FRS2 (Wang et al., 2000; Cheung et al., 2000; Weng et al., 1999; Mooney et al., 1997). These
observations suggest that Lar may negatively regulate transmembrane signalling by inhibiting the tyrosine phosphorylation of signal transducing adaptor proteins and enzymes activated by the plasma membrane associated tyrosine kinases. Lar is also sensitive to increased levels of oxidative stress. Low levels of oxidative stress reversibly deactivate the phosphatase domain of Lar (Denu and Tanner, 1998), while higher levels permanently deactivate Lar (Takakura et al., 1999).

Lar knockout mice have been generated with different phenotypic characteristics. An earlier Lar knockout mouse was demonstrated to be apparently phenotypically normal, with the exception of some development defects in the mammary gland that prevented lactation (Schaapveld et al., 1997). Another Lar knockout mouse line showed profound disturbances in glucose homeostasis (Ren et al., 1998). This could be expected as Lar is thought to negatively regulate the insulin receptor. Over expression of Lar has also been reported to render muscle cells resistant to insulin (Zabolotny et al., 2001). TNFα stimulated HepG2 cells increase their expression of Lar and show increased resistance to insulin (Cheung et al., 2000). Shc is also involved in insulin signalling, as Shc is phosphorylated at serine, threonine and tyrosine residues in response to insulin stimulation (Kao et al., 1997; Ishihara et al., 1998) and directly associates with insulin receptor substrate 1 (IRS1) (Kasus-Jacobi et al., 1997). A complex between Shc, IRS1, PI3 kinase and Insulin receptor has been reported (Dupont and Blank, 1998). If p66Shc were present in these complexes, it could also recruit Lar.

Little is known about the control of Lar activity. Lar has a large extracellular domain containing immunoglobulin like domains and fibronectin type III repeats. The extracellular domain of Lar shows homology to cell adhesion molecules such as N-CAM, which may explain its role in neuron spreading (Gershon et al., 1998; Zhang et al., 1998). This may suggest that Lar plays a role in transducing extracellular matrix derived signals. Lar is produced as polypeptide of 190 kDa and is processed to a mature form by a proteolytic cleavage in the extracellular domain (Yu et al., 1992). The resulting two fragments stably interact anchoring the extracellular domain to the intracellular domain. The extracellular domain of Lar can be shed under certain conditions such as TPA stimulation and reduction of cellular calcium levels (Aicher et
The consequence of this processing is not clear but suggests that the extracellular domain of Lar may be important in the regulation of Lar activity.

The intracellular domain consists of a small juxtamembrane region followed by two tandem phosphatase domains, the first of which is catalytically active, while the second is not. It has been suggested that the second phosphatase domain may be important in determining substrate specificity. The liprin family of proteins have been shown to bind the second phosphatase domain (Pulido *et al*., 1995; Serra-Pages *et al*., 1998) and also Trio, a nucleotide exchange factor for rac and rho, containing a serine/threonine kinase domain (Debant *et al*., 1996). The role of these proteins in the action of Lar is unclear. Lar also contains a juxtamembrane region, which spans between the transmembrane segment and the first phosphatase domain. This region may be important in the regulation of the receptor tyrosine phosphatases as it allows receptor tyrosine phosphatases to dimerise and this appears to have an inhibitory role in their activity (Blanchetot and den Hertog, 2000).

Lar over-expression has been reported to induce apoptosis in a p53 independent manner, which requires caspase 3 activation (Weng *et al*., 1998). Lar over-expression has been shown to trigger the dephosphorylation of p130cas and with this reduce its stability leading to a decrease in p130cas levels (Weng *et al*., 1999). Interestingly Lar over-expression has little affect on cell adhesion suggesting that Lar is directly triggering apoptosis. It appears that Lar regulates signals important for cell survival and the over-expression of Lar tilts the balance of phosphorylation of the signalling proteins towards the dephosphorylated state, which triggers apoptosis.

Proteins have been identified which either stabilise (e.g. MAP4, MAP2 and Tau) or destabilise (e.g. XKCM1 or oncoprotein 18) microtubules allowing the microtubule structure to be tightly controlled (Anderson, 2000). Microtubules are dynamic structures made up from alternating α and β tubulin monomers. The α - β tubulin dimers associate linearly to form protofilaments that associate laterally to create the hollow cylindrical wall of the microtubule. Microtubule stability is regulated to give relatively stable structures during interphase or more dynamic structures during the formation of the mitotic spindle. Tubulin monomers bind GTP, which is hydrolysed to
GDP following polymerisation, creating a relatively unstable structure. This relatively unstable microtubule structure can undergo a process termed dynamic instability, where microtubules undergo growth or shrinkage with rapid transitions between the two states known as catastrophe and rescue (Reviewed in Downing, 2000).

Colonic and hepatic over expressed gene (Ch-Tog, XMAP215 in *Xenopus* or msps in *drosophila*) is involved with the regulation of microtubule dynamics (Charrasse *et al.*, 1995; Cullen *et al.*, 1999). Ch-Tog is highly expressed in human tumours and in brain. Immunofluorescence microscopy shows Ch-Tog localised to the perinuclear cytoplasm co-localising with ER markers and with the microtubule network, especially during mitosis (Charrasse *et al.*, 1998). Ch-Tog has now been demonstrated to associate directly with tubulin (Spittle *et al.*, 2000) and to stabilise and promote the growth of microtubules by between 7 and 10 fold at the plus end. Ch-Tog also stimulates an increase in the shortening rate of three fold (Tournébize *et al.*, 2000). The C terminal region of Ch-Tog is thought to target Ch-Tog to microtubules, while the N terminal region is involved in microtubule dynamics. Ch-Tog also appears to protect microtubule plus ends from catastrophe induced by the Kin I kinesin, XKCM1 (Popov *et al.*, 2001). It would therefore appear that Ch-Tog functions to increase microtubule plus end growth, while still allowing rapid microtubule turnover and preventing catastrophe. Ch-tog has been visualised by electron microscopy using shadowing techniques and appears to be an elongated molecule at around 60nm long. This means that Ch-tog could span between 7-8 tubulin dimers along a protofilament. Ch-Tog appears to be a flexible molecule and does not appear to alter the flexural rigidity of the microtubule (Casseimeris *et al.*, 2001). Ch-Tog is also phosphorylated by the cyclin B/cdc2 complex (Vasquez *et al.*, 1999) and has been reported to locate this complex to the microtubule network through an interaction between Ch-Tog and cyclin B (Charrasse *et al.*, 2000). The region of Ch-Tog cloned in the yeast two-hybrid screen contains the cdc2 phosphorylation site, which may be significant in controlling the interaction of p66Shc with Ch-Tog. The transforming acidic coil-coiled protein (TACC1) has also been demonstrated to associate with Ch-Tog. TACC1 family members are known to be involved in growth and differentiation and are up regulated in some cancers (Lauffart *et al.*, 2002). D-TACC has been reported to be complexed with aurora A kinase. Mutation of this kinase impairs the ability of D-TACC and Msps to associate with the centrosome. D-TACC has been shown to be a substrate of Aurora
A kinase, which could implicate this phosphorylation event in mediating the localisation of D-TACC/Msps to the centrosome (Giet et al., 2002).

An interaction between p66Shc and Ch-Tog could implicate p66Shc as being important in the regulation of some aspect of microtubule regulation. The interaction between the CH2 domain of p66Shc and Ch-Tog is of interest as it is the first time that p66Shc has been linked to the microtubule network. Although the Shc adaptor protein family have never been linked to microtubule dynamics before, Shc has been shown to associate with the actin cytoskeleton (Thomas et al., 1995). The PTB domain of Shc can associate with F actin, although the exact function of this observation remains unclear. It has also been reported that an intact actin cytoskeleton is essential for efficient activation of Shc (Tsadkiridis et al., 1998).

It has been reported that Shc constructs with mutated Grb2 binding sites inhibited progression of the cell cycle at progression of not only G1 to S phase but also G2 to M phase (Stevenson et al., 1999). The role of Shc in these processes was thought to be limited to signalling from activated growth factor receptors, stimulating progression through the cell cycle. A possible interaction between p66Shc and Ch-Tog could implicate p66Shc in microtubule dynamics and therefore directly in control of the cell cycle, as major microtubule rearrangements are seen during mitosis. The interaction between p66Shc and Ch-tog correlates well with an observation that the microtubule disrupting drugs such as Nocodazole, or microtubule stabilising drugs such as Taxol trigger the serine phosphorylation of p66Shc (Yang and Horowitz, 2000).

The interaction between clone 23.2 and p66Shc was potentially interesting as it could be a novel protein. Comparison of the binding affinity of this interaction to those of the Ch-Tog and Lar interactions with p66Shc show it to be weak. The interaction is also only just stronger than the interaction with an unrelated bait, so it was decided this sequence was unlikely to be a real binding partner for p66Shc. Data obtained from the EST clones also indicated that there was a potential splice site present; suggesting that the sequence cloned in the yeast two-hybrid screen may not be contained in the mature protein or at least in the majority of the protein expressed. Recently, the majority of clone 23.2 has been cloned (KIAA1856 accession number AB05879.1). Sequence alignment shows that clone 23.2 was cloned in the antisense orientation confirming
that this sequence was indeed a false positive generated by the yeast two-hybrid system.

Alignment of the cloned sequences identified in the yeast two-hybrid screens show some homology. Close homology is seen between Ch-Tog and Lar over a six amino acid sequence of SSKXXQ. This also shows homology with the artefactual peptides cloned in the library screens. This confirms that the artefactual peptide sequences that were generated during the library screen probably are indeed mimicking Ch-Tog and Lar, the real binding partners for p66Shc. To prove this hypothesis, it is necessary to mutate this binding site to show that loss of these key residues does indeed block the binding of p66Shc to Ch-Tog and Lar. The yeast two-hybrid system has proved an effective method for characterising both known protein interactions as well as identifying potentially new interactions. Further experiments are required to ascertain the validity of these interactions and hopefully to further characterise these interactions if found to be accurate.
Chapter 4. Further characterisation of the interaction of Ch-tog and Lar with the CH2 domain of p66Shc.

4.1 Introduction.

Semi quantitative analysis using the yeast two-hybrid system showed the interaction between Lar and the CH2 domain to be weak. As no other binding partners for this domain have been identified it is hard to predict or compare the binding affinity of this interaction. Strength of interaction as predicted by the yeast two-hybrid system generally correlates with affinity measured by \textit{in vitro} methods (Estojak \textit{et al.}, 1995). No reporter gene expression linearly matches \textit{in vitro} measurements, but is possible to distinguish low, intermediate and high affinity interactions, using the yeast two-hybrid system. Co-operative binding effects of proteins within a complex have been shown to alter the affinity of interactions within protein complexes containing Shc (Ravichandran \textit{et al.}, 1995). These effects cannot be accounted for in the yeast two-hybrid system so the affinity of interaction is not a good measure for predicting the viability of these interactions \textit{in vivo}. To validate this potential interaction requires a mammalian cell based approach, which can provide possible additional necessary factors consistent with a more relevant model environment. To this end we attempted to confirm the interaction using mammalian cells as the source of Ch-tog and Lar, using either GST fusion protein precipitation or co-immunoprecipitation. The identification of a common SSKXXQ motif in the cloned sequences suggests that this motif may be significant in mediating interactions with the CH2 domain. Using site directed mutagenesis techniques it is possible to disrupt this sequence and evaluate its importance in binding the CH2 domain of p66Shc. If successful, this technique could be used to generate non-binding mutants to investigate the physiological role of these associations with the CH2 domain of p66Shc. To preserve the correct folding of the proteins, mutations were made to change key residues within Lar to similar amino acids that should disrupt binding without significantly affecting over all protein structure. For example, Serine residues were mutated to Alanine residues that remove the hydrophilic hydroxyl group. This will prevent any hydrogen bonds from forming at these residues that could mediate binding between Lar and p66Shc. The positively charged Lysine residue was mutated to Threonine, which maintains the hydrophilic nature of this group but removes the positive charge. Introduction of negatively charge
residue could potentially be more disruptive to the protein fold so this was avoided. This change could block the formation of any ionic interactions between Lar and p66Shc. Finally the Glutamine residue was mutated to Alanine. This should disrupt any dipole–dipole interactions between Lar and p66Shc hopefully without affecting protein folding.

4.2 Results

4.2.1 Preparation of a GST-CH2 domain fusion protein.

The sequence encoding the CH2 domain of p66Shc (Amino acids 1-110) fused to the N terminus of Gutathione-S-Transferase (GST) was available in a pGEX vector. (Constructed by J.A. Brown).

---

![Image of gel showing degradation patterns](image)

Figure 4.1: GST-CH2 fusion protein is partially degraded during purification of the GST-CH2 fusion protein to yield three forms of the fusion protein.

The GST-CH2 fusion protein vector was transformed into DH5α bacteria and the exponentially growing bacteria were induced to produce the fusion protein with the addition of IPTG. The fusion protein was then purified from the bacterial cell lysate and analysed on SDS polyacrylamide gels to ascertain purity and yield by coomassie blue staining.
GST/CH2 fusion protein is cleaved during purification to yield three forms. The highest mass form (43 kDa) is the unprocessed full-length form. The two lower mass forms are truncations of the fusion protein. Unconjugated GST is shown for comparison. The truncation of the GST-CH2 fusion protein was variable and was partially dependent on the purification procedure. With smaller cultures and shorter induction times it was possible to obtain equal amounts of the full length and the intermediate mass truncated form, although the overall fusion protein yield was low. Larger scale preparations with longer induction times gave a higher fusion protein yield but at the cost of generating more truncated protein. The procedure described in the methods section gave an adequate protein yield with minimal truncation of the fusion protein. As the truncation of the fusion protein was quite variable, all GST-CH2 fusion protein used was analysed on SDS polyacrylamide gels to ensure the presence of the full-length fusion protein. The bands at 66 kDa are bacterial contaminants seen with both GST and GST-CH2 fusion protein preparations.

4.2.2 Lar associates with p66She in Lar transfected Hek293 cells.

Western blot analysis showed very low levels of Lar expression in Hek293 and Hela cells. Hela cells have been reported to express relatively large amounts of Lar protein, which may mean that the anti Lar mouse monoclonal antibody used may not be able to detect Lar sufficiently well at the levels expressed in these cell types. A full length Lar construct was made available from Dr T S Pillay, (University of Nottingham, United Kingdom) in the pcDNA3 vector. Transient transfection of this construct using Fugene transfection reagent (Roche) gave high levels of Lar expression. After transfection, Lar protein was easily detectable using this antibody, which identifies two bands of 150 and 190 kDa with Western blotting. High levels of Lar protein are toxic and it has been reported that Lar over expression triggers apoptosis (Weng et al., 1998). Some cell death was observed with expression in Hek293 cells, but sufficient protein was expressed under experimental conditions. To demonstrate an association between p66She and Lar, co-immunoprecipitation was attempted using a rabbit polyclonal anti She antibody directed against the SH2 domain of She. This antibody recognises all of the She isoforms. Secondly, a GST fusion protein of the CH2 domain was used to attempt to precipitate Lar. To investigate whether the interaction between p66She and
Lar was influenced by tyrosine or serine phosphorylation, cells were stimulated with the growth factor EGF and hydrogen peroxide.

Figure 4.2: Lar binds to a GST fusion protein of the CH2 domain of p66Shc and co-immunoprecipitates with Shc from Hek 293 cells.

(A) Whole cell lysates were prepared from Lar transfected (+) or untransfected (-) Hek 293 cells. Shc and its associating proteins were precipitated using a rabbit anti-Shc antibody. A parallel protein precipitation with an unrelated rabbit polyclonal antibody recognizing Fli1/VEGFR-1 was used as a negative control. Proteins interacting directly with the CH2 domain were purified from whole cell lysates prepared from Lar transfected and untransfected Hek 293 cells using a GST fusion protein of the CH2 domain (GST-CH2). GST alone was used for comparison. (B) Reprobing the blot with an anti GST antibody confirms equal loading of GST and GST-CH2 protein.
A GST fusion protein of the CH2 domain precipitated Lar protein from Lar transfected 293 cells. This fusion protein will also precipitate Lar from untransfected cells, which is visible after prolonged exposure of the blot. Co-immunoprecipitation experiments using an anti-Shc antibody show that transfected Lar associates with endogenous Shc. Co-immunoprecipitation experiments in untransfected cells did not manage to detect Lar association with p66Shc. Hek293 cells show low expression of Lar protein, which can be detected with the antibody from Transduction Labs. This may limit the ability to detect the endogenous Lar protein. Stimulation of the Hek293 cells with EGF or treatment of the cells with hydrogen peroxide did not result in a change in the association of Lar with the CH2 domain of p66Shc.

4.2.3 Mutational analysis of the CH2 domain-binding site in Lar.

To further characterise the interactions between Ch-Tog and Lar with the CH2 domain of p66Shc, mutations were used to map the binding site. A conserved sequence had been identified in both Ch-Tog and Lar that was predicted to be the potential binding site. As a full-length construct was available for Lar, Lar was chosen as a model to analyse the binding interaction. Using site directed mutagenesis, it is possible to change the residues within the predicted binding motif, with the aim of disrupting the association as detected in the yeast two-hybrid system or by co-immunoprecipitation and GST fusion protein precipitation. All mutant constructs were analysed by DNA sequencing to confirm that only the desired mutation had occurred.

Sequence alignment of the Ch-Tog and Lar and protein sequences cloned in the yeast two-hybrid system show homology over a short sequence. Both sequences contain an SSKXXXQ motif, where X is any amino acid. This sequence is surprisingly rare when used to search protein sequence databases. This also correlates well with artefactual peptides created in the yeast two-hybrid screens. Frame shifted and antisense sequences gave false positive results in the library screen. The yeast two-hybrid system is notorious for creating false positives from in frame sequences as well as antisense and frame shifted clones. Several false positives that were sequenced during the library screen gave artefactual serine rich peptides with basic residues close by to serine repeats. This could suggest that the artefactual peptides were mimicking real proteins that interact with the CH2 domain. This information suggested that the SSK sequence
could be responsible for the majority of the CH2 domain Lar / Ch-Tog interaction. What had not been predicted in the yeast two-hybrid artefactual peptides was the presence of the glutamine residue, upstream of the SSK motif. This could mean that this residue is not important per se for the binding affinity, although it could be important for specificity in vivo as the SSK motif is much more common.

4.2.4 The SSKXXQ motif of human Lar is required for its association with p66Shc.

The results of the mutation of the SSKXXQ motif are shown in figure 4.3. Point mutation of the SSKXXQ motif in Lar shows a drop in binding affinity of between 20 and 58 %. Relatively modest decreases in binding affinity were observed with the mutation of the serine and glutamine residues in the region of 25 and 20 % respectively. The lysine mutant shows the greatest decrease in binding affinity of around 58 %.

Figure 4.3: Individual point mutations of the SSKXXQ motif reduce the affinity of the CH2 domain for LAR in the yeast two-hybrid system.
Mutations were introduced into the Lar_{4190-4472}-VP16 construct to selectively change the double serine motif to alanines (SS>AA), the lysine to threonine (K>T) and glutamine to alanine (Q>A). A deletion was also introduced to delete the whole SSKXXQ motif (Deletion). The mutant Lar_{4190-4472}-VP16 and WT construct was then transformed into the L40 reporter yeast strain with constructs encoding for the CH2 domain of p66Shc fused to LexA (CH2). The ability of the mutant proteins to interact with the CH2 domain of p66Shc was then assayed by ONPG solution assay, and the β-galactosidase activity measured as nmol min^{-1} mg^{-1}. Error bars represent mean ± S.D.
These data suggest that the lysine residue is critical for binding. The serine repeat and the glutamine are less important in determining binding affinity. This data suggests this that this sequence is indeed mediating the interaction between p66Shc and Ch-Tog / Lar. None of the mutations eliminates binding completely. From this data it is hard to determine whether this is due to some residual binding or whether this is non-specific background activity. To confirm the importance of the lysine 1285 in the association with p66Shc, a different assay is required. To achieve this, the lysine mutant was made in the full-length Lar construct as the yeast two-hybrid data suggests this to be the most significant mutation in attenuating binding. As the full deletion of the motif is no more effective in blocking binding than the lysine mutant, it was decide to test the affect of the lysine mutant on the ability to co-immunoprecipitate with p66Shc as this should have a less drastic effect on Lar protein structure than the deletion.
To test the effect of the lysine mutation on the expression of Lar, the mutated Lar lysine mutant was expressed in Hek293 cells in parallel to wild type Lar.

![Western Blot Analysis](image)

**Figure 4.4: The lysine 1285 mutant of human LAR is post translationally processed and expressed at similar levels to WT LAR.**

A point mutation of Lysine 1285 to threonine was introduced in the pcDNA3 construct encoding the full-length sequence of human LAR. Whole cell lysates were then prepared from Hek 293 cells transfected with either the WT LAR construct or the 1285 K>T mutant LAR and LAR expression was analysed by western blot use a mouse monoclonal anti-LAR antibody. The blot was the reprobed with an anti-FAK antibody to show equal protein loading.

Western blot analysis shows that the lysine mutant form of Lar is expressed at comparable levels to that of wild type Lar. The mutant Lar protein undergoes post-translational processing from the 190 kDa pre-protein to the mature 145 kDa form as normal. This shows that the mutant protein is being successfully transported through the endoplasmic reticulum and golgi network to the surface of the cell as this is the site where the cleavage event occurs forming mature Lar protein. This would indicate that there are no significant problems with the mutant Lar construct as it is largely indistinguishable from the wild type protein by Western blot analysis. As the lysine
mutant of Lar appears to be expressed and processed normally, it was used to test whether p66Shc was still capable of associating with this mutant form of Lar.

Figure 4.5: Lysine 1285 is essential for the interaction of LAR with the CH2 domain of p66Shc.

Whole cell lysates were prepared from Hek293 cells transfected with either the WT LAR construct or the 1285 K>T mutant LAR and proteins associating with Shc were co-immunoprecipitated using an anti-Shc polyclonal antibody or a GST fusion protein of the CH2 domain of p66Shc. The precipitated proteins were analysed by SDS-PAGE and immunoblotting using a monoclonal antibody recognising LAR. Wt Lar protein is efficiently precipitated from Hek293 cells by GST-CH2 fusion protein and by co-immunoprecipitation using a rabbit anti Shc antibody. No association was detected between the lysine 1285 mutant of Lar and the GST-CH2 fusion protein, nor could such an interaction be demonstrated in Shc immunoprecipitates. Whole cell lysates from Hek293 cells transfected with wild type WT Lar and the lysine 1285 mutant Lar show even expression. Reprobing with an anti-GST antibody confirms equal amounts of GST-CH2 fusion protein.

This result demonstrates the requirement of the lysine residue for the association of Lar with p66Shc. It also demonstrates that all binding is lost with this mutant.
suggesting the incomplete loss of binding seen in the yeast two-hybrid experiments is due to background reporter gene activation. This is confirmed with the fusion protein precipitation result, as this method is more sensitive than the co-immunoprecipitation method and should detect any possible binding. This result confirms the observations of the yeast two-hybrid system data, which suggests that the Lysine residue is vital for the interaction. The role of the Serine and Glutamine residues is less clear as these residues are involved with the interaction as shown by the yeast two-hybrid data, but may play more of a role in determining the specificity of the interaction. This is probably true for the Glutamine residue, as this was not predicted to be part of the motif from the yeast two-hybrid library screen sequences.

4.2.5 Tyrosine phosphorylated p66Shc is not a substrate for Lar.

The association of Lar with the CH2 domain of p66Shc has some parallels with previous observations. It has already been shown that T cell protein tyrosine phosphatase associates with p52Shc at its extreme N terminal sequence, outside the PTB domain (Tiganis et al., 1998). This association is not seen in p46Shc which lacks this sequence. Lar could be associating with the CH2 domain of p66Shc in order to specifically dephosphorylate p66Shc. To test this hypothesis it is necessary to see whether p66Shc is a substrate for Lar. It is possible to purchase recombinant Lar phosphatase domain, but this does not contain the juxtamembrane sequence, which may be necessary for the recruitment of p66Shc for dephosphorylation by Lar. A more reliable approach is to immunoprecipitate p66Shc from stimulated Lar transfected and untransfected cells and to compare tyrosine phosphorylation levels.
Figure 4.6: p66Shc is not a substrate of LAR.

Shc was immunoprecipitated using a rabbit polyclonal antibody from Lar transfected and untransfected, EGF stimulated Hek293 cells. Immunoprecipitated p66Shc from Lar transfected cells shows comparable levels of tyrosine phosphorylation to that of untransfected cells. Re-probing the blot with a mouse monoclonal antibody to Shc shows equal amounts of immunoprecipitated Shc confirming equal levels of tyrosine phosphorylation. Immunoblotting of whole cell lysates confirm expression of Lar.

EGF stimulation induced phosphorylation of all three Shc isoforms, but no changes in p66Shc tyrosine phosphorylation were observed in Lar transfected Hek293 cells when compared to untransfected Hek293 cells. This would indicate that p66Shc is not a substrate for Lar under these conditions.

4.2.6 The microtubule binding protein Ch-Tog interacts with p66Shc.

Rabbit polyclonal anti serum raised against Ch-Tog was kindly provided by Dr A Hyman, as no anti Ch-Tog antibody is available commercially (Tournebize et al., 2000). Unlike Lar, transient transfection is not required, as western blot analysis of Ch-Tog expression in Hek293 cells showed large amounts of Ch-Tog protein to be present. Co-immunoprecipitation and GST fusion protein precipitation experiments were attempted to show association between p66Shc and Ch-Tog in Hek293 cells. To investigate whether the interaction between p66Shc and Ch-Tog was dependent on the condition of the cellular microtubule network, Hek293 cells were stimulated with the microtubule stabilising drug Taxol.
Figure 4.7: Ch-Tog interacts specifically with the CH2 domain of p66Shc in Hek 293 cells.

(A) Whole cell lysates were prepared from Hek293 cells. Shc and its associating proteins were precipitated using a rabbit anti-Shc antibody. A parallel protein precipitation with an unrelated rabbit polyclonal antibody recognizing Flt1/VEGFR-1 was used as a negative control. Proteins interacting directly with the CH2 domain were purified from whole cell lysates prepared from Hek293 cells using a GST fusion protein of the CH2 domain (GST-CH2). GST alone was used for comparison. (B) Reprobing the blot with an anti-GST antibody confirms equal loading of GST and GST-CH2 protein.

Hek293 cells express large amounts of Ch-Tog protein, detectable in the whole cell lysates as a 200 kDa band. A GST fusion protein of the CH2 domain can precipitate
Ch-tog from Hek 293 cells and co-immunoprecipitation with the anti Shc antibody also precipitates Ch-tog from Hek 293 cells. Taxol treatment does not seem to affect the Ch-Tog CH2 domain interaction. Treatment with EGF or hydrogen peroxide also failed to alter the association of the CH2 domain of p66Shc with Ch-Tog.

Ch-Tog can be co-immunoprecipitated with Shc demonstrating the validity of the yeast two-hybrid system observations. Ch-Tog is efficiently precipitated using the GST fusion protein of the CH2 domain localising the interaction exclusively to p66Shc. Microtubule disruption using Taxol or Nocodazole fails to alter this interaction, suggesting that the association is independent of the state of the microtubule network. These results match closely with the observed interaction with Lar. Both interactions appear to be constitutive. No alteration in affinity for p66Shc is detectable in response to stimuli for Lar and Ch-Tog. The yeast two-hybrid data shows little difference in the affinity of interaction between Ch-Tog and Lar with the CH2 domain of p66Shc, and both proteins use a common motif to associate with p66Shc. This could mean that there is some competition for p66Shc binding between these two proteins. Little detail is known about the expression of Ch-tog other than it appears to be fairly constant throughout the cell cycle. Lar expression is variable (Cheung et al., 2000), so it could be possible that the levels of expression of Lar and Ch-Tog determine the relative distribution of p66Shc between them.

4.2.7 Co-localisation studies between Lar, Ch-Tog and p66Shc.

Co-immunoprecipitation studies have demonstrated that p66Shc associates with both Lar and Ch-tog. To further characterise these interactions, it would be advantageous to study the sub-cellular localisation of these individual proteins. This would further validate the observed interactions if co-localisation could be visualised and may also give valuable clues to the function of these interactions. It could be expected that a fraction of the p66Shc in the cell would be associated with the microtubule network in association with Ch-Tog and also with focal adhesions where Lar has been reported to be located. Human and mouse p66Shc constructs tagged with ds Red (RFP) for co-localisation studies have been made for use with green fluorescent protein (GFP) tagged proteins already available. Red fluorescent protein was cloned from the reef coral Discosoma, and gives strong fluorescence with emission maxima at 583nm. This
wavelength is considerable longer than those used with GFP, allowing co-localisation studies between GFP and RFP tagged proteins.

Figure 4.8: Expression of a full-length dsRed protein tagged human p66Shc construct in Hek293 cells.

Whole cell lysates were prepared from Hek293 cells (A) and Hek293 cells transfected with the red fluorescent protein tagged p66Shc construct and Shc expression was analysed by western blotting with an anti-Shc antibody.

A construct of the full-length human p66Shc protein fused to red fluorescent protein expresses in Hek293 cells to give a 78 kDa protein. Unfortunately the tagged p66Shc fails to give a detectable red fluorescence in vitro. Serious failing have recently been reported with red fluorescent protein, as it has been shown to form tetramers and has very slow folding kinetics (Garcia-Parajo et al., 2001). These limitations could explain the inability to detect red fluorescence from the constructs, even though tagged p66Shc protein is detectable by western blot analysis. To remedy this, a human p66Shc construct was made fused to green fluorescent protein. This construct does not express. Sequencing of the construct has not shown any errors. This could indicate that expression of p66Shc from these vectors is toxic or that there is some problem with
transcription or translation of the construct. GFP tagged p66Shc has been constructed before, although in this construct the GFP fusion was at the N-terminus of p66Shc (Sato et al., 2000). This could overcome any problem with the p66Shc start codon, but is not ideal as it could effect folding of the CH2 domain.

To avoid the problems involved with using tagged constructs and over expression, it was attempted to raise an antibody to the CH2 domain in rabbits (figure 4.9).

![Figure 4.9: Production of polyclonal antibodies against the CH2 domain of p66Shc.](image)

Two RB/NZW rabbits were immunised with 0.4 mg of GST / CH2 domain fusion protein in Freund's complete adjuvant. Two booster doses of 0.2 mg GST / CH2 domain fusion protein in incomplete adjuvant were given, with titre checks 7 day after each booster. The sera was then analysed for ability to detect p66Shc in whole cell lysates by western blot (lanes 4-6) and with commercial rabbit polyclonal anti-Shc antibodies for comparison (Transduction labs, lanes 1-3). Lane 1 and 4 = Porcine endothelial cell whole cell lysate. Lane 2 and 5 = 3T3 fibroblast whole cell lysate. Lane 3 and 6 = Hek293 whole cell lysate.

The production of polyclonal anti sera against the CH2 domain of p66Shc also proved problematic, as the rabbits reacted badly to the injection of the GST CH2 domain fusion protein. Analysis of the antibodies generated from the rabbits revealed that they recognised GST / CH2 domain fusion protein (Data not shown) but its inability to
detect p66Shc in whole cell lysates suggests that the anti sera was recognising the GST portion of the fusion protein. The anti sera does recognise a protein in the whole cell lysates but comparison with commercial anti-Shc antibodies confirms this is not p66Shc. These set backs have prevented the visualisation of an interaction between p66Shc with Ch-Tog and Lar in intact cells.

4.3 Discussion.

Lar is expressed as precursor protein 190 kDa, which is proteolytically cleaved to generate the mature protein. Lar is cleaved into two fragments, an N terminal 145 kDa fragment containing the extracellular domain and a 85 kDa C terminal fragment containing the phosphatase domains and juxtamembrane domain. The two fragments stably associate to form the mature protein (Yu et al., 1992). An antibody available from Transduction Labs was generated against the N terminus of the protein so detects the precursor protein and the extracellular portion of the mature protein giving bands of 200 and 145 kDa when used for western blotting. The interaction between the two subunits of Lar is stable under IP conditions allowing p66Shc to precipitate the detectable extracellular fragment of Lar through its interaction with the intracellular Lar fragment, which cannot be detected by western blot using this antibody. The GST precipitation and the co-immunoprecipitation experiments both suggest more of the mature form of the protein interact with p66Shc than the unprocessed form indicating that p66Shc associates preferentially with the mature form of Lar. The significance of this is unclear as little is known about how the proteolytic processing event affects the activity of Lar (Aicher et al., 1997). The extracellular domain of Lar has been reported to be shed under certain conditions such as protein kinase C activation via TPA treatment. Lar also is expressed as several different isoforms dependent on tissue type and developmental period (Yang et al., 1999). Splicing also controls the substrate specificity of the extracellular domain (O'Grady et al., 1998), which may affect the activity of Lar.

The co-immunoprecipitation experiments and GST fusion protein precipitations show that Lar interacts specifically with the CH2 domain of p66Shc in transfected cells which confirms the observations from the yeast two hybrid system. The inability to detect the interaction through co-immunoprecipitation in untransfected cells could be
due to the limitations of the Lar antibody. Hela cells have been reported to express high levels of Lar expression, but we have had difficulties detecting Lar protein using this antibody in this cell type. This could mean that the antibody is not sensitive enough to detect the small amounts of Lar protein involved in the interactions under investigation. Several studies have shown that Lar protein levels can be increased in HepG2, by increasing cell density or through TNF treatment (Cheung et al., 2000). Lar appears to be active depending on its levels of expression. Modest increases in Lar expression have been reported to trigger events such as apoptosis and inhibit cellular growth (Weng et al., 1998; Weng et al., 1999; Wang et al., 2000). This could mean that we are not triggering the cells with the correct stimulus to increase Lar expression to allow this interaction to occur.

This observation correlates well with the results from the co-immunoprecipitation experiments that demonstrate that stimulation with various different factors does not alter the interaction between Lar and p66Shc. If Lar were a substrate for p66Shc it could be expected that an increase in association between Lar and p66Shc would be visible after stimulation. Otherwise p66Shc tyrosine phosphorylation would be consistently low or absent after stimulation. This could mean that the second hypothesis may be more likely, which could mean that instead of p66Shc being a substrate for Lar, it is actually used to recruit Lar to its substrates. p66Shc would be an ideal way for a tyrosine phosphatase to be recruited to its substrates as the Shc family are recruited to most tyrosine kinase receptors, some G protein coupled receptors as well as receptors without any intrinsic enzymatic activity. Shc also forms large protein complexes consisting of many signalling adaptor proteins and cytoplasmic signalling enzymes (Bonfini et al., 1996). This would allow Lar to be targeted to a variety of different targets through its association with p66Shc.

5.1 Introduction

Recently it has been demonstrated that Taxol and other microtubule disrupting drugs such as vinblastine and nocodazole can trigger a serine / threonine phosphorylation of the CH2 domain of p66Shc (Yang and Horowitz, 2000). Three main classes of microtubule disrupting drugs have been identified: the taxanes (Includes Taxol and derivatives), vincas (Includes Vinblastine and Vincristine) and Colchicine analogues. Taxol is a natural diterpene compound derived from the stem bark of *Taxus brevifolia*. Taxol is a broad-spectrum anticancer agent, used in the treatment of ovarian, breast, lung, head and neck, bladder and oesophageal cancers. Taxol functions by binding to a hydrophobic pocket in the luminal side of the microtubule protofilament within the β tubulin subunit. This stabilises the structure of the microtubule by strengthening lateral interactions between protofilaments (Ngooles *et al.*, 1995). This causes kinetic suppression of microtubule dynamics, leading to mitotic arrest. This has been shown to induce apoptosis in numerous cancer cell lines. Other classes of drugs have different binding sites on the microtubules and tend to destabilise microtubule structure. Overall this has the same effect of blocking mitosis, giving an overall effect similar to Taxol (Reviewed in Downing, 2000).

By interfering with microtubule dynamics, Taxol and other microtubule disrupting drugs triggers arrest at the metaphase – anaphase transition, preventing chromosome segregation. Arresting cells at this phase of the cell cycle is detrimental to cell viability, with prolonged mitotic arrest leading to apoptosis (Burke, 2000; Gardiner and Burke, 2000). The exact signalling pathways involved in initiating apoptosis and the other cellular responses associated with drug treatment are not fully understood. Dose and exposure time are important factors in the outcome of Taxol treatment. Short exposure to microtubule disrupting drugs is associated with a reversible M phase arrest that does not lead to apoptosis. Longer treatments with low concentrations of Taxol (10nM) lead to prolonged cell cycle arrest and apoptosis. With higher doses of Taxol (0.2 - 30μM), stress kinase activation is observed, as are changes in gene expression.
These events are thought to be cell cycle independent and are probably induced by chronic disruption of the microtubule cytoskeleton (Wang et al., 2000). High dose Taxol treatment of macrophage cell lines induces a response similar to lipopolysaccharide treatment. This is mediated by the MyD88 / TLR4 pathway and is independent of the microtubule damage (Byrd-Lleifer et al., 2001).

The stress kinases are a subfamily of mitogen activated protein kinases superfamily, which have a proline directed serine/threonine kinase activity and are associated with apoptosis (Ichijo, 1999; Ronai, 1999). They include c-jun N terminal kinase (Jnk) and p38 (Figure 5.1). Microtubule disrupting drugs have been shown to activate Jnk and p38, but it remains unclear what significance this has to the initiation of apoptosis. Inhibitors and dominant negative studies have implicated both Jnk and p38 as being important factors in Taxol mediated apoptosis (Bacus et al., 2001; Wang et al., 1999; Stadheim et al., 2001). Very different profiles of activation for p38 and Jnk have been reported after treatment with different microtubule disrupting drugs and in different cell types (Shtil et al., 1999). It has been reported that activation of the stress kinases is responsible for the early phase of Taxol induced apoptosis. This is cell cycle independent and may only be a factor in cells exposed to very high doses of Taxol. The involvement of the stress kinases in Taxol mediated apoptosis has been questioned (Okana and Rustgi, 2001), so stress kinase activation per se may not be a major factor in the apoptotic process.

Controversy also surrounds the role of MAPK activity in Taxol mediated apoptosis. MAPK activity has been reported to be important for inducing apoptosis (Bacus et al., 2001) and important for preventing apoptosis (Stadheim et al., 2001). Raf activity is reported to be important for Taxol mediated apoptosis and appears to be distinct from the MAPK signalling pathway (Hayne et al., 2000). Pak activation is thought to be responsible for Raf activity during Taxol induced mitotic arrest. Dominant negatives of Pak or the upstream small GTPase Rac/cdc42 inhibit microtubule disrupting drugs ability to activate Raf. Raf activation by EGF is unaffected (Zang et al., 2001).
Figure 5.1: Simplified scheme of Jnk and p38 signal transduction pathways.

This uncoupling of Raf-1 activity and MAPK activity may be significant to p66Shc serine phosphorylation which shows a similar time course to Raf-1 phosphorylation after drug treatment (Yang and Horowitz, 2000). Both p66Shc phosphorylation and Raf-1 phosphorylation start about 4 hours after Taxol treatment before returning to a basal levels around 18 hours after treatment. This is in contrast with the stress kinase activation profiles after drug treatment, which appears to be much more transient than the time course for p66Shc phosphorylation (Shtil et al., 1999). Taxol treatment has been reported to induce an increase in Jnk activity 30 minutes after treatment, which returns to basal after four hours in MCF-7 cells. Jnk activity is only detectable after a
500nM dose in MCF-7 cells. These observations may link p66Shc serine phosphorylation to a Jnk independent pathway as both the dose required and the time scale of Jnk activation make Jnk an unlikely candidate.

Taxol also triggers an increase in the activity of the cyclin dependent kinase 1 (Cdk1) (Donaldson et al., 1994). Cell cycle progression and apoptosis appear to be closely linked as the activity of the cyclin dependent kinases controlling the apoptotic response (Meikrantz and Schlegel, 1996). Taxol triggers an increase in Cdk1 activity through inhibiting proteosome-dependent degradation of the regulatory cyclin B subunit, which controls Cdk1 activation. This leads to an increase in Cdk1 activity in Taxol arrested cells, which is thought to be pro-apoptotic. It has also been shown that there is also an increase in the synthesis of new Cdk1 protein, as treatment with the protein synthesis inhibitor, Cycloheximide, prevents an increase in Cdk1 activity in Taxol treated cells (Chadebech et al., 2000). This increase in activity is only seen in M phase arrested cells, as preventing entry to M phase through the use of cyclin dependent kinase inhibitors, or premature release from mitosis using non specific kinase inhibitors abolishes, and interestingly also prevents p66Shc serine phosphorylation in Taxol treated cells. The increase in the activity of Cdk1 after Taxol treatment continues for up to 48 hours.

Members of the Bcl-2 family have been shown to be involved in mediating apoptosis after Taxol treatment (Scatena et al., 1998). Phosphorylation of the anti-apoptotic Bcl-2 protein is thought to be important in Taxol treated cells. The phosphorylation of Bcl-2 at serine 70 serves to inhibit its anti-apoptotic activity and mutation of this site to prevent phosphorylation has been reported to totally block Taxol induced apoptosis in MDA-MB-231 cells (Srivastava et al., 1999). Taxol also has been shown to down-regulate the expression of the anti-apoptotic Bcl-XL and to up-regulate the pro-apoptotic Bak and Bax. Raf-1 has been implicated in the phosphorylation of Bcl-2 after Taxol treatment but this remains controversial. The overall role of Bcl-2 phosphorylation remains unclear.

p66Shc phosphorylation by Taxol and other microtubule binding drugs is slower than the serine phosphorylation event seen after growth factor treatment and cellular stresses, suggesting it may be functionally different (Yang and Horowitz, 2000;
Migliaccio et al., 1999). This observation correlates well with the observation that Ch-Tog associates with the CH2 domain of p66Shc. This could link p66Shc to the microtubule network where Ch-Tog is thought to exclusively locate. Ch-Tog has been shown to be a regulator of microtubule dynamics, and importantly, a component of the spindle checkpoint (Garcia et al., 2001). Drugs such as Taxol activate the spindle checkpoint, as they block attachment of replicated chromosomes to the mitotic spindle and prevent their segregation. Ch-tog could act to recruit p66Shc to the microtubule network where it is phosphorylated following microtubule injury possibly in response to the activation of the spindle checkpoint.

5.2 Results

5.2.1 Taxol induces p66Shc serine / threonine phosphorylation.

Taxol induced p66Shc phosphorylation was investigated in Hela cells. Hela cells are highly sensitive to the effects of Taxol with doses as low as 8nM blocking cell cycle progression in Hela cells, by blocking mitotic spindle formation. To cause major stabilisation of the whole microtubule network in Hela cells requires a dose in the region of 330nM (Wang et al., 2000). Phosphorylation of p66Shc can be detected through a shift in the electrophoretic mobility of p66Shc on SDS PAGE gels, where phosphorylated p66Shc migrates marginally slower than unphosphorylated p66Shc. The shift in electrophoretic mobility is slight but causes a broadening of the p66 band or a full shift to apparent higher mass.
Figure 5.2: p66Shc is phosphorylated after Taxol treatment.

Hela cells were treated with 100 nM Taxol for 4, 8, 16 or 24 hours and used to prepare whole cell lysates. The phosphorylation of p66Shc was analysed using SDS-PAGE and immunoblotting using a polyclonal anti-Shc antibody recognising all three Shc isoforms. Phosphorylation is detected as a shift in the electrophoretic mobility during the SDS-PAGE separation towards an apparently higher mass. Whole cell lysates from untreated, asynchronous cells are included for comparison.

As p66Shc serine phosphorylation occurs at clinically relevant doses, it may be an important factor in Taxol toxicity. Nocodazole (Methyl (5-[2-thienylcarbonyl]-1H-benzimidazol -2-YL) carbamate) treatment (500ng/ml) gives similar results (Data not shown.) indicating that both microtubule stabilisation and depolymerisation trigger this phosphorylation event.

5.2.2 Taxol induced p66Shc phosphorylation is specific to M phase.

Taxol induced p66Shc phosphorylation may be occurring as a response to the microtubule disruption induced by the drug, or by the mitotic arrest that follows drug treatment. Microtubule drug treatment has not been reported to induce significant oxidative stress so it would be unlikely that oxidative stress is the stimulus for p66Shc phosphorylation after Taxol treatment. The Taxol induced phosphorylation event
occurs over longer time courses compared to the mobility shifts reported with hydrogen peroxide, UV light, osmotic stress and growth factor treatment (Kao et al., 1997; Migliaccio et al., 1999) The four-hour delay between treatment and the detection of the mobility shift could suggest that a different pathway is active compared to the previously reported stress response.

To confirm whether p66Shc phosphorylation was an M phase specific event, it was tested to see whether arrest of the cell cycle at any of the different phases also triggers p66Shc phosphorylation.

Figure 5.3: Taxol induced p66Shc phosphorylation is specific to M phase.
Asynchronous Hela cells were arrested at S phase with hydroxyurea and released to proceed to G1 or G2 phase or arrested at S or M phase. Whole cell lysates were then prepared from Hela cells at G1, S, G2 and M phase and p66Shc phosphorylation assessed by electrophoretic mobility shift on Western blots. Asynchronous and Taxol treated (100nM) Hela cells are shown for comparison. Reprobing the blot with an antibody raised against cyclin B confirms the correct phase of the cell cycle.

No mobility shift in p66Shc is detectable in asynchronous cells. Cells in G1 phase, S phase or G2 phase show no change in electrophoretic mobility indicative of phosphorylation. This is not unexpected as Taxol and other microtubule binding drugs have not been observed to have major effects at any other phases of the cell cycle other
than M phase at these concentrations. Cell experiencing mitotic arrest do show an
electrophoretic mobility shift suggestive of phosphorylation. Cell cycle analysis
confirms that the cells are arrested in the correct cell cycle phase as demonstrated by
Cyclin B expression. Although this result shows that p66Shc phosphorylation is
specific to cells arrested in M phase, it does not discount the possibility that
phosphorylation could be induced by the microtubule disruption induced by Taxol.

5.2.3 Microtubule disruption alone does not induce p66Shc phosphorylation.

To distinguish between microtubule disruption and M phase arrest is more difficult as
microtubule disruption will induce M phase arrest. It is possible to distinguish between
the two events by disrupting microtubules transiently, or by disrupting microtubules in
cells incapable of reaching mitosis. This is possible through either cold shocking the
cells, which has the effect of depolymerising the cellular microtubule network
transiently, allowing the microtubule network to reform after the normal temperature is
restored or by arresting cells in S phase before disrupting the microtubule network by
drug action. After Taxol treatment, it is also possible to identify and separate cells
undergoing mitotic arrest from cells that are still cycling. Treatment of an
asynchronous population of Hela cells will cause a percentage of cells to reach arrest
after a short Taxol treatment. Shaking off the rounded up cells can separate the fraction
of cells undergoing mitotic arrest. The phosphorylation of p66Shc was then
investigated in both the mitotic and non-mitotic cell populations.
Figure 5.4: Phosphorylation of p66Shc is M phase specific and occurs independently of microtubule injury.

A) Whole cell lysates were prepared from asynchronous and S phase arrested cells after treatment with 500nM Taxol for 16 hours or from Hela cells cold shocked at 4°C for thirty minutes and allowed to recover for four hours. p66Shc phosphorylation was then analysed by mobility shift on western blots. Taxol treated cells are shown as a positive control to demonstrate the mobility shift. (B) Asynchronous Hela cells were treated with 100nM Taxol for 8 hours and cells arrested in mitosis were shaken off separating mitotic and non-mitotic cells. Whole cell lysates were prepared and p66Shc phosphorylation analysed by mobility shift on western blots.

No shift in the electrophoretic mobility of p66Shc can be detected in whole cell lysates prepared from Hela cells arrested in S phase prior to Taxol treatment. Cells released from an S phase block or with an asynchronous cell cycle show a shift in p66Shc electrophoretic mobility after Taxol treatment. This experiment demonstrates that microtubule disassembly does not induce p66Shc phosphorylation. It may require longer periods of microtubule disruption than the thirty minutes used to trigger p66Shc phosphorylation. This hypothesis can be tested in Taxol treated cells that have been synchronised and arrested in S phase by hydroxyurea treatment. These data would suggest that microtubule disruption alone is not sufficient for the electrophoretic
mobility shift of p66Shc observed after drug treatment. If microtubule damage was the prerequisite for the mobility shift, it could be expected that a mobility shift would be detectable after cold shock disruption or drug induced disruption of the microtubule network, independent of the cell cycle phase. This is clearly not the case and would suggest that p66Shc phosphorylation is purely an M phase specific event.

5.2.4 The cyclin dependent kinase inhibitor Roscovitine inhibits p66Shc phosphorylation after Taxol treatment.

The cyclin dependent kinase Cdk1 / Cyclin B complex has been reported to interact with and phosphorylate Ch-Tog at a PTP motif during mitosis (Charasse et al., 1999; Vasquez et al., 1999). This Cdk1 phosphorylation site was located within the fragment of Ch-Tog cloned in the yeast two hybrid screen and therefore may be functionally important. The S36 phosphorylation site of p66Shc is also flanked by proline residues, which may mean that a common kinase may phosphorylate both Ch-Tog and p66Shc during mitosis. To investigate the significance of Cdk1 activation in the phosphorylation of p66Shc during mitosis, the cyclin dependent kinase inhibitor Roscovitine (2)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine) was used to inhibit the activity of the Cdk1 / cyclin B complex. Roscovitine inhibits Cdk1 by competing with ATP for the ATP binding site in Cdk1 as well as other related cyclin dependent kinases such as Cdk2. This allows Roscovitine to inhibit progression of the cell cycle at the G1/S and G2/M phases.
Figure 5.5: The cyclin dependent kinase inhibitor Roscovitine inhibits Taxol induced p66Shc phosphorylation.

Asynchronous Hela cells were treated with 100nM Taxol for 12 hours. Roscovitine (10μM or 100μM) was added with the Taxol (12 hours), 4 hours after Taxol treatment (8 hours) or 8 hours after treatment (4 hours). Whole cell lysates were then prepared and p66Shc phosphorylation was analysed by mobility shift on western blots.

At high concentrations of Roscovitine, phosphorylation of p66Shc is inhibited as shown by the gel shift assay. Roscovitine blocks the shift in p66Shc electrophoretic mobility for up to 8 hours after Taxol treatment. Lower concentrations of Roscovitine do not inhibit the phosphorylation of p66Shc. Roscovitine inhibits p66Shc phosphorylation in the 40 - 50μM range (Figure 5.5). It is unclear as to whether this effect is direct or indirect, as Roscovitine will be disrupting many processes during mitosis. Previous experiments demonstrated the requirement of the entry to mitosis for the phosphorylation of p66Shc. It is therefore important to distinguish whether or not the effect of Roscovitine on Shc phosphorylation is due simply to the inhibition of the progression to mitosis. To achieve this it is necessary to synchronise the cell cycle and to allow entry to mitosis before addition of the Roscovitine. This should minimise any interferences generated by the Roscovitine treatment and ensure that any inhibition of p66Shc phosphorylation is not due to cell cycle disruption.
Figure 5.6: Roscovitine inhibits p66Shc phosphorylation at concentrations greater than 40 μM.

Asynchronous HeLa cells were arrested at G1/S phase with hydroxyurea. The synchronised HeLa cells were released from the S phase block in the presence of 100nM Taxol and allowed to proceed to a mitotic arrest. Cells were then either treated with a range of Roscovitine concentrations or with a control of DMSO alone for 12 hours. Whole cell lysates were then prepared and the electrophoretic mobility of p66Shc assayed by western blot.

To eliminate the possibility of cell cycle interferences in this assay, FACS analysis was used to confirm that the Roscovitine treatment was not blocking entry to mitosis. Using FACS analysis it is possible to distinguish the cell cycle phase of individual cells in a population. FACS analysis detects differences in the cellular DNA content by the intensity of fluorescence of cells stained with the DNA binding dye propidium iodide. FACS analysis was used to confirm that cell population had been successfully synchronised after hydroxyurea treatment and that the cells had been fully released from the hydroxyurea block. FACS analysis was also used to demonstrate that Roscovitine addition was not blocking entry to mitosis.
Figure 5.7: Cell cycle analysis confirms that Roscovitine treatment will disrupt entry to mitosis.

Asynchronous Hela cells were arrested at G1/S phase with hydroxyurea. The synchronised Hela cells were released from the S phase block in the presence of 100nM Taxol and allowed to proceed to a mitotic arrest at which point 100µM Roscovitine was added. Cell cycle phase synchronisation was monitored by FACS analysis. (a) asynchronous cells, (b) cells arrested at G1/S phase and (c) cells released in the presence of 100nM Taxol to a mitotic arrest with the addition of Roscovitine. (d) Shows asynchronous cells treated with 100µM Roscovitine.

The FACS analysis confirms that the synchronisation of the cell population was successful. Importantly, it also demonstrates the ability of the Roscovitine to disrupt the cell cycle, confirming the necessity of synchronising the cell cycle and ensuring entry to mitosis before addition of the Roscovitine. These results indicate that Cdk1 activity is important in the phosphorylation of p66Shc, but since inhibition of Cdk1 will disrupt many of the normal mitotic processes. It is therefore difficult to distinguish whether this is a direct or indirect response.
5.2.5 The CH2 domain of p66Shc is a substrate for up to five unidentified protein kinases in an in gel kinase assay.

From the yeast two-hybrid system it has been possible to identify two binding partners to the CH2 domain of p66Shc. Both Lar and Ch-tog stably associate with the CH2 domain, in an apparent constitutive interaction, making it a good candidate for detection in the yeast two-hybrid system. Kinase and phosphatase enzymes that directly modify the CH2 domain may also be detected by the yeast two-hybrid method, but due to their transient nature and the possible requirement for post-translational modifications to the CH2 domain, they are less likely to be detected using the yeast two-hybrid system. It has therefore been necessary to use different methods to attempt to identify the kinase responsible for the stress-induced phosphorylation of the CH2 domain. It is possible to detect kinase activity after separation on SDS Page gels. The in gel kinase assay uses this principle and is an approach to identify kinases capable of phosphorylating a given substrate. The theory behind this is that a very small percentage of the proteins run on the SDS Page gel can be renatured, and used to phosphorylate a bait protein also incorporated in the Gel.

For our experiments whole cell lysates were prepared in SDS containing sample buffer and run on polyacrylamide gels containing CH2-GST fusion protein. The gel contains no SDS, which prevents the GST-CH2 bait protein from migrating on the gel. After separation, the gel is soaked in a 20% isopropanol buffer to remove SDS and is then denatured using 6 M Guanidine hydrochloride. The Gel is then soaked in buffer overnight to refold the proteins within the gel. The gel is then incubated in reaction buffer containing $^{32}$P γATP and is then dried and exposed to film. Bands can then be detected where a kinase capable of phosphorylating the CH2 domain of p66Shc has refolded. This technique allows kinases that phosphorylated the CH2 domain to be identified, but will only provide information on their mass as determined by their migration on the SDS gel. The aim of this experiment is to identify candidate kinases that will be further investigated through other techniques such as *in vitro* kinase assays.

It has been necessary to optimise the conditions of this experiment to achieve best results. The GST-CH2 fusion protein was purified from bacterial lysates using
glutathione coated sepharose beads. Although high yields can be achieved, it is
difficult to elute the fusion protein from the beads without using harsh conditions.
Using the eluted GST-CH2 fusion protein for the in gel kinase assay gives satisfactory
results, but better results can be obtained by incorporating the GST-CH2 coated
sepharose beads into the gel directly. The use of glycerol in the gel mixture prevents
the beads from sedimenting and after polymerisation there is an even distribution of
beads throughout the gel. One possible problem would be that the beads might alter the
mobility of the samples run on the gel, by possibly acting like a gel filtration system
where larger proteins would be excluded from the beads while smaller proteins pass
through. Experiments show that this does not affect the resolution of bands of the gel.
Resolution is a problem with this system due to the low levels of SDS used. Increasing
SDS concentrations improves resolution, but may reduce sensitivity as remaining SDS
in the gel inhibits refolding of the proteins. Preparation of the samples for the in gel
kinase is also important. To give best resolution it is important to fully denature and
fragment genomic DNA in the sample. Use of mild denaturing conditions results in
much better activity in the assay, but at the cost of resolution. It has therefore been
important to find a balance between resolution and activity. As p66Shc has been
reported to be phosphorylated in response to a range of stresses, kinase activation was
studied using the in gel kinase assay after Taxol and hydrogen peroxide treatment.
These compounds have been reported to induce the phosphorylation of p66Shc as
shown by the gel shift assay. As a control, GST protein alone was used alone as a
substrate in the assay to demonstrate specificity of phosphorylation to the CH2
domain.

GST containing gels show no phosphorylation, demonstrating that GST alone is not
phosphorylated by Hela cell extracts in this assay (data not shown). Unstimulated Hela
cell extracts show low levels of kinase activity, as shown by two faint bands of 70 kDa
and 116 kDa. Hydrogen peroxide stimulated cells show a substantial increase in the 70
kDa and 116 kDa bands as well as bands in the 45 kDa range (Figure 5.8).
Polyacrylamide gels were prepared containing a GST fusion protein of the CH2 domain of p66Shc or GST protein alone as a substrate. Whole cell lysates from 500μM hydrogen peroxide (A) or 100nM Taxol (B) stimulated Hela cells were prepared in 1X SDS sample buffer and resolved by SDS-PAGE. The resolved proteins were then renatured in the gel and kinase activity assayed by detecting the phosphorylation of the GST/CH2 substrate with $^{32}$PγATP with X-ray film. (C) A kinase of around 45 kDa phosphorylates the CH2 domain after treatment with hydrogen peroxide.
This demonstrates that the CH2 domain can be specifically phosphorylated in the assay. This would indicate that the CH2 domain might have some basal level of phosphorylation. This is increased after hydrogen peroxide treatment, which also induces two or possibly three bands in the 40 – 50 kDa range. This kinase is only active towards the CH2 domain of p66Shc after treatment with H$_2$O$_2$, or Taxol, suggesting this may be involved with a stress response.

This might indicate that the phosphorylation of p66Shc is activated through a conserved stress response signalling pathway that is activated in response to these compounds. One key serine phosphorylation site (S36) has been identified as being vital for the stress response. It would appear from this result that the CH2 domain of p66Shc is phosphorylated by multiple kinases after stress. This could be due to the ability of several kinases to phosphorylated the same site, or more likely that there are several phosphorylation sites on the CH2 domain. The robust gel shift suggests it is likely that there could be more than one phosphorylation site as one addition of a phosphate group is unlikely to cause such as significant retardation in electrophoretic mobility. Sequence analysis of the CH2 domain gives few clues to identify potential phosphorylation sites. The CH2 domain contains three potential casein kinase II phosphorylation sites at Serine 20, threonine 29 and serine 80. A proline directed kinase might be responsible for phosphorylating the S36 phosphorylation site as proline residues flank it. Members of the MAPK family could be candidates for phosphorylating this site. Also of interest are three potential SH3 binding motifs. There is only one tyrosine residue present, and the motif around it makes it unlikely to be a phosphorylated. The CH2 domain is however rich in serine and threonine residues, giving many potential phosphorylation sites.

One other potential draw back of this system is that it will not detect kinase enzymes that require regulatory subunits or other quaternary structure for activity. Most kinases tend to be relatively small single subunit proteins that should be detected by this system. Earlier work indicated that Cdk1 might be important in phosphorylating the CH2 domain. This would not be detected in this assay, as it requires the regulatory Cyclin B to be in complex with it. Casein kinase II would also not be detected, as it exists as a tetramer of two $\alpha$ and two $\beta$ subunits.
5.2.6 The CH2 domain of p66Shc is not phosphorylated by p38, Jnk, Erk1, Erk2 or Cdk1 in vitro.

The in gel kinase assay identifies five possible kinases that are capable of phosphorylating the CH2 domain. Of interest are the three lower mass bands that are in the possible mass range for many members of the MAPK family. ERK1 and 2, p38, Jnk and Cdk1 were assayed using the GST-CH2 fusion protein as a substrate. P38 and Jnk are activated by a variety of stresses including oxidative stress, UV irradiation, osmotic stress and Taxol treatment. Strong Erk activation is seen after hydrogen peroxide, growth factor, PMA and Taxol treatment. Cdk1 activity is seen during mitosis and is strongly up regulated during mitotic arrest. These are good candidates for the kinases that phosphorylate the CH2 domain after stress.

![Figure 5.9: The CH2 domain of p66Shc is not phosphorylated by Jnk, p38, Cdk1, Erk1 or Erk2.](image)

Active Cdk1, p38α, Jnk1, ERK1 and 2 were immunoprecipitated from Hela cells stimulated with either Taxol, PMA or Anisomycin. The immune complexes were incubated with the GST fusion protein of the CH2 domain of p66Shc or with the appropriate positive control substrate, myelin basic protein (Erk1 and 2), Histone H1 (Cdk1), a GST fusion protein of c-jun (Jnk) or a GST fusion protein of ATF-2 (p38). Phosphorylation was analysed by incorporation of $^{32}$P into the respective substrates by autoradiography. Protein loading was confirmed by staining the gel with coomassie blue stain (Data not shown).
Strong activation of the positive controls is seen for each kinase demonstrating kinase activation. These results show that it is unlikely that any of these kinases is directly responsible for the phosphorylation of the CH2 domain after stress. Erk 1 and 2 were good candidates for being capable of phosphorylating the CH2 domain as they are activated strongly by the stresses that trigger serine phosphorylation of Shc and ERK inhibitors have been shown to partially block this phosphorylation. Jnk and p38 are not thought to be activated at the low doses of Taxol used in generating the p66Shc electrophoretic mobility shift so were unlikely to be important in the Taxol induced shift but could have been important in triggering the shift induced by osmotic and oxidative stress and UV irradiation. Other work using dominant negative constructs has also indicated that p38 and Jnk are unlikely to trigger p66Shc serine phosphorylation. The protein synthesis inhibitor Anisomycin (2-[p-Methylbenzyl]-3,4-pyrrolidinediol 3-acetate), which has been traditionally used to activate p38 and Jnk, and also to trigger p66Shc serine phosphorylation, also activates ERK strongly. Blocking p38 and Jnk activation through dominant negatives does not prevent Anisomycin induced p66Shc serine phosphorylation, whereas ERK inhibitors do.

5.3 Discussion.

A mobility shift can be detected in p66Shc after four hours of Taxol treatment, which peaks at around 16 hours. Taxol or Nocodazole treatment failed to disrupt the interaction between p66Shc and Ch-Tog but treatment with these compounds does however result in a substantial shift in the electrophoretic profile of p66Shc suggesting the occurrence of a phosphorylation event. It has already been demonstrated that this is due to a serine / threonine phosphorylation event although the exact phosphorylation sites are not yet known (Yang and Horowitz, 2000). Analysis of this phosphorylation event shows that the serine phosphorylation of p66Shc occurs at doses that are clinically relevant (50 – 200 nM) in Hela cells. Higher doses of Taxol (3 – 100 μM) have been shown to initiate effects similar to Lipopolysaccharide (LPS) and tumour necrosis factor. This includes wide spread changes in gene expression, through activation of AP-1 and NK-κB transcription factors and activation of the MAPK pathway and tyrosine phosphorylation of Shc. The most dramatic effects are seen in macrophages and occur within 15 minutes of treatment. Although high plasma
concentrations of Taxol are achievable ($5 - 10 \mu M$), it has been reported that these doses are relatively short lived, with Taxol levels falling to several hundred nanomolar or less. Cells growing in multicellular spheroids are also much less sensitive to Taxol than cells grown in a monolayer. This is thought to mimic conditions similar to those found in a tumour and would suggest that Taxol does not efficiently penetrate into the tumour, resulting in lower doses of Taxol within the tumour. This would suggest that the observations relating to high Taxol doses may not be clinically relevant, or are less important in Taxol killing (Blagosklonny and Fojo, 1999).

Analysis of cell populations exposed to Taxol shows that only the cells arrested in mitosis show a change in electrophoretic mobility, which would indicate that p66Shc serine phosphorylation is an effect of mitotic arrest as opposed to being a microtubule damage-induced event. No electrophoretic mobility shift is observed upon disruption of the microtubule network by drug or cold shock, confirming this theory. This correlates well with the early observation that like Lar, Ch-Tog appears to be constitutively associated with p66Shc. Treatment with Taxol failed to alter the interaction of p66Shc with Ch-Tog, meaning that microtubule damage probably does not recruit p66Shc to the microtubule network, but microtubule damage does trigger serine phosphorylation of p66Shc. A potential model for the role of p66Shc in this process could be that some p66Shc is constitutively associated with the microtubule network through its interaction with Ch-Tog. Microtubule damage does not activate p66Shc phosphorylation, but prolonged microtubule injury arrests the cell cycle in mitosis activating a kinase capable of phosphorylating the CH2 domain of p66Shc. This could be induced by the spindle checkpoint, of which Ch-Tog is a part, and is potently activated by microtubule disrupting drugs. The pro-apoptotic nature of p66Shc would make it a good candidate for playing a part in Taxol mediated apoptosis.

The kinase responsible for the phosphorylation of p66Shc after Taxol treatment may only be active under mitotic conditions, and in particular during abnormal mitosis. During mitosis, microtubule-binding proteins are phosphorylated to regulate their activity and control microtubule function. Ch-tog is such an example as it has been reported to recruit the cyclin B / Cdk1 complex which then phosphorylates Ch-Tog.
Detecting p66Shc serine phosphorylation under normal mitosis conditions was not possible, which suggests that this response may be due exclusively to mitotic arrest. It is not clear however how sensitive the gel shift assay is. If a small percentage of p66Shc is serine phosphorylated normally during mitosis it could be hard to detect. Ideally an antibody-based approach would be more useful to distinguish between serine phosphorylated and non-phosphorylated p66Shc. As there are several potential serine/threonine phosphorylation sites within the CH2 domain and the exact site phosphorylated during mitotic arrest is not known it would not be possible to generate a phospho-specific antibody.

Cdk1 was identified as a candidate kinase for phosphorylating the CH2 domain in response to Taxol treatment through the use of its inhibitor Roscovitine. The gel shift experiments show that Roscovitine totally blocks the electrophoretic mobility shift of p66Shc induced by Taxol. This only occurs at relatively high dose, which may indicate that this is not a specific effect. Cdk1 has been shown to phosphorylate sequences similar to the PSP motif of p66Shc, but requires the presence of a basic residue to the C terminus of the potential phosphorylation site. p66Shc lacks such a motif, making it an unlikely substrate for Cdk1. In vitro kinase assay data confirms that Cdk1 does not directly phosphorylate p66Shc. This could mean that Cdk1 activation is upstream of p66Shc serine phosphorylation, or that at these concentrations, Roscovitine is inhibiting a related enzyme that phosphorylates p66Shc. Another possibility is that inhibiting Cdk1 is deregulating mitosis in some way. This could lead to progression through mitosis or arrest of mitosis in a stage where the kinase responsible for phosphorylating p66Shc is not active. Further experiments would be required to elucidate the exact mechanisms by which Roscovitine inhibits p66Shc phosphorylation.

It is difficult to identify candidate kinase(s) responsible for phosphorylating p66Shc. The in gel kinase assay will however be a powerful tool in mapping the phosphorylation sites. Mutation of individual serine and threonine residues could be used to map the kinase phosphorylation sites. This would allow identification of the kinase responsible for phosphorylating each site, which would be of particular interest with the S36 site. It would appear that p66Shc is phosphorylated in response to a variety of stresses that would indicate a conserved stress response pathway could be
involved. The ERK pathway remains a good candidate for triggering p66Shc phosphorylation although it would appear that ERK 1 and 2 are not directly involved.

Jnk and p38 do not phosphorylate the GST-CH2 domain fusion protein in these experiments. It has subsequently been reported that both p38 and Jnk can phosphorylate p66Shc at the S36 residue (Le et al., 2001). These experiments used full-length calmodulin binding protein tagged and myc tagged p66Shc constructs in in vitro kinase assays and gel shift assays. ERK was reported not to phosphorylate p66Shc. The differences observed may be due to the nature of the constructs used. The GST-CH2 construct can be truncated at the N terminus, which may affect the phosphorylation at the S36 site. The full-length construct may also allow additional cofactors to be associated with the CH2 domain, which may be required for the phosphorylation by Jnk and p38. To observe the p66Shc gel shift induced by active Jnk in SH-SY5Y cells, over expression of myc tagged p66Shc construct was used. Wild type SH-SY5Y cells exposed to UV show a very modest retardation in electrophoretic mobility. It has been very difficult to reproducibly show any shift in electrophoretic mobility of p66Shc in Hek293 and Hela cells with any other stimuli other than Taxol or related compounds. Over-expression of p66Shc could help study the phosphorylation of p66Shc induced by oxidative stress.

Other candidates could include kinases activated by the spindle checkpoint. These could be important in microtubule drug induced phosphorylation of p66Shc, but could not be responsible for the rapid phosphorylation induced by oxidative stress and growth factor treatment. In accordance with the in-gel kinases results, it is tempting to speculate that multiple kinases could phosphorylate p66Shc. Also of importance could be the observation that very different stresses seem to induce the same pattern of phosphorylation in the in-gel kinase assay, which could implicate a conserved stress signalling pathway. Three potential CK2 phosphorylation sites are present as mentioned earlier. Reagent limitations have prevented examination of CK2 as a possible candidate kinase. Potential CK2 phosphorylation sites are relatively common and CK2 has not been implicated in stress signalling.
Chapter 6. p66S6c expression is required for efficient induction of apoptosis by Taxol and other microtubule disrupting drugs.

6.1 Introduction.

6.1.1 Taxol efficiently induces apoptosis in rapidly proliferating cells.

Taxol is a potent broad-spectrum anticancer drug that efficiently kills cancer cells by inducing apoptosis. Apoptosis is the process by which cells undergo a programmed cell death during which a variety of distinct morphological and biochemical changes can be observed. Apoptosis functions to destroy cells in a controlled manner. This can be because of damage sustained by the individual cell or for the development of tissues as seen during embryogenesis. Apoptosis is necessary to maintain a healthy population of cells within an organism by preventing damaged cells progressing towards becoming cancerous and to destroy virally infected cells. Cell death triggered by cancer chemotherapy and radiotherapy has been demonstrated to occur via apoptosis, so understandably there is great interest in this process. This differs from necrosis, which describes an unregulated death triggered by direct lethal injury. The two processes can be distinguished both morphologically and biochemically. Cells undergoing apoptosis show shrinkage of the cell and blebbing of the plasma membrane. Apoptotic cells also show distinct nuclear events, including chromatin condensation and hypersegmentation of the nuclear chromatin that buds off to form structures termed apoptotic bodies. DNA from apoptotic cells shows a characteristic laddering on agarose gels as cellular DNA is cleaved between nucleosomes. Neighbouring cells phagocytose these apoptotic bodies to efficiently recycle cellular components from the dying cell. Necrosis differs in that swelling of the cell is seen before membrane lysis occurs. Nuclear changes also occur in necrotic cells, as the nucleus swells and lyses. DNA from necrotic cells runs as a smear on agarose gels as it is randomly broken up during necrosis. The end result is that the cellular contents are released during necrosis, and are not packaged in vesicles for efficient removal (Reviewed in Steller, 1995).
6.1.2 Taxol mediated apoptosis is independent of the tumour suppressor p53.

Taxol and other microtubule disrupting drugs are thought to function by arresting the cell cycle in mitosis by preventing normal segregation of the chromosomes. At higher doses, these drugs can also cause major disruption of the microtubule cytoskeleton in a cell cycle independent manner, which may contribute to the toxicity of these compounds (Blagosklonny and Fojo, 1999). At the molecular level, the events controlling apoptosis after Taxol treatment are not fully understood. The apoptotic response to Taxol appears to be dose dependent. The responses to low doses of Taxol are summarised in figure 6.1.

**Figure 6.1: Apoptotic pathways induced by low doses of Taxol.**

Low doses of Taxol appear to disrupt the mitotic spindle leading to mitotic arrest and apoptosis. Apoptosis may occur rapidly in mitotically arrested cells, or more slowly in cells that have escaped mitosis to form multinucleated cells.
Chapter 6

The cellular responses to higher Taxol doses are summarised in Figure 6.2. The apoptotic pathways common to mitotic arrest shown in Figure 6.1 will be active, but with higher Taxol doses, more significant disruption of the microtubule network as a whole is observed. This contributes to the activation of the stress kinases p38 and Jnk and changes in gene expression.

![Diagram of cellular responses to high doses of Taxol]

**Figure 6.2: Cellular responses to high doses of Taxol.**

In addition to the apoptotic pathways observed with low doses of Taxol, higher Taxol doses induce changes in gene expression and increasing activation of the stress kinases p38 and Jnk. Some microtubule independent effects may be apparent in macrophage cell lines.

Taxol induced apoptosis is independent of the tumour suppressor p53, but the p53 status of the cells may influence the apoptotic pathways utilised and the sensitivity of the cell to Taxol (Bacus et al., 2001; Giannakakou et al., 2001; Sorger et al., 1997). p53 up regulates the cell cycle inhibitor p21\(^{waf1/cip1}\), which has been demonstrated to protect against apoptosis after mitotic arrest (Sionov and Haupt, 1999). Mitotic slippage into G1 phase may occur after a prolonged mitotic block. If a cell escapes a mitotic block without division it can become multinucleated leading to apoptosis.
(Cross et al., 1995) Some tumour cells with wt p53 and wt p21 status can however progress to become multinucleated after Taxol treatment (Long and Fairchild, 1994, Torres and Horowitz, 1998). p53 functions in part to ensure that DNA replication occurs before mitosis. Cell expressing Wt p53 are probably less likely to become multinucleated after such an event as they arrest in G1 phase. Mutations in p53 are common in many cancers so this could be a distinct advantage and also as it makes untransformed cells less susceptible to the toxic effects of Taxol. The induction of p53 by Taxol is variable and often weak so it is unclear whether p53 status will have a major effect on the treatment outcome. Clinically, it would appear that there is a poor correlation between p53 status and clinical outcome of Taxol treatment (O'Connor et al., 1997). A reported signalling pathway between p53 and p66Shc has been reported to control apoptosis in response to oxidative stress (Trinei et al., 2002). Induction of p53 increases p66Shc stability after UV and hydrogen peroxide treatment and over expression of p66Shc and p53 shows a synergistic increase in the rates of apoptosis. p53-dependent cell cycle arrest is unchanged in Wt and p66Shc -/- cells. As p53 induction by Taxol is thought to be unimportant, it is unlikely that the p53 / p66Shc apoptotic pathway will be utilised for Taxol mediated apoptosis.

As Ch-Tog has been demonstrated to function within the spindle checkpoint, it is conceivable that p66Shc could be recruited by Ch-Tog to function within a mitotic arrest induced apoptosis pathway (Garcia et al., 2001). Knockout studies have confirmed that p66Shc expression is a major factor in resisting a variety of cellular stresses, particularly oxidative stress. Oxidative stress is not thought to be a major contributing factor in Taxol mediated apoptosis as treatment with anti-oxidants has little protective effect (Shtil et al., 1999). Low levels of oxidative stress can induce mitotic arrest, which correlates with p38 activation (Karuta, 2000). Taxol treatment does activate many of the stress kinase pathways including Jnk, p38 and ERK kinases, which are thought to mediate apoptosis, and have been reported to be active after oxidative injury. It is therefore important to determine whether the serine / threonine phosphorylation of p66Shc induced by Taxol treatment is indeed involved in an apoptotic pathway as could be assumed. As p66Shc appears to be constitutively associated with Ch-Tog, association with Ch-Tog is unlikely to promote apoptosis.
itself. It would therefore be likely that p66Shc is a target for proteins activated or 
induced by mitotic spindle damage and or mitotic arrest.

6.2 Results.

6.2.1 Taxol efficiently induces apoptosis in mouse embryo fibroblast cells.

The capacity of Taxol to induce apoptosis in two Shc knockout cell lines was 
investigated. Mouse embryo fibroblasts derived from a Shc knockout mouse were 
available from Dr A Pawson (University of Toronto, Canada). In these cells the Shc 
gene is disrupted to eliminate expression of the p46, p52 and p66 isoforms of Shc 
(Shc−/−). This gives an embryonic lethal phenotype, although cells derived from the 
embryonic mouse are viable in tissue culture (Lai and Pawson, 2000). Also available 
from Dr A Pawson was a cell line derived from the knockout mouse that have been 
transfected to expresses both p46 and p52 Shc isoforms at levels comparable to the 
wild type mouse embryo fibroblasts (p66Shc−/−). These cells are essentially a p66Shc 
knockout cell line. A true p66Shc knockout mouse has been created through targeted 
deletion of the exon coding for the CH2 domain of p66Shc (Migliaccio et al., 1999). 
We have been unable to obtain any of these cells to confirm our observations.

Several methods are available for detection and analysis of apoptosis. The nuclear 
changes seen during apoptosis are a reliable indicator of apoptosis (Collins et al., 
1997; Bhalla et al., 1993). Stains such as Hoechst, bind tightly to DNA and can be 
detected by fluorescence microscopy. Changes in nuclear structure can easily be 
detected using this stain, allowing easy quantification of apoptotic cells. Apoptotic 
degradation of the genomic DNA can also be detected with FACS analysis, which is 
apparent as a sub G1 peak. Cells undergoing apoptosis show a variety of nuclear 
changes as demonstrated in figure 6.3. Figure 6.3 (A) shows several non-apoptotic 
cells with clear well defined nuclei and a single cell in an advanced apoptotic state. 
The cell undergoing apoptosis shows extensive nuclear blebbing as the nuclear 
material is packaged into apoptotic bodies. Figure 6.3 (B) shows several apoptotic cells 
at different stages in the apoptotic process. A non-apoptotic cell is present at the top 
left.
Figure 6.3: MEF cells undergo distinct apoptotic nuclear changes after Taxol treatment.
MEF cells cultured on microscope cover slips were incubated with 100nM Taxol for 16 hours. The cells were stained with Hoechst and fixed and mounted. Nuclear changes were then analysed use fluorescence microscopy (X680 magnification). (A) Apoptotic cell shown next to non-apoptotic cell (1). (B) Cells in early (2), middle (3) and late (4) stages of apoptosis. An healthy cell is located to the top left hand corner.
Figure 6.4: FACS analysis confirms G2/M phase cell cycle arrest leading to apoptosis in Taxol treated MEF cells.

Asynchronous MEF cells (A) were arrested at G1/S phase with 10mM hydroxyurea treatment (B). The cells were then released in the presence of 100nM Taxol to G2/M phase arrest (C) leading to apoptosis (D).

Apoptotic cells are easily detected by fluorescence microscopy and can be readily distinguished from non-apoptotic cells. FACS analysis demonstrates a sub G1 peak indicative of apoptosis confirming the observations by fluorescence microscopy.

6.2.2 p66Shc -/- MEF cells show resistance to Taxol induced apoptosis.

Apoptosis induced by Taxol treatment can be detected in MEF cells using nuclear staining and fluorescent microscopy. To investigate the effect of p66Shc expression on apoptosis induced by Taxol, Shc -/-, p66Shc -/- and WT MEF cells were exposed to a range of doses of Taxol and nocodazole over a time course of up to 48 hours.
Figure 6.5: She knockout MEF cells show an increased resistance to Taxol induced apoptosis.
Exponentially growing wt MEF and She -/- MEF cells were exposed to 100nM Taxol over a 48 hour time course and the percentage of cells undergoing apoptosis scored by detection of Hoechst stained apoptotic nuclei by fluorescence microscopy. Error bars represent mean ± S.D.

Figure 6.6: Puromycin does not affect apoptosis levels in the p66Shc -/- MEF cell line.
p66Shc -/- cells were grown in the presence or absence of puromycin overnight. The percentage of p66Shc -/- cells undergoing apoptosis was compared after 100nM Taxol treatment for 18 hours. WT MEF cell are included for comparison. Error bars represent mean ± S.D.
The low level of apoptosis seen in the p66Shc -/- cells would suggest that p66Shc expression increases the susceptibility of MEF cells to Taxol mediated apoptosis. This correlates well with the observation that the p66Shc -/- mouse has greatly increased resistance to cellular stress (Migliaccio et al., 1999). This would indicate that p66Shc is probably a major factor in apoptotic susceptibility. Nocodazole gives similar results although the levels of apoptosis induced by Nocodazole are much lower. This might be expected as Taxol is known to be a one of the most potent microtubule based anticancer drugs. The differences seen in toxicity between the microtubule drugs are thought to be due to the pharmacodynamics of the compounds as the basic method of action is very similar. The effects of Taxol treatment are harder to reverse compared to those of the Vinca alkaloids such as vinblastine, which remain effective anticancer drugs. The effects of Nocodazole are comparatively easy to reverse, and as a consequence Nocodazole has less toxic effect. The lower toxicity and the reversibility of Nocodazole action make it useful experimentally, but little use in cancer therapy (Giannakakou et al., 1998). Differences have also been reported in the profiles of kinase activation after treatment with different microtubule disrupting drugs (Shtil et al., 1999). These may also play a factor in their overall toxicity.

P66Shc -/- cells are grown in puromycin containing medium to select for p46 and p52 Shc expression. Apoptosis levels were compared with and without puromycin addition to confirm that it does not protect against apoptosis by slowing the rate of cell division. Comparable levels of apoptosis were seen between cells growing with or without puromycin confirming that this does not affect Taxol induced cell death. Although puromycin addition has no obvious effects on the levels of apoptosis it was omitted during the experiments and was only present during the culturing of the cells. Short-term removal of puromycin should not affect the expression the p52 and p46 Shc isoforms, which is confirmed by Western blot analysis (Fig 6.7).
Figure 6.7: Shc expression in Wt MEF cells, Shc -/- and p66Shc -/- MEF cells.

Whole cell lysates from Wt MEF cells, Shc -/- and p66Shc -/- MEF cells were separated by SDS PAGE and immunoblotted for Shc using a polyclonal anti Shc antibody. p66Shc -/- cells were cultured in puromycin free medium for 24 hours prior to analysis.

6.2.3 p66Shc -/- cells are resistant to high concentrations of Taxol.

Previous experiments have suggested that p66Shc phosphorylation is a product of mitotic arrest as opposed to microtubule damage. It has been reported that some microtubule disrupting drugs at high levels can trigger apoptosis independently of the cell cycle. To investigate whether p66Shc plays a role in this, apoptosis rates were compared for high doses of Taxol.

Figure 6.8: p66Shc -/- MEF cells are resistant to high doses of Taxol.

Exponentially growing WT MEF and p66Shc -/- MEF cells were exposed to a range of Taxol doses from 1 - 1000 nM for 24 hours and the percentage of cells undergoing apoptosis was scored by nuclear staining. Error bars represent mean ± S.D.
P66Shc -/- cells are protected against high doses of Taxol. At these doses significant Jnk and p38 activation has been reported to occur and major microtubule disruption will be present. Interestingly there is little additive effect in the levels of apoptosis as the Taxol dose increases. This could mean that p66Shc protects against a range of apoptotic responses induced by Taxol, or that the observed stress response to high doses of Taxol plays a minor role in apoptosis.

6.2.4 Blocking entry to mitosis prevents apoptosis in MEF cells.

To separate microtubule damage from cell cycle arrest, either asynchronous cells or cells arrested at G1/S phase by 1 mM hydroxyurea treatment were treated with 1μM Taxol. The G1/S phase-arrested cells will undergo major microtubule disruption in response to Taxol, but will not experience mitotic arrest. This will demonstrate whether microtubule disruption plays a major role in the apoptosis process and whether p66Shc is involved.

![Graph showing percentage of cells undergoing apoptosis](image)

**Figure 6.9: p66Shc MEF cells do not show increased resistance to microtubule damage induced apoptosis.**

Exponentially growing MEF and Shc -/- MEF cells were treated with either 1mM hydroxyurea, 1mM hydroxyurea with 1μM Taxol after 16 hours or 1μM Taxol alone. The percentage of cells undergoing apoptosis was scored by nuclear staining. Error bars represent mean ± S.D.
Preventing entry into mitosis efficiently blocks Taxol mediated apoptosis for wild type and knockout cell lines. Similar low levels of apoptosis are apparent after S phase block, suggesting no significant protection is achieved through the loss of p66Shc. The observed levels of apoptosis are very low suggesting that mitotic arrest is essential for the toxic effects of Taxol. Similar results were achieved using Nocodazole. This correlates well with the previous observation that high Taxol doses do not significantly increase levels of apoptosis. This could be explained by the fact that low doses of Taxol (10nM) efficiently induce mitotic arrest. Any further increase in dose has little effect, which is apparent from the earlier data.

![Image: Figure 6.10: High doses of Taxol induce Jnk and p38 activation in MEF cells.](image)

WT MEF cells were arrested in S phase and treated with 1μM Taxol for 30 minutes. Whole cell lysates were prepared and Jnk and p38 immunoprecipitated for immune complex kinase analysis. Jnk and p38 activation was assayed using GST fusion proteins of Jun and ATF2 as substrates.

Strong activation of p38 and Jnk was observed after 1μM Taxol treatment of S phase arrested cells demonstrating their activation during microtubule injury. No significant apoptosis was observed in these cells (figure 6.9) suggesting p38 and Jnk activity do not contribute to the apoptosis process.
6.2.5 The cyclin dependent kinase inhibitor Roscovitine protects MEF cells against Taxol mediated apoptosis.

As many of the kinases activated by Taxol are known, the role of individual kinases in the apoptotic process can be analysed using inhibitors. It could be possible to identify a signalling pathway responsible for the phosphorylation of p66Shc during Taxol treatment by comparing the effects of a particular inhibitor on rates of apoptosis in p66Shc -/- and wild type cells. Candidates include Cdk1, Jnk, p38 and Erk kinases, which have been reported to be involved in the apoptotic response to Taxol. Inhibitors are available to block Erk, cdk1 and p38 activity. At the time of carrying out the experiment an inhibitor to Jnk was not available. SB20190 has been reported to inhibit p38. PD90859 (2’-Amino-3’-methoxyflavone) is a selective inhibitor of MAP kinase kinase (MEK). Roscovitine (2-[R]-[1-ethyl-2-hydroxyethylamino]-6-benzylamino-9-isopropylpurine) inhibits cyclin dependent kinases Cdk1 and Cdk2.

![Graph showing the effects of Roscovitine on Taxol mediated apoptosis in MEF cells.](image)

**Figure 6.11: Roscovitine inhibits Taxol mediated apoptosis in MEF cells.**

Exponentially growing MEF and She-/- MEF cells were synchronised at S phase with the addition of 1mM hydroxyurea and released in the presence of 100nM Taxol to arrest at mitosis. Roscovitine (Final concentration 10μM and 100μM), PD90589 (50μM) and SB202190 (50μM) were then added and the percentage of cell undergoing apoptosis was scored after 16 hours. Error bars represent mean ± S.D.
Previous results have demonstrated that mitotic arrest is essential for efficient apoptosis, so it is important that the inhibitors used do not block the entry of the cells to mitosis. To overcome this problem, cells are synchronised in S phase by 1 mM hydroxyurea treatment and released in the presence of Taxol to arrest at mitosis. This can be seen as the cells round up upon arrest in mitosis. The cells can then be treated with the various inhibitors, avoiding disruption of entry into mitosis.

With the exception of the Cdk1 inhibitor, Roscovitine, inhibitor treatment had little effect. This contradicts other reported observations. As mentioned before, the role of the MAPK pathway and the stress kinases is contentious. Roscovitine has already been shown to block the phosphorylation of p66Shc by Taxol. It could be predicted that this should trigger a reduction in apoptosis. Low doses of Roscovitine that do not prevent p66Shc phosphorylation protect against apoptosis and Roscovitine also protects the p66Shc -/- cells. CDK1 activity is evidently important in initiating apoptosis after exposure to microtubule disrupting drugs, but whether p66Shc is involved in this is not so clear. Down regulation of Cdk1 activity could be triggering early release from mitosis. This has been reported to induce apoptosis over a longer period of several days as premature release from mitosis leads to the formation of aneuploid cells. This could be masking the quick apoptotic response detected after spindle checkpoint activation. Further experiments are required to distinguish between these possibilities.

At the doses used in these experiments it can be concluded that stress kinase activation is unimportant in the induction of apoptosis. Stress kinase activation has been reported to be proportional to Taxol dose, and is probably a response to major microtubule disruption, which occurs at higher Taxol doses. Previous experiments have shown that p66Shc phosphorylation is most likely a result of mitotic arrest, as opposed to microtubule damage. This would suggest that stress kinase activation is probably not a major factor in triggering p66Shc phosphorylation. Inhibitor studies suggest that this is probably the case, confirming the observations from the gel shift experiments. Also of interest, is the observation that the p66Shc -/- cells are resistant to high doses of Taxol. At these concentrations, major microtubule disruption is seen triggering substantial stress kinase activation, and changes in gene expression. As the p66Shc -/- cells are still resistant to these effects, it could be concluded that these effects do not play such a major role in inducing apoptosis as mitotic arrest.
6.3 Discussion.

The observation that Taxol is much less efficient at killing tumours with low levels of p66Shc expression could be of importance clinically as it has already been reported that several breast cancer cell lines (Xie and Hung, 1996) and an erythroleukaemia cell line have reduced p66Shc expression (Leslie et al., 1998). This could be expected, as loss of the growth inhibitory isoform of Shc would be advantageous to a transformed cell. This observation has been linked to over expression or constitutively active growth factor receptors, which are present in many tumours. Conversely, it has been reported that some of the most aggressive tumours have high expression of p66Shc (Jackson et al., 2000). Tumour cell lines selected for metastatic potential and breast metastases from lymph nodes showed higher p66Shc expression in a significant number of cases compared to the parent cell line or primary tumour. EGF signalling was reported to be unaffected by the increased p66Shc expression. This may correlate with the observation that highly metastatic breast tumours express high levels of abnormally spliced Lar (Yang et al., 1999). Lar has been shown to be important in sensing the extracellular matrix and in aiding cellular migration. These characteristics could make increased Lar expression useful for tumour metastasis. We have predicted that p66Shc may be required for recruiting Lar to its substrates; so high levels of p66Shc might be required for the possible function of Lar in metastasis. It would therefore be of interest to investigate whether there is a link between p66Shc expression and Taxol susceptibility in tumour samples. It would also be of interest to investigate whether there is a correlation between high p66Shc and high Lar expression in aggressive tumours. If similar results were obtained from tumour samples as seen in the apoptosis experiments reported here, it would confirm that p66Shc expression is a major factor in Taxol mediated apoptosis and that this would be a major factor in the outcome of any treatment. It has also been reported that p66Shc expression can be induced in cell lines that normally suppress p66Shc with the use of histone deacetylase inhibitors and demethylating agents (Venture et al., 2002). These compounds could be important in sensitising cells to the cytotoxic action of Taxol and related drugs in cell lines with low or absent p66Shc expression.

Interestingly, the Shc -/- cells also show some increased resistance to apoptosis. This is not however as great as the p66Shc -/- cells resistance to apoptosis. The early induction of apoptosis observed in the Shc -/- cells is of interest. This could be due to
differences in the rate of progression through the cell cycle. MEF cells grow rapidly, making them acutely susceptible to the effects of Taxol. The Shc -/- cells grow marginally faster than either the Wt of p66Shc -/- cells. This may mean that a larger percentage of cells will reach mitotic arrest in the earlier time points. Alternatively, p46 and p52Shc may have an anti-apoptotic effect that is required to survive the early effects of Taxol. Shc tyrosine phosphorylation is well characterised in activating the MAPK pathway and Taxol treatment does also induce tyrosine phosphorylation of Shc (Wolfson et al., 1997). It has been reported that Erk inhibition potentiates killing by Taxol (Stadheim et al., 2001), and oncogenic growth factor receptors that strongly activate Erk can inhibit Taxol mediated apoptosis through an up regulation of p21 (Yu et al., 1998). Shc phosphorylation is an important factor in anti-apoptotic signalling from v-Abl where p52Shc and to a lesser degree p46Shc are tyrosine phosphorylated by v-Abl (Owen-lynch et al., 1995). Shc phosphorylation at residues 239/240 has been demonstrated to be essential for survival against the pro-apoptotic activity of IL-3 in Ba/F3 cells (Gotoh et al., 1996). p46Shc and p52Shc could play an anti-apoptotic role against a variety of pro-apoptotic insults, possibly explaining the early onset of apoptosis in the Shc-/- cells upon Taxol treatment. The absence of p66Shc still however gives them considerable protection against the main action of Taxol in apoptosis. Shc tyrosine phosphorylation has been reported as being necessary for the G0-G1 transition as well as the G2-M transition (Stevenson et al., 1999). This may mean that Shc is required for efficient transition through mitosis, which may facilitate the action of Taxol. These factors may play a role in determining the susceptibility of the Shc-/- knockout to Taxol.

Recently it has been reported that the expression of individual Shc isoforms can be manipulated with small interfering RNA techniques (Kisielow et al., 2002). This technique involves the use of double stranded RNA sequences to post transcriptionally silence a gene of interest. Using this technique it is possible to repress the expression of individual Shc isoforms, either transiently or for longer periods using repeated transfections. This would allow the observations in the MEF cell line to be studied in other more relevant cell lines. The MEF cells are also difficult to transfect, limiting the experiments possible with these cell lines.
p66Shc -/- and to a lesser degree Shc-/- cells show increased resistance to Taxol across a range of concentrations, up to concentrations difficult to achieve medicinally. Interestingly, there is not a linear relationship between dose and effect. This would suggest that doses of Taxol above 50nM have little additional affect. This would also suggest that the additional affects of high Taxol doses are in MEF cells at least, irrelevant. This would indicate that mitotic arrest is the basic requirement for apoptosis, and the effects of microtubule disruption are largely unimportant. This is also apparent in G1/S phase arrested cells that show low levels of apoptosis with high doses of Taxol that should cause significant disruption of the microtubule network. Significant Jnk and p38 activity is observed under these conditions without significant apoptosis. This is significant clinically where drug combinations are frequently used. This correlates with the observation that combinations of G1/S arresting agents such as 5-fluorouracil and hydroxyurea interfere with microtubule disrupting drugs ability to induce apoptosis (Johnson et al., 1999).

Different profiles of stress kinase activation have been reported after treatment and it has also been shown that individual drugs have different stress kinase activation profiles. Some of these differences are likely to be due to different cell types and doses used. The many differences observed and the contradictory nature of some reports makes it unlikely that the stress kinase response is a fundamental step in the induction of apoptosis. Inhibitor experiments show little effect in attenuating the Taxol response with the exception of Roscovitine. Strong Jnk and p38 activity was observed in the MEF cells after high dose Taxol treatment but this does not correlate with increased levels of apoptosis. It would therefore appear that in MEF cells, stress kinase activation is unimportant in the induction of apoptosis by Taxol. Cdk1 activity would appear to be an important factor in Taxol mediated apoptosis, as its inhibition protects Wt MEF and the Shc knockout MEF cells against Taxol mediated apoptosis. Inhibition of Cdk1 also blocks p66Shc phosphorylation, which maybe significant. Roscovitine and related compounds do significantly disrupt cell cycle progression. It is possible that Roscovitine is triggering an early release from mitosis leading to multinucleated cells. It has been reported that Flavopiridol, another cyclin dependent kinase inhibitor can promote Taxol induced apoptosis, by promoting mitotic slippage (Motwani et al., 1999). It is therefore possible that Roscovitine is just giving a temporary reprieve from apoptosis.
Chapter 7. General discussion.

Recent studies have demonstrated the ability of p66Shc to negatively regulate MAPK activity and to sensitize cells to oxidative stress although the mechanisms by which p66Shc elicits these effects remain unclear (Migliaccio et al., 1997; Migliaccio et al., 1999). An important approach to identifying signalling pathways responsible for p66Shc activity would be to identify proteins which can associate with the second collagen homology domain of p66Shc, the unique domain in p66Shc thought to be responsible for the alternative effects of the p66 isoform. Utilising the yeast two-hybrid system, the leukocyte common antigen related protein (Lar) and the colonic and hepatic tumour up regulated gene (CH-Tog) have been identified as binding partners for p66Shc. The observations of the yeast two-hybrid system results could be verified in GST fusion protein pull down experiments and by co-immunoprecipitation, suggesting the observed interactions are physiologically relevant.

The transmembrane tyrosine phosphatase Lar interacts with the second collagen homology domain through a SSKXXQ motif found in the juxtamembrane region of Lar. Mutational analysis confirms that lysine residue 1285 in the SSKXXQ motif is essential for binding. The full length Lar lysine 1285 mutant will be a useful tool in studying the role of the p66Shc / Lar interaction. Early investigations have yet to show the significance of this interaction. The juxtamembrane region has been implicated to be important in Lar regulation, as this region maybe important in Lar dimerisation, which has an inhibitory role. (Blanchetot and den Hertog, 2000). This may link p66Shc with the regulation of Lar activity. Lar has been shown to negatively regulate a range of receptor tyrosine kinases and adaptor proteins, which correlates with the ability of p66Shc to negatively regulate the MAPK pathway (Wang et al., 2000; Kulas et al., 1996; Mooney et al., 1997).

Lar appears to be a multifunctional protein, potentially implicating p66Shc activity in a role of different processes. p66Shc looks to be constitutively bound to Lar. This may suggest that p66Shc is not a substrate for Lar as EGF triggers a strong tyrosine phosphorylation of all the Shc isoforms. Analysis of p66Shc tyrosine phosphorylation
in Lar over expressing cells showed no decrease in tyrosine phosphorylation levels. It has also been reported that p66Shc is inefficiently phosphorylated by EGF in human bronchial epithelial cells, grown on collagen gels despite achieving high levels of p46/52Shc tyrosine phosphorylation (Moghal and Neel, 1998). This correlates with an inability of EGF to activate the MAPK pathway. When these cells are grown on plastic this effect is not seen suggesting that the extracellular matrix may be important to regulate levels of p66Shc tyrosine phosphorylation. As Lar has homology to cellular adhesion molecules it could mean that certain extracellular matrix components such as collagen could activate Lar causing the dephosphorylation of p66Shc. This could implicate p66Shc as being important in determining whether a cell continues to grow or differentiates. As little is known about the activation of Lar and the receptor tyrosine phosphatases it is unclear at present what conditions may trigger p66Shc to be dephosphorylated by Lar. Hydrogen peroxide is known to trigger serine phosphorylation of p66Shc, which triggers p66Shc to promote apoptosis. This too also fails to disrupt the interaction between p66Shc and Lar. This data suggests that the interaction of p66Shc and Lar may not be directly important to the ability of p66Shc to sensitize cells to oxidative stress.

As p66Shc is constitutively associated with Lar, it could be that Lar is using p66Shc to recruit potential substrates. As p66Shc does not appear to be a substrate for Lar it could also be speculated that p66Shc may function to recruit Lar to many of the multi protein complexes formed by the Shc family. Cytoplasmic tyrosine phosphatases have specific domains, which localise them with their substrates, for example SHP-1 and SHP-2 have a PTB domain and two SH2 domains, which target them to tyrosine-phosphorylated proteins. The receptor tyrosine phosphatases do not have these motifs so may have to recruit substrates by different means. p66Shc appears to be activated in a similar manner to the p46 and p52 Shc isoforms and is thought to form similar multi protein complexes to the other Shc isoforms. The unique CH2 domain allows p66Shc to recruit extra proteins to the standard complexes formed by Shc. In this case it recruits Lar, which may function to deactivate the signalling complexes through dephosphorylation of target proteins within the complex (Figure 7.1).

The p66Shc / Lar interaction could therefore function to attenuate signals from growth factor receptors that use Shc as a signalling intermediate. To validate this hypothesis,
several experiments were attempted. Phosphorylation of the known Lar substrate of pl30Cas was investigated in Hek293 cells transfected with either Wt Lar or the lysine 1285 mutant. Lar has been reported to dephosphorylate pl30Cas, which has the effect of targeting it for destruction (Weng et al., 1999). Unfortunately, this observation could not be repeated in Hek293 cells (data not shown). Differences in global phosphorylation were also investigated in cells expressing either the Wt Lar receptor or the lysine 1285 mutant. Differences in phosphorylation are detectable but are difficult to reproduce. This experiment is technically difficult to achieve as small differences in expression can have a big effect. Further work is required to obtain convincing data for this experiment but some observations have been promising.

Figure 7.1: p66Shc acts to recruit Lar to its substrates.
The tyrosine phosphatase Lar is constitutively associated with the CH2 domain of p66Shc. The p66Shc / Lar complex is recruited to activated growth factor receptors by the PTB or SH2 domains of p66Shc (1). The phosphatase domain of Lar is then able to dephosphorylate the activated receptor (2), and/or proteins complexed with the receptor (3).

Analysis of the p66Shc / Ch-Tog interaction has linked p66Shc to the microtubule network. This correlates well with the report that microtubule disrupting drug Taxol can induce serine phosphorylation of p66Shc (Yang and Horowitz, 2000). Ch-Tog is
not well characterised but it is known to be important in microtubule dynamics. This suggests that p66Shc might be recruited to the microtubule network by Ch-Tog where it functions to monitor microtubule integrity. The toxic effects of Taxol are thought to act through several different signalling pathways. Dose is very important in the control the observed effects of Taxol treatment. Doses between 10 – 200 nM disrupt the mitotic spindle but do not cause major disruption of the microtubule cytoskeleton. Doses of 200nM to 30µM trigger significant microtubule damage and induce changes in gene expression (Wang et al., 2000). Phosphorylation of the CH2 domain is observed under 100nM suggesting that major microtubule disruption is not required. Although high doses of Taxol can induce phosphorylation of the CH2 domain, no phosphorylation is observed in S phase arrested cells treated with high doses of Taxol or Nocodazole. These observations suggest strongly that microtubule damage per se is not a stimulus for p66Shc phosphorylation. It is instead an M phase specific event triggered by an abnormal Shc mitosis.

Further evidence for this hypothesis comes from apoptosis studies. Cells lacking p66Shc appear resistant to the apoptotic effects of Taxol. This would suggest that p66Shc is an important factor in Taxol mediated apoptosis. Cells deficient in p66Shc are resistant to Taxol even at high doses, higher than that which could be achieved clinically. This could mean that p66Shc protects against the effects of major microtubule disruption. Importantly there is no additional effect on apoptosis of doses above 100nM. This would imply that the toxic effects of Taxol are due exclusively to its ability to arrest the cell cycle in mitosis in MEF cells. Secondly blocking cells before entry to apoptosis severely attenuates Taxol mediated apoptosis even at high doses. This evidence would suggest that mitotic arrest is the major factor in Taxol mediated apoptosis and that microtubule disruption plays only a very minor role in apoptosis in MEF cells.

This does contradict some of the published reports into Taxol mediated apoptosis. Several groups have reported that the activation of Jnk and p38 are vital for the apoptotic response (Bacus et al., 2000; Wang et al., 1999). Jnk and p38 are activated primarily through microtubule disruption and occur independently of the cell cycle. ERK activation has also been reported to be vital. Studies using the p38 inhibitor
SB202190 have no effect on apoptosis in this system. ERK activation has been reported to be important for inducing apoptosis (Bacus et al., 2000) and repressing apoptosis (Stadheim et al., 2001). The ERK inhibitor PD98059 does have a slight effect in reducing apoptosis in the wild type MEF cells. Jnk and p38 activation is occurring in the MEF cells after high dose Taxol treatment but does not appear to have any obvious effect on the rates of apoptosis. It has been reported that there are differences in the activation profiles of Jnk, p38 and ERK elicited by different microtubule disrupting drug treatment (Shtil et al., 1999). The contradictory reports of p38, Jnk and Erk activation and involvement in Taxol mediated apoptosis could be due to cell type specific differences. It is also possible that the inhibitors used may inhibit other related enzymes. It is also possible that the inhibitors and dominant negative constructs may disrupt the progression of the cell cycle, which has been demonstrated to block Taxol mediated apoptosis. Higher doses of Taxol are unlikely to be achieved clinically so the strong activation of p38 and Jnk observed at these concentrations are unlikely to be significant. The role of ERK, Jnk and p38 in Taxol mediated apoptosis has also been reported to be unimportant (Okano and Rustgi, 2001), which correlates with the observations in MEF cells.

Taken together our observations point towards a signalling pathway specific to mitosis or more specifically to mitotic arrest. During Taxol induced mitotic arrest several changes are observed. A key factor seems to be the sustained activation and transcriptional up regulation of the cyclin B / Cdk1 complex (Chadebech et al., 2000; Donaldson et al., 1994; Makino et al., 2001). The Cdk1 inhibitor Roscovitine, potently blocks the phosphorylation of the CH2 domain seen after Taxol treatment. Cdk1 does not directly phosphorylate the CH2 domain as shown by in vitro kinase assays but may be upstream of the signalling pathway leading to p66Shc phosphorylation. C-raf1 phosphorylation also occurs and its profile of activation closely matches that of p66Shc serine phosphorylation (Blagosklonny et al., 1995; Zang et al., 2001). Bcl-2 phosphorylation seems important in altering the balance of the cell away from survival and towards apoptosis. Induction of the cyclin dependent kinase inhibitor p21 is also seen after Taxol treatment (Barboule et al., 1997; Giannakakou et al., 2001; Yu et al., 1998). Interestingly, it has been reported that p66Shc -/- cells are deficient in the induction of p21.
This suggests that the serine phosphorylation of p66Shc may be a component of a signalling pathway involving the raf-1 and cdc2 activation and Bcl-2 phosphorylation. The association of p66Shc with Ch-tog could link p66Shc phosphorylation to the spindle checkpoint. It would therefore be of interest to study whether raf-1 activation and Bcl-2 phosphorylation occurs normally in p66Shc -/- cells and whether raf-1 or its activating kinase Pak can phosphorylate the CH2 domain of p66Shc.

Figure 7.2: p66Shc phosphorylation is involved in the apoptotic process induced by mitotic arrest.

Microtubule damage initiates a cascade of events leading to apoptosis. p66Shc phosphorylation appears to be a major factor in inducing apoptosis after microtubule damage. The exact kinase(s) and signalling pathway responsible for the phosphorylation of p66Shc are still unclear. Jnk or p38 do not appear to be responsible for the phosphorylation of p66Shc. The association of p66Shc with Ch-Tog will probably locate p66Shc to the microtubule network and possible to components of the spindle checkpoint.

It has been reported that p66Shc expression can be suppressed or up regulated in tumours (Xie and Hung, 1996; Jackson et al., 2000). It would therefore be of interest to investigate whether these tumours are more or less susceptible to Taxol, as predicted by their p66Shc status. LAR is also reported to be up regulated in aggressive tumours and it would be interesting to see if this correlates with high levels of p66Shc expression.
To further the understanding of physiological role of p66Shc / Ch-Tog it would be useful to link p66Shc phosphorylation to other events occurring in mitotic arrest. p66Shc phosphorylation occurs with a similar profile to that of Raf phosphorylation during mitotic arrest. This is thought to be a component of a signalling pathway from Pak which activates Raf leading to Bcl-2 phosphorylation. It would therefore be of interest to investigate whether p66Shc is involved in this process. The phosphorylation of p66Shc after Taxol treatment would appear to be linked to the apoptotic processes induced by Taxol. The phosphorylation site is not yet known, but could be expected to be serine 36 which has been linked to apoptosis after oxidative stress. It would therefore be of interest to map the phosphorylation pattern induced by Taxol. Cells expressing p66Shc lacking the phosphorylation sites could then be used in the apoptosis assays to determine whether the phosphorylation of p66Shc is required for its pro-apoptotic activity. The physiological consequences of the p66Shc / Lar complex remain unclear. The nature of receptor tyrosine phosphatases make them difficult proteins to study. A tagged Lar construct would be of use experimentally as the commercially available antibodies to Lar prevent co-immunoprecipitation and may not detect low levels of Lar protein. This would allow protein complexes containing Lar to be investigated. Substrate trapping mutants can be constructed for Lar and could be used to identify other substrates for Lar. This could be used to confirm that p66Shc is not a Lar substrate.
SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN
References.


of leukocyte antigen-related tyrosine phosphatase with focal adhesion kinase. *Diabetes* 49 (5), 810-819.


tyrosine residues 239/240 of Shc, contributing to suppress apoptosis by IL-3. The EMBO Journal 15 (22), 6197-6204.


insulin receptors. *Biochemical and Biophysical Research Communications* **252**, 139-144.


kinetochoore proteins in fission yeast: Microtubule-associating Dis1 and Mtc1 display rapid separation and segregation during anaphase. Current Biology 11, 539-549.


Ohmori, T., Yatomi, Y., Asazuma, N., Satoh, K. and Ozaki, Y. (2000). Involvement of proline-rich tyrosine kinase 2 in platelet activation: tyrosine phosphorylation mostly dependent on αIIβ3 integrin and protein kinase C translocation to the cytoskeleton and...


Takenaka, K., Moriguchi, T. and Nishida, E. (1998). Activation of the protein kinase p38 in
the spindle assembly checkpoint and mitotic arrest. Science 280, 599-602.


