The Regulation of Integrin Vesicular Trafficking

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

by

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Abstract
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Growth factors are able to influence cell adhesion and migration by regulating the function of integrins. Integrins engage in endo/exocytic cycling and I have investigated the possibility that growth factors influence integrin function by controlling their endocytosis and/or recycling. In serum starved mouse fibroblasts αvβ3 and α5β1 integrins are internalised, trafficked to the perinuclear recycling compartment, and then returned to the cell surface in a Rab11-dependent fashion. In contrast, following addition of PDGF, αvβ3 integrin (but not α5β1) was returned directly from the early endosomes to the plasma membrane via a pathway dependent on Rab4, and not Rab11. Moreover, growth factor regulated integrin recycling was not restricted to fibroblasts, but occurred in human endothelial cells in response to VEGF. Inhibition of αvβ3 recycling using dominant negative Rab4 mutants compromised cell adhesion and spreading on vitronectin (a ligand for αvβ3), but adhesion to fibronectin (a ligand for α5β1 and αvβ3) was unchanged indicating that Rab4-dependent recycling is essential for αvβ3 function.

PDGF and VEGF are known to activate the PI(3)K/ PKB/Akt signalling axis and recent evidence indicates that this pathway is involved in modulating integrin function. Recycling of both αvβ3 and α5β1 was inhibited by expression of dominant negative PKB and, furthermore, constitutively active PKB stimulated the flux of αvβ3 from the early endosome to the plasma membrane. Blockade of PKB/Akt by dominant negative mutants compromised cell spreading on vitronectin and fibronectin, consistent with a requirement for recycling in the function of both αvβ3 and α5β1 integrins.

To gain insight into the biochemical events occurring during integrin activation I looked for active signalling molecules that are recruited to αvβ3 following PDGF treatment. A 44kDa protein rich in phosphotyrosine and phosphothreonine communoprecipitated with αvβ3 integrin and western blotting revealed this protein to be active pp44 ERK1. PD98059, an inhibitor of MEK, ablated the association of ERK1 with αvβ3 and this led to reduced cell spreading on vitronectin. By contrast, PD98059 had no effect on the PDGF-regulated Rab4-dependent flux of αvβ3 integrin from early endosomes to the plasma membrane. I propose that αvβ3 integrin must recycle to the plasma membrane via the Rab4 pathway and recruit active ERK1 in order to function efficiently.
Acknowledgements

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ANK</td>
<td>Ankyrin repeat</td>
</tr>
<tr>
<td>ARE</td>
<td>Apical recycling endosome</td>
</tr>
<tr>
<td>ARF</td>
<td>ADF-ribosylation factor</td>
</tr>
<tr>
<td>ARNO</td>
<td>Arf nucleotide binding site opener</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAE</td>
<td>Bovine aortic endothelial</td>
</tr>
<tr>
<td>CAS</td>
<td>CRK-associated substrate</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco Modified Eagle Medium</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td><em>E. Coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemi-luminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EEA</td>
<td>Early endosomal antigen</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular related kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin, radixin and moesin</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAT</td>
<td>Focal adhesion targeting sequence</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-----------</td>
<td>----------------------------------------------------------------------------</td>
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<tr>
<td>Flt1</td>
<td>Vascular endothelial growth factor receptor-1</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GDF</td>
<td>GDI-displacement factor</td>
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<td>GDI</td>
<td>guanine nucleotide dissociation inhibitors</td>
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<td>Guanosine diphosphate</td>
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<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GGTase</td>
<td>geranylgeranyl transferase</td>
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<td>Glucose transporter</td>
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<td>GSK</td>
<td>Glycogen synthase kinase</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>IAP</td>
<td>Integrin associated protein</td>
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<td>Integrin-linked kinase</td>
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<td>I.P.</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin related substrate</td>
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<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
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<tr>
<td>KDR</td>
<td>Vascular endothelial growth factor receptor-2</td>
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<tr>
<td>LFA-1</td>
<td>Lymphocyte-function associated antigen</td>
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<tr>
<td>LIBS</td>
<td>Ligand induced binding site</td>
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<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MARCKS</td>
<td>myristoylated alanine rich C kinase substrate</td>
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<td>MEK</td>
<td>MAPK/ERK kinase</td>
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<tr>
<td>MIDAS</td>
<td>Metal ion dependent adhesion site</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>--------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>min</td>
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<tr>
<td>MLCK</td>
<td>Myosin Light chain kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>PAE</td>
<td>Porcine aortic endothelial</td>
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<tr>
<td>PAG1</td>
<td>Paxillin associated ARF GAP protein</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor type-1</td>
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<tr>
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<td>Rac-activated serine/threonine kinase p21</td>
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<tr>
<td>PBS</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDK</td>
<td>3-phosphoinositol dependent kinase</td>
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<tr>
<td>PI(3)K</td>
<td>Phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PI4(5)K</td>
<td>Phosphatidyl 4-phosphate 5 kinase</td>
</tr>
<tr>
<td>PIF</td>
<td>PDK interacting fragment</td>
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<tr>
<td>PIP</td>
<td>Phosphatidylinositol 4 phosphate</td>
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<tr>
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<td>Phosphatidylinositol 3,4,5-triphosphate</td>
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<tr>
<td>PIX</td>
<td>PAK-interacting exchange factor</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKL</td>
<td>Paxillin-kinase linker</td>
</tr>
<tr>
<td>PI GF</td>
<td>Placental growth factor</td>
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<td>PLC</td>
<td>Phospholipase C</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>PMQ</td>
<td>Primaquine</td>
</tr>
<tr>
<td>PRK</td>
<td>PKC-related kinase</td>
</tr>
<tr>
<td>RACK1</td>
<td>Receptor for activated PKC</td>
</tr>
<tr>
<td>REP</td>
<td>Rab escort protein</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-kinase</td>
</tr>
<tr>
<td>SCAR</td>
<td>Suppressor of cAMP receptor</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>Ser or s</td>
<td>serine</td>
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<tr>
<td>SH2 and SH3 domain</td>
<td>Src homology region</td>
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<td>SOS</td>
<td>Son of sevenless</td>
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<tr>
<td>Thr or T</td>
<td>threonine</td>
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<tr>
<td>TGN</td>
<td>Trans Golgi network</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator stimulated phosphoprotein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VN</td>
<td>vitronectin</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiscott-Aldrich syndrome Protein</td>
</tr>
<tr>
<td>WMN</td>
<td>Wortmannin</td>
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<tr>
<td>Y or Tyr</td>
<td>tyrosine</td>
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Chapter 1: Introduction

1.1 INTEGRIN STRUCTURE.................................................................................1
  1.1.1 Integrin cytoplasmic domains .................................................................2

1.2 INTEGRIN CONTAINING STRUCTURES ............................................  4

1.3 INTEGRIN BINDING PROTEINS .................................................................5
  1.3.1 Cytoskeletal proteins ......................................................................................6
  1.3.2 Integrin associations with kinases ...............................................................7
  1.3.3 Adaptor proteins and other cytoplasmic factors ......................................8
  1.3.4 Lateral associations ....................................................................................10

1.4 INTEGRIN SIGNALLING .............................................................................11
  1.4.1 Outside-In signalling .................................................................................11
    1.4.1.1 Focal adhesion Kinase (FAK) ..................................................................11
    1.4.1.2 Cas/CRK pathway ....................................................................................12
    1.4.1.3 MAP kinase pathway ............................................................................15
    1.4.1.4 PI(3)K/PKB signalling axis ...................................................................15
    1.4.1.5 Integrin linked kinase ............................................................................17
    1.4.1.6 Syk pathway ............................................................................................20
  1.4.2 Inside-out signalling ....................................................................................20
    1.4.2.1 Affinity modulation ................................................................................20
    1.4.2.2 Avidity modulation ................................................................................22

1.5 FOCAL ADHESION /COMPLEX ASSEMBLY ............................................23
  1.5.1 Rho induced focal adhesion assembly and actin organisation ....................23
  1.5.2 Rac and Cdc42 induced focal complex assembly and actin reorganisation 24
  1.5.3 ARFs and Focal complex formation ...........................................................24
  1.5.4 Focal adhesion turnover ..............................................................................27

1.6 CELL MIGRATION .........................................................................................27
  1.6.1 Actin cytoskeleton ......................................................................................28
  1.6.2 Membrane dynamics ...................................................................................28

1.7 ENDOCYTOSIS ...............................................................................................31
  1.7.1 Rabs in endo/exocytic cycling ....................................................................33
    1.7.1.1 Rab5 ........................................................................................................35
    1.7.1.2 Rab4 ........................................................................................................37
    1.7.1.3 Rab11 ......................................................................................................39
    1.7.1.4 Rab 27 .....................................................................................................41
  1.7.2 The ADP-Ribosylation factors (ARFs) .......................................................42
  1.7.3 The Rho subfamily .....................................................................................43

1.8 REGULATION OF INTEGRIN TRAFFICKING........................................45

Chapter 2: Materials and Methods

2.1 ANTIBODIES AND REAGENTS ...................................................................49
  2.2 MOLECULAR BIOLOGY ..............................................................................49
    2.2.1 Plasmids ....................................................................................................49
    2.2.2 Bacterial Transformation of Plasmid DNA ..............................................50
    2.2.3 Large scale preparation of plasmid DNA ...............................................51

2.3 CELL CULTURE ..............................................................................................51
  2.3.1 NIH and Swiss 3T3 cells .............................................................................51
2.3.2 Human Umbilical Vein Endothelial Cells (HUVECs) ...........................................51

2.3.3 Transient transfection ..........................................................................................52

2.4 PROTEIN ANALYSIS .............................................................................................52

2.4.1 Immunoprecipitations and western blotting ....................................................52

2.4.2 Western blotting ................................................................................................52

2.4.3 Immunofluorescence ..........................................................................................53

2.4.3.1 Surface only integrin labeling .......................................................................53

2.4.3.2 Tracking of internal integrin .........................................................................53

2.4.3.3 ERK staining ..................................................................................................54

2.4.4 Capture ELISA ..................................................................................................54

2.5 CELL BASED ASSAYS .........................................................................................54

2.5.1 Total surface expression, internalisation and recycling assays .......................54

2.5.1.1 Total surface expression ................................................................................55

2.5.1.2 Internalisation ................................................................................................55

2.5.1.3 Recycling .......................................................................................................56

2.5.2 Cell adhesion and spreading assays ....................................................................57

Chapter 3: Integrin Trafficking in Fibroblasts

3.1 INTRODUCTION ....................................................................................................59

3.2 RESULTS ...............................................................................................................61

3.2.1 Surface expression of v3 and 51 integrins in Swiss 3T3 Fibroblasts ................61

3.2.2 Integron internalisation .......................................................................................63

3.2.3 Integrin endosomal compartments ....................................................................63

3.2.4 Recycling of integrins .......................................................................................65

3.2.5 Rab4-dependence of avp3 recycling ..................................................................65

3.2.6 Visualisation of PDGF-dependent avp3 recycling ............................................70

3.2.7 Involvement of Rab4 in cell adhesion and spreading .......................................70

3.3 DISCUSSION .........................................................................................................75

3.3.1 Regulation of recycling from early endosomes ...............................................75

3.3.2 avp3 recycling and the regulation of cell adhesion and motility .......................77

Chapter 4: ERK1 associates with avp3 integrin and regulates cell spreading on vitronectin

4.1 INTRODUCTION ....................................................................................................80

4.2 RESULTS ...............................................................................................................83

4.2.1 Active Erk1 is associated with avp3 integrin ....................................................83

4.2.2 Recruitment of avp3 and ERK to plasma membrane puncta ...............................84

4.2.3 Association of ERK1 with avp3 integrin requires the activity of MEK ...............87

4.2.4 Association of ERK1 with avp3 integrin is not necessary for integrin recycling .90

4.2.5 Recycling of avp3 is not a prerequisite for association of ERK1 with v3 ..........90

4.2.6 Recycling of avp3 and active ERK are required for cell spreading on vitronectin ....................................................................................................................93

4.3 DISCUSSION .........................................................................................................96

4.3.1 Summary ............................................................................................................96
4.3.2 Role of integrins in ERK translocation ..........................................................96
4.3.3 A role for ERK at the plasma membrane.......................................................99

Chapter 5: Vascular endothelial growth factors (VEGFs) regulate v3 Recycling in Human Umbilical Vein Endothelial Cells (HUVECs) .................................................................104
5.1 INTRODUCTION ............................................................................................104
5.1.1 Integrins and angiogenesis ........................................................................104
5.1.2 Angiogenic growth factors ........................................................................106
5.1.3 VEGF receptor signalling .........................................................................106
5.1.4 VEGF and migration .................................................................................107
5.1.5 VEGFs and integrin activation ..................................................................108
5.2 RESULTS .........................................................................................................109
5.2.1 αvβ3 and α5β1 integrins, but not αv51 take part in an endo/exocytic cycle ...109
5.2.2 VEGF does not affect integrin endocytic rates .........................................109
5.2.3 VEGF stimulates the recycling of αvβ3 but not α5β1 .........................109
5.2.4 Wortmannin inhibits VEGF-stimulated αvβ3 recycling .........................112
5.2.5 Surface distribution of αvβ3 in HUVECs ................................................112
5.3 DISCUSSION ..................................................................................................118
5.3.1 Summary ...................................................................................................118
5.3.2 VEGFs stimulate αvβ3 recycling .............................................................118
5.3.3 Assembly of αvβ3 puncta and subsequent focal complex formation 120
5.3.4 Endo-/exocytic cycles and angiogenesis ....................................................121
5.3.5 αvβ3 and cell-survival ..............................................................................122

Chapter 6: PKB/Akt and Integrin Trafficking .................................................................125
6.1 INTRODUCTION .....................................................................................125
6.1.1 PI(3)K and Endosomal Trafficking ..........................................................127
6.1.2 PKB/Akt and Integrin Trafficking ............................................................128
6.2 RESULTS .........................................................................................................130
6.2.1 Integrin endocytosis does not require PI(3)K or PKB/Akt .......................130
6.2.2 Integrin recycling requires PI(3)K and PKB/Akt .....................................130
6.2.3 Membrane targeted PKB/Akt drives αvβ3 recycling ...............................132
6.2.4 PKB/Akt is necessary for cell spreading ..................................................132
6.3 DISCUSSION ..................................................................................................138
6.3.1 Summary ...................................................................................................138
6.3.2 PKB/AKT and Integrin Recycling ............................................................138
6.3.3 PKB/AKT and Integrin function ...............................................................139

Chapter 7: General Discussion ............................................................................142
7.1 SUMMARY .................................................................................................142
7.2 HOW COULD THE ENDOCYTIC PATHWAY CONTRIBUTE TO MAINTAINENCE OF V3 ACTIVITY? ..........................................................143
7.2.1 Endosome acidification and ligand-receptor dissociation .................. 143
7.2.2 Retrieval of damaged integrins from the cell surface ....................... 145
7.2.3 The role of endo-exocytosis in generating polarised distribution of surface integrin ................................................................. 147
7.2.4 Lipid rafts ........................................................................................... 148

7.3 HOW IS SELECTIVITY ACHIEVED IN THE ENDOSONAL PATHWAY? .............................................................................................................. 152
7.3.1 Sorting Nexins .................................................................................... 152
7.3.2 Endosomal lipid domains ................................................................. 153
7.3.3 Rab microdomains ........................................................................... 153
7.3.4 Other microdomains ........................................................................ 154
7.3.5 Integrin sorting motifs .................................................................... 155
7.3.6 Recruitment of kinases ..................................................................... 156
7.3.7 SNARE complexes .......................................................................... 156
7.3.8 Motor proteins .................................................................................. 157

Bibliography ................................................................................................. 158

Appendix: Publications .............................................................................. 198
List of figures

Chapter 1
Fig. 1.1 A domain structure of the straightened extracellular domain of αvβ3.............3
Fig. 1.2 Integrin binding proteins.................................................................6
Fig. 1.3 Paxillin Interactions................................................................................. 9
Fig. 1.4 FAK Effectors.........................................................................................13
Fig. 1.5 The role of Cas in integrin mediated signalling........................................14
Fig. 1.6 MAPK signalling....................................................................................16
Fig. 1.7 Downstream Effectors of PKB/Akt........................................................18
Fig. 1.8 Integrin Linked Kinase (ILK)......................................................................19
Fig. 1.9 Rho induced focal adhesion assembly.................................................25
Fig. 1.10 Rac and Cdc42 and actin reorganisation..............................................26
Fig. 1.11 Model of cell adhesion and actin cytoskeleton during cell migration...29
Fig. 1.12 Integrin trafficking and the formation of focal adhesions..................30
Fig. 1.13 Vesicular trafficking pathways and intracellular compartments...........32
Fig. 1.14 Endosomal Rab proteins.........................................................................34
Fig. 1.15 The Rab cycle.........................................................................................36

Chapter 3
Fig. 3.1 Effect of various agents on surface expression of α5β1 and αvβ3 integrins.. .62
Fig. 3.2 Internalisation of integrins......................................................................64
Fig. 3.3 Visualisation of integrin endosomal compartments..............................66
Fig. 3.4 Recycling of α5β1 and αvβ3 integrins....................................................67
Fig. 3.5 Rab4-dependence of αvβ3 recycling......................................................69
Fig. 3.6 Visualisation of PDGF-dependent recycling of αvβ3 integrin.................71
Fig. 3.7 Effect of dominant negative Rab4s on cell adhesion and spreading....73
Fig. 3.8 Effect of S22NRab4 on cell spreading.....................................................74
Fig. 3.9 Integrin recycling pathways..................................................................76

Chapter 4
Fig. 4.1 Active ERK1 is associated with endogenous mouse αvβ3 integrin in Swiss 3T3 fibroblasts.................................................................85
Fig. 4.2 Active ERK1 is associated with human αvβ3 expressed in NIH 3T3 fibroblasts.................................................................86
Fig. 4.3 Recruitment of αvβ3 integrin to ERK and focal complexes...............88
Fig. 4.4 αvβ3 integrin and ERK localise to punctate plasma membrane complexes...89
Fig. 4.5 PD98059 inhibits association of ERK with αvβ3 integrin.......................91
Fig. 4.6 PDGF-stimulated recycling of αvβ3 does not the activity of MEK........92
Fig. 4.7 Dominant negative Rab4 does not block association of ERK1 with αvβ3...94
Fig. 4.8 Cell spreading on vitronectin requires the activity of MEK and Rab4......95
Fig. 4.9 Scheme for association of active ERK with integrin in plasma membrane complexes.................................................................97
Fig. 4.10 Pathways invovled in modulating integrin function..............................98

Chapter 5
Fig. 5.1 VEGF receptors and their ligands........................................................105
Fig. 5.2 Internalisation of integrins..................................................................110
Fig. 5.3 VEGFs do not regulate integrin internalisation.................................111
Fig. 5.4 Recycling of αvβ3 and α5β1 integrins.............................................113
Fig. 5.5 The effect of wortmannin on αvβ3 internalisation and recycling........114
Fig. 5.6 Visualisation of αvβ3 containing puncta...........................................116
Fig. 5.7 Visualisation of αvβ3 containing puncta in response to VEGFD and PI GF...117
Fig. 5.8 VEGFs and actin cytoskeleton............................................................119

Chapter 6
Fig. 6.1 Integrin internalisation is not affected by wortmannin or dominant negative
PKB..................................................................................................................131
Fig. 6.2 PKB is necessary for both αvβ3 and α5β1 recycling..............................133
Fig. 6.3 Constitutively active PKB stimulates αvβ3 recycling..........................135
Fig. 6.4 Adhesion of cells expressing mutant PKBs.........................................136
Fig. 6.5 PKB is necessary for cell spreading.....................................................137

Chapter 7
Fig. 7.1 Lateral diffusion of integrins...............................................................149
Fig. 7.2 The formation of focal complexes by vectoral vesicular transport of
integrins..............................................................................................................150
Fig. 7.3 Migrating fibroblasts in a chemotactic gradient.................................151
Chapter One: Introduction
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The ability of a eukaryotic cell to sense its environment is a key process for the development and homeostasis of a multicellular organism. Cells are able to detect their environment via interactions with neighbouring cells or by contact to the extracellular matrix (ECM) and these interactions are essential for cell function. Cell:ECM interactions are necessary for the regulation of cell shape, growth, proliferation, differentiation and the ability of a cell to migrate. The adhesion of cells to the ECM is a dynamic yet tightly regulated process and is primarily mediated by the integrin family of cell adhesion receptors.

Integrins are a family of heterodimeric glycoproteins that mediate cell to ECM and some cell-cell interactions. Each integrin receptor is made up of an alpha and a beta subunit, each of which has a single transmembrane domain, a large extracellular domain, and a short intracellular domain (the exception being β4 integrin which has an extended cytoplasmic domain). To date, approximately 18 α subunits and 8 β subunits have been identified and these can combine to form 24 different integrin receptors. Some integrin are widely expressed such as αvβ3 and α5β1, whereas, others are tissue specific i.e. αIIbβ3 is expressed only on haemopoietic cells. The extracellular domain of the integrin contains a ligand binding site which recognises specific, short peptide, sequences in ECM proteins, such as the RGD sequence in fibronectin (Pierschbacher & Ruoslahti, 1984 and Yamada & Kennedy, 1984). α5β1 binds to fibronectin and denatured collagen, and vitronectin is a ligand for αvβ3 (Cheresh & Spiro, 1987).

1.1 INTEGRIN STRUCTURE

Integrin structure has been extensively studied using structural predictions, biochemical dissection and crystallography (Tuckwell & Humphries 1997, Springer 1997, Emsley & Liddington 2000 and Xiong et al., 2001). The recent resolution of the crystal structure of the extracellular portion of αvβ3 has revealed that the 12 domains assemble into an ovoid "head" and two "tails" (Xiong et al., 2001) (see Fig. 1.1), and as previously predicted (Tuckwell & Humphries, 1997 and Springer, 1997), the head of the integrin contains a seven bladed β propeller corresponding to the seven amino-terminal repeats of the α subunit. The upper face of the α subunit β propeller interacts with the β subunit A
domain, and ligand binding is thought to take place between blades 2 and 3 of the β propeller and the Metal ion-dependent adhesion site (MIDAS) of the βA domain (Xiong et al., 2001). The α subunit leg of the integrin contains three large β sandwich domains which have been termed thigh and calf domains, and the region between the thigh and the first calf domain exhibits a highly flexible 'knee' which is severely bent in the crystal structure, reflecting an unusual flexibility (Xiong et al., 2001). This knee region also has a metal ion- binding site and the binding of cations to this region suggests a mechanism by which integrin conformational changes could be induced during ligand binding.

1.1.1 Integrin cytoplasmic domains

The β cytoplasmic tails of integrins are alternatively spliced with various splice variants being expressed in different tissues or stages of embryonic development. For example, β1 integrin has 4 cytoplasmic splice variants; β1A is ubiquitously expressed and targeted deletions of this variant results in embryonic lethal conditions. β1B is found in skin, liver and small areas of skeletal and cardiac muscle (Balzac et al., 1993), β1C is found in blood cell lines, platelets monocytes and in liver and kidney (Languino & Ruoslathi, 1992), and β1D is found in adult skeletal and cardiac muscle and is up-regulated during myoblast differentiation (Zhidkova et al., 1995). β3 integrins are also alternatively spliced resulting in a number of cytodynamic variants. β3A is expressed on endothelial cells and platelets and is the most studied, β3B was isolated from a human placental library and β3C was identified as a novel osteoclast integrin subunit (van Kuppevelt et al., 1989 and Kunnar et al., 1997).

The highly conserved β cytoplasmic tails contain several important sequences which are necessary for subunit pairing, the targeting of integrins to focal adhesions and cytoskeletal protein binding. The β1 C integrin tail contains 3 clusters of amino acids, termed cyto-1 (residues 764-774), cyto-2 (residues 785-788) and cyto-3 (residues 797-800), which are essential for focal adhesion targeting and these are highly conserved are amongst the β subunits (except β4) (Reszka et al., 1992). Cyto-1 contains a LLVIHDR sequence, which is proposed to form a salt bridge with a KXGFFKR sequence found in α chains, and mutations in this region of the α chain have been found to prevent subunit association and transport to the membrane (Reszka et al., 1992, Briesewitz et al., 1995 and Hughes et al., 1995). Cyto-2 and Cyto-3 contain NPXY motifs (where X represents a
Fig. 1.1 A domain structure of the straightened extracellular domain of αvβ3 (adapted from Humphries & Mould 2001). The α subunit leg of the integrin contains three large β sandwich domains which have been termed thigh and calf domains, and the region between the thigh and the first calf domain is a highly flexible area. An area of flexibility is also seen in the β chain between the EGF repeats and the PSI domain. The upper face of the α subunit β propeller interacts with the β subunit A domain, and ligand binding is thought to take place between blades 2 and 3 of the β propeller and the Metal ion-dependent adhesion site (MIDAS) of the βA domain. Abbreviations: MIDAS- metal ion-dependent adhesion site, PSI -Plexins, semaphorins and integrins.
non-conserved residue), which are thought to function in the binding of cytoskeletal proteins such as talin and are necessary for the recruitment of integrins to focal adhesions (Rezska et al., 1992). Upon integrin binding to the ECM it is thought that cytoskeletal protein binding domains in the integrin tail are unmasked and this results in the recruitment of integrin binding proteins and the establishment of integrin containing structures such as focal adhesions.

1.2 INTEGRIN CONTAINING STRUCTURES

Several types of integrin-containing macromolecular structures exist. These include fibrillar adhesions, which are elongated dot like central structures that contain α5β1 integrin, tensin and parvin/actopaxin and are attached to fibronectin fibrils (Katz et al., 2000, Olski et al., 2001 and Zamir et al., 1999). Integrins are also found in plasma membrane microdomains rich in tetraspannin proteins. These structures are devoid of FAK, paxillin and vinculin, but contain myristoylated alanine rich C kinase substrate (MARCKS), one of the prominent substrates for different members of the protein kinase C family (Berditchevski & Odintsova, 1999 and Berditchevski, 2001). Another type of integrin containing adhesion has been termed ‘integrin clusters’. These structures contain full length β3 integrin, the protease calpain, calpain cleaved β3, vinculin and the myosin-associated protein skelemin (Bialkowska et al., 2000 and Reddy et al., 2001). Macrophages and osteoclasts have unique integrin containing cell:ECM adhesions known as podosomes and these are small cylindrical structures containing ανβ3 integrin, paxillin, vinculin, talin and the FAK related kinase PYK2 (Pfaff & Jurdic, 2001). However, the most studied integrin containing structures are known as focal adhesions and focal complexes.

Focal adhesions (F.A.s) are complex structures containing a plethora of structural and signalling proteins and are situated at the end of actin stress fibres to which their formation is intimately linked. The association of integrins with actin binding proteins and signalling proteins promotes the assembly of actin filaments, and conversely, the organisation of actin filaments into stress fibres encourages integrin clustering into focal adhesions and thus enhances ECM binding. F.A.s have been reported to contain numerous cytofacial components (talim, paxillin, vinculin, tensin and α-actinin) some of which can bind directly to actin, tyrosine kinases (FAK/Src), serine/threonine kinases (ILK, PKC, PAK), tyrosine phosphatases (PTP-PEST, SHP-2) and modulators of small
GTPases (ASAP-1, Graf). In addition to integrins, which serve to link the ECM and the actin cytoskeleton, other transmembrane proteins have been reported to associate with focal adhesions such as the syndecans (Woods & Couchman, 1994), and these may be important in the recruitment and activation of protein kinase C (PKC). Integrin containing Focal complexes are smaller than F.A.s and are found at the tips of lamellopodia and filopodia rather than being associated with stress fibres (Nobes & Hall, 1995). The molecular composition of focal complexes is less clear than that of F.A.s, but they are known to contain αvβ3, paxillin, vinculin and FAK (Kiosses et al., 2001 and Nobes & Hall, 1995).

1.3 INTEGRIN BINDING PROTEINS

1.3.1 Cytoskeletal proteins

Integrins have been shown to bind to a number of cytoskeletal, adaptor and signalling proteins and these are illustrated in Fig. 1.2. The association of integrins with the actin cytoskeleton occurs via both direct and indirect interactions of β cytoplasmic tails with actin binding proteins. This has been extensively reviewed in Critchley (2000) and Calderwood (2000) and therefore will be only briefly discussed here.

Talin, a major structural component of focal adhesions, has been shown to bind to β1, β2, β3 and less strongly to β7 integrin tails (Pfaff et al., 1998, Sampath et al., 1998 and Moulder et al., 1996). Talin consists of a 50 kDa head, which includes a 200 stretch of amino acids with homology to the ezrin, radixin and moesin (ERM) family proteins, and a 220 kDa C-terminal rod containing actin binding domains. Both the head and the rod contain integrin-binding regions and reduced expression of talin disrupts cell surface expression of integrins and export from the golgi, and this impairs focal adhesion formation and cell migration (Priddle et al., 1998 and Martel et al., 2000). This suggests that talin-integrin interactions are important for integrin function.

The actin binding protein filamin, composed of 2 anti-parallel 280kDa subunits, binds to β1A, β2, β7 integrins, with a weaker interaction to β1D (Pfaff et al., 1998) and localises to the cortical actin cytoskeleton and to focal adhesions. Filamin-1 is required for neuronal migration (Walsh & Goffinet, 2000) and cells defective for this isoform have impaired migration and reduced levels of β integrin surface expression (Meyer et al.,
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<td>Caveolin-1</td>
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**Fig. 1.2 Integrin binding proteins** (adapted from and references within Liu et al 2000, see text for additional references)
α-actinin exists as homodimers of rodlike monomers of 100kDa and binds to β1, β2 and β3 integrins, and also to the focal adhesion proteins vinculin and zyxin (Burridge & Chrzanowska-Wodnicka, 1996 and Jockusch et al., 1995). The α-actinin binding site has been localised to the membrane proximal half of β1 and β2 integrin tails and mutations in this region alter the formation of focal adhesions and stress fibres (Otey et al., 1990).

Integrins have also been shown to bind to myosin and the myosin-related protein skelemin. Myosin was found to bind to di-phosphorylated peptides corresponding to the amino acids 740-762 of the β3 tail, but did not interact with the corresponding un-phosphorylated peptides indicating the association is phosphorylation-dependent (Jenkins et al., 1998). A yeast two-hybrid screen to isolate β3 binding partners indicated that skelemin associated with β3 and, furthermore, skelemin was shown to interact with GST fusion proteins of β1 and β3 integrin cytoplasmic tails but not with β2 integrin (Reddy et al., 1998). Additionally, a skelemin-like protein was found to co-localise with both β1 and β3 integrins in CHO cells (Reddy et al., 1998).

1.3.2 Integrin associations with kinases

Integrins lack any intrinsic enzymatic activity and, therefore, must form associations with other proteins to elicit signals. Integrins can bind to and activate non-receptor tyrosine kinases such as focal adhesion kinase (FAK), Src family kinases and integrin-linked kinases (ILKs). The cytoplasmic domain of β1 integrin has been shown to bind to the NH2-terminal, noncatalytic domain of FAK (Schaller et al., 1995) and FAK co-immunoprecipitates with β1 integrin differentiating Schwann cells (Chen et al., 2000). Integrin Linked kinase (ILK) has also been demonstrated to associate with β1 and β3 integrin subunits by yeast two hybrid and co-immunoprecipitation studies (Hannigan et al., 1996) and it is thought that this may phosphorylate downstream effectors such as PKB and thus mediate some of the consequences of α5β1 and αvβ3 integrin engagement. The tyrosine kinase Syk, which is expressed in haemopoietic cell lineages, binds to four C-terminal residues of the β3 cytoplasmic tail and disruption of integrin-Syk binding in the αIIbβ3 integrin disrupts lamellipodia formation upon cell adhesion to fibrinogen (Woodside et al., 2001).
1.3.3 Adaptor proteins and other cytoplasmic factors

Paxillin is a multidomain intracellular signalling adaptor molecule found in focal adhesions. Paxillin binds to \( \alpha 4 \) and \( \alpha 9 \) integrins (Liu & Ginsberg, 2000, Liu et al., 2001 & Young et al., 2001) and to peptides corresponding to the 17C terminal amino acids of the chicken \( \beta 3 \) cytoplasmic tail (Pfaff & Jurdic 2001), and specifically co-immunoprecipitates with \( \beta 1 \) integrin in differentiating neurons (Chen et al., 2000). Furthermore, a co-localisation of paxillin and \( \alpha v \beta 3 \) has been demonstrated in osteoclast podosomes (Pfaff & Jurdic, 2001). Paxillin acts as an adaptor providing a platform for protein tyrosine kinases such as FAK and Src (Tachibana et al., 1995 Hildebrand et al., 1995 and Weng et al., 1993), and binds to the downstream effector CRK via Cas (Schaller & Parsons, 1995) and this is important for activation of downstream signalling pathways. Paxillin also binds to the structural proteins, vinculin and actopaxin, to regulators of the actin cytoskeletal dynamics such as ARF GAPS, PKL, PIX and PAK (Turner, 2000), and to clathrin, Poly(A)-binding protein 1 and integrin linked kinase (ILK) (Turner, 2000, Woods et al., 2002) (see Fig. 1.3). Paxillin is recruited to focal adhesions via LIM domain associated kinases and negative regulators of these pathways such as PTP-PEST bind directly to paxillin (Shen et al., 1998). The association of both protein-tyrosine kinases and protein-tyrosine phosphatases with paxillin suggests that paxillin may play a critical role in the regulation of the phosphotyrosine content of focal adhesions.

Other adaptor proteins are known to associate with integrins, for instance in aggregated platelets, Shc binds in a phosphorylation dependent manner to residue Y759 of the \( \beta 3 \) tail of the platelet integrin \( \alpha IIb\beta 3 \) (Cowan et al., 2000). Moreover, in vascular cells, shear stress was found to prolong the association of Shc with \( \alpha v \beta 3 \) and with \( \beta 1 \) integrin (Chen et al., 2000) and Shc co-immunoprecipitates with \( \alpha 5\beta 1 \) when overexpressed in a breast cancer cell line (Mauro et al., 1999). The association of Shc with integrins is likely to recruit Grb2, an adaptor which links to many signalling pathways including the Ras/Raf/ERK cascade (Mainiero et al., 1995) and thus mediate cell cycle progression in response to matrix engagement. The role of other cytodomain-associated proteins is less clear. The \( \beta 3 \) cytoplasmic domain has also been shown to bind \( \beta 3 \)-endonexin by yeast two-hybrid analysis (Shattil et al., 1995 and Eigenthaler et al., 1997). It was found that both membrane-proximal and membrane-distal residues of the
Fig. 1.3 Paxillin Interactions. Paxillin acts as an adaptor providing a platform for protein tyrosine kinases such as FAK and Src and binds to the downstream effector CRK via Cas and this is important for activation of downstream signalling pathways. Paxillin also binds to the structural proteins, vinculin and actopaxin, to regulators of the actin cytoskeletal dynamics such as ARF GAPS, PKL, PIX and PAK and to clathrin, Poly-(A) binding protein 1 (Poly(A)BP1) and integrin linked kinase (ILK). Paxillin is recruited to focal adhesions via LIM domain associated kinases and negative regulators of these pathways such as PTP-PEST also bind directly to paxillin.
β3 cytoplasmic domain are involved in binding to β3-endonexin, and in particular, the membrane-distal NITY motif of β3 (756-759) was critical for the interaction (Shattil et al., 1995, Eigenthaler et al., 1997). The NITY motif is present in β3 cytoplasmic domains but in β1 integrins, and this could explain the selective interaction of β3-endonexin with β3 integrins. β3-endonexin has been shown to increase PAC1 binding and result in fibrinogen-dependent aggregation of platelets, indicating that β3-endonexin modulates the affinity state of αIIbβ3 (Kashiwagi et al., 1997).

1.3.4 Lateral associations

Integrins are now well established to form stable complexes with proteins of the transmembrane 4 superfamily. α3 and α6 integrins have been shown to interact with CD151 tetraspannin, whereas α3,α6 and ανβ3 associate with CD9, CD63 and CD81 (Berditchevski et al., 1996). Tetraspanins have also been proposed to function as an adaptor for the association of tyrosine kinases (Skubitz et al., 1996), phosphotidylinositol 4-kinase (Berditchevski et al., 1997) and conventional protein kinase C (Zhang et al., 2001) and the observed modulatory effects of integrin-tetraspannin complexes on adhesion-dependent signalling may involve these associations. Integrins have also been shown to associate with caveolin-1, a protein found in glycospingolipid-cholesterol rich domains of the plasma membrane also known as rafts (Wary et al., 1998). This interaction is mediated by the transmembrane region of the integrin and it is likely to be important in linking integrins to the tyrosine kinase, Fyn, and the adaptor protein, Shc (Wary et al., 1998).

The extracellular domains of integrins are also known to form associations with other cell surface receptors. β1, β2 and β3 integrins have been shown to interact with the glycosyl phosphatidylinositol-anchored urokinase plasminogen activator receptor (uPAR) using immunoprecipitation, immunocolocalisation and resonance energy transfer (Xue et al., 1994, Xue et al., 1997 and Wei et al., 1996). A further study shows that uPAR specifically binds to α4β1, α6β1, α9β1, and ανβ3 integrins on chinese hamster ovary (CHO) cells in a cation-dependent manner, and that anti-integrin and anti-uPAR antibodies effectively block the association of uPAR and integrin (Tarui et al., 2001). It has been proposed that uPAR: integrin interactions activate signal transduction cascades
such as the Cas/Rac-dependent signalling that leads to actin remodelling and increased cell motility (Kjoller & Hall, 2001).

The extracellular domains of some integrins interact with integrin-associated protein (IAP). IAP contains five transmembrane domains and a large extracellular immunoglobulin domain that mediates the interaction with αvβ3 (Lindberg et al., 1993). It has been suggested that interactions between integrins and IAP can modulate cell motility, and α2β1 and IAP are found to regulate cell migration on collagen in the presence of thrombospondin which acts as a ligand for IAP (Wang & Frazier, 1998). It has also been reported that receptor tyrosine kinases can physically associate with integrins. For instance, the platelet derived growth factor receptor β (PDGFR-β) associates with αvβ3 integrin, (but not β1 integrins), via the β3 extracellular domain, and the association of the vascular endothelial growth factor receptor 2 (KDR/VEGFR2) with αvβ3 requires the α subunit (Borges et al., 2000). Additionally, integrins have been shown to bind to the insulin receptor (Vuori & Ruoslahti, 1994), and the EGF receptor forms a complex with β1 integrin after cells attach to fibronectin leading to ERK/MAP kinase induction.

1.4 INTEGRIN SIGNALLING

Integrins mediate bi directional signalling, whereby integrins can activate various signalling cascades in the cell (termed outside-in signalling), and integrins can themselves be activated by signalling cascades from within the cell (inside out signalling).

1.4.1 Outside–In signalling

Integrin outside-in signalling often activates the same pathways as do growth factor receptors. For instance, integrins can activate the Ras/Raf/MEK/ERK signalling axis, the stress activated p38 and JNK cascades, and the Rho subfamily of GTPases that regulate the actin cytoskeleton.

1.4.1.1 Focal adhesion Kinase (FAK)

FAK is a central player in integrin-mediated signalling and it is known to bind to and activate a number of proteins involved in migration, survival and cell growth. FAK is a 125 kDa tyrosine kinase consisting of an N-terminal domain, containing a putative
integrin binding domain, an ERM domain which binds to growth factor receptors such as the PDGFR, kinases such as Src, Etk and PI(3)K, the adaptor GRB7, and a central kinase domain. The targeting of FAK to focal adhesions is mediated by a 100 amino acid domain situated at the C-terminus, which is termed the focal adhesion-targeting domain (FAT), and this domain binds to Cas, Grb2, GRAF (a Rho GAP), in addition to the focal adhesion proteins talin and paxillin (See Fig. 1.4). Upon integrin engagement, FAK is autophosphorylated on Tyr 397 creating binding site for Src or Fyn (Schaller et al., 1994 and Schlaepfer et al., 1994). The interaction of Src with FAK results in phosphorylation of FAK at additional tyrosine phosphorylation sites and thereafter extensive tyrosine phosphorylation of numerous other focal adhesion components for instance paxillin and Cas.

1.4.1.2 Cas/CRK pathway

Cas is a docking protein that can recruit SH2 containing molecules such as CRK or NCK (Burnham et al., 1996 and Schlaepfer et al., 1997). The SH3 domain of Cas interacts with proline rich regions of FAK (Polte & Hanks, 1997) and becomes tyrosine phosphorylated and leads to the recruitment of CRK (Sattler et al., 1997). Additional kinases may then be recruited to this complex leading to further phosphorylation events (Abl) (Mayer et al., 1995), or phosphatases (PTP1B, PTP-PEST and SHP2) (Rock et al., 1997 and Garton et al., 1997) which would lead to attenuation of integrin signalling. The overexpression of FAK is found to stimulate migration in a Src/Fyn dependent manner (Cary et al., 1996) and the coupling of FAK to Cas/CRK pathways is necessary for cell migration (Klemke et al., 1998 and Cheresh et al., 1999). Furthermore, fibroblasts devoid of Cas have reduced motility (Honda et al., 1998) and overexpression of Cas-CRK enhances migration via mechanisms involving Rac and the CRK SH2 domain. It has been suggested that Cas-CRK exerts its effects on migration by the recruitment of Dock180 to the complex and the subsequent recruitment of Rac (see Fig. 1.5), in coordination with a parallel induction of the MAPK kinases ERK1/2 that act to regulate actin-myosin contraction and cellular contraction of collagen (Cheresh et al., 1999) (See Fig. 1.5).
**Fig. 1.4 FAK Effectors.** FAK is a 125 kDa tyrosine kinase consisting of an N-terminal domain, containing a putative integrin binding domain, an ERM domain which binds to growth factor receptors such as the PDGFR, kinases such as Src, Etk and PI(3)K, the adaptor GRB7, and a central kinase domain. The targeting of FAK to focal adhesions is mediated by a 100 amino acid domain situated at the C-terminus, which is termed the focal adhesion-targeting domain (FAT), and this domain binds to Cas, Grb2, GRAF (a Rho GAP), and the focal adhesion proteins talin and paxillin. Upon integrin engagement, FAK is autophosphorylated on Tyr 397 creating binding site for Src or fyn. The recruitment of FAK effectors has implications for cell survival, migration and growth.
Fig. 1.5 The role of Cas in integrin mediated signalling. Upon binding to the ECM a signalling complex comprising of FAK, Cas and Src family kinases assembles. CRK is recruited to this complex and the cells are stimulated to migrate probably from the subsequent recruitment of DOCK180 and Rac which results in actin rearrangement.
1.4.1.3 MAP kinase pathway

The engagement of integrins to the matrix also leads to activation of elements of the Mitogen Activated Protein Kinase cascade (MAPK). Upon integrin engagement Src is recruited to FAK and further phosphorylates FAK on several positions including Y925. This leads to the recruitment of the SH2 containing adaptor protein Grb2 and the membrane localization of the exchange factor Sos, which in turn promotes the GTP loading of Ras (Schlaepfer et al., 1994). Once active, Ras is able to bind to the kinase Raf and localise it to the membrane where Raf, then phosphorylates and activates the kinase MEK, which in turn can then phosphorylate the ERKs leading to activation of transcription factors and cell cycle progression (see Fig. 1.6).

There are several lines of evidence to suggest that integrin activation of the MAP kinase cascade can occur independently of FAK. Barberis et al. (2000) show that in cells where FAK is inhibited, either by cytochalasin treatment or in cells carrying a mutant β1 which is unable to bind FAK, that ERK can be activated by the adaptor protein Shc, which becomes tyrosine phosphorylated and associates with Grb2 upon matrix adhesion (Barberis et al., 2000). Studies in Swiss 3T3 fibroblasts show that Shc mediates the early phase, whereas FAK, Cas and CRK contribute to the late phase of ERK activation and these pathways work synergistically (Barberis et al., 2000). Additionally, integrin α chains have been shown to bind to caveolin-1 that acts as an adaptor to link the tyrosine kinase Fyn to integrins (Wary et al., 1998). Upon integrin engagement Fyn becomes activated and this allows the association of its SH3 domain to interact with the proline rich region in Shc. Shc then undergoes phosphorylation by Fyn at tyr317 and combines with Grb2 and Sos (Wary et al., 1996 and Wary et al., 1998) and ERK activation then occurs via Ras-Raf mediated events.

1.4.1.4 PI(3) Kinase/PKB signalling axis

Upon integrin engagement to the ECM, FAK becomes activated and can lead to the activation of PI(3)K, which results in the generation of the membrane restricted second messengers of polyphosphatidylinositides that contain a 3’ phosphate. The serine/threonine kinase Protein Kinase B (PKB/Akt) can be recruited to the membrane via an association of its PH domain with the PIP2 and PIP3 created by PI(3)K activity (Klippel et al., 1997). Two additional 3-phosphoinositide dependent kinases are involved
Fig. 1.6 MAPK kinase signalling. The engagement of integrins to the matrix leads to activation of elements of the Mitogen Activated Protein Kinase cascade (MAPK). Upon integrin engagement Src is recruited to FAK and further phosphorylation of FAK leads to the recruitment of the SH2 containing adaptor protein Grb2 and the membrane localization of the exchange factor Sos, which in turn promotes the GTP loading of Ras. Active, Ras is able to bind to the kinase Raf and localise it to the membrane where Raf, then phosphorylates and activates the kinase MEK, which in turn can then phosphorylate the ERKs. FAK activation can also recruit Cas/CRK and this can lead to activation of JNK. These events lead to the activation of transcription factors and to cell cycle progression.
in the full activation of PKB, and these are PDK1 and PDK2, which phosphorylate Thr 308 and Ser 473 respectively (Alessi et al., 1997). PKB/Akt has many effectors involved in cellular processes such as survival, proliferation, metabolism, migration and GLUT4 trafficking (see Fig.1.7).

1.4.1.5 Integrin linked kinase

Integrin Linked kinase (ILK) associates with the β1 and β3 integrin subunits (Hannigan et al., 1996) and can regulate integrin mediated signalling in response to ECM binding and growth factor treatments (Dedhar, 2000) and has also been proposed to be a component of the WNT pathway and the PKB signalling axis. ILK may act to phosphorylate PKB/Akt on serine 473 and, therefore, may directly contribute to PKB/Akt activation (Persad et al., 2001)(See Fig. 1.8), however, the role of ILK as a kinase is controversial. In *Drosophila*, the absence of ILK gives a wing blister phenotype indicative of the loss of integrin adhesion, but not to the loss of the fore-mentioned signalling pathways (Zervas et al., 2001). Additionally, mutations in the kinase domain of ILK result in aberrant phenotype in *Drosophila* (Zervas et al., 2001) suggesting a kinase independent function for ILK. As it has been shown to bind to the adaptor protein paxillin (Nikolopoulos & Turner 2001) and the LIM domain protein PINCH (Tu et al., 1999), ILK may act as an adaptor protein that could recruit other signalling proteins to integrin complexes. The binding of ILK to paxillin is found to be necessary for its focal adhesion targeting (Nikolopoulos & Turner 2001) and the inhibition of the PINCH-ILK interaction is found to reduce cell spreading and motility (Zhang et al., 2002) suggesting ILK adaptor functions are important. Other adaptor proteins such as NCK2 can bind to PINCH which links this complex to growth factor signalling (Tu et al., 1998). The SH3 domain of NCK2 has 75% homology to the closely related NCK-1 and this protein has been shown to bind to the Wiscott Alldrich syndrome protein (WASP) (Snapper & Rosen 1999). An interaction of PINCH with NCK-2 could act to recruit WASP and Arp2/3 localising actin polymerization to sites of ECM adhesion in the lamellipodia of migrating cells (see Fig. 1.8).
Fig. 1.7 Downstream effectors of PKB/Akt. Protein Kinase B (PKB/Akt) can be recruited to the membrane via an association of its PH domain with the PIP2 and PIP3 created by PI(3)K activity. Two additional 3-phosphoinositide dependent kinases are involved in the full activation of PKB/Akt, and these are PDK1 and PDK2, which phosphorylate Thr 308 and Ser 473 respectively (Alessi et al 1997). PKB/Akt has many effectors involved in cellular processes such as survival, proliferation, metabolism, migration and GLUT4 trafficking (Adapted from Downward 1998)
Fig. 1.8 Integrin Linked kinase (ILK). Integrin Linked kinase (ILK) associates with β1 and β3 integrins and may act to phosphorylate PKB/Akt on serine 473. ILK binds to the adaptor protein paxillin and the LIM domain protein PINCH. NCK2 can bind to PINCH which links this complex to growth factor signalling. An interaction of PINCH with NCK-2 could act to recruit WASP and Arp2/3 and result in actin re-organisation.
1.4.1.6 Syk pathway

The non-receptor tyrosine kinase Syk, which is essential for the development and function of several haemopoietic cells, becomes activated through tandem SH2 interactions with ITAM motifs in immune response receptors (Johnson et al., 1995), or via the binding of Syk to the four C-terminal residues of the β3 cytoplasmic tail (Woodside et al., 2001). Syk activates the guanine nucleotide exchange factor Vav1, and together Syk and Vav1 are able to trigger lamellipodia formation in fibrinogen-adherent cells. Both Syk and Vav1 have been co-localised with αIIbβ3 in lamellipodia, but not in focal adhesions (Miranti et al., 1998).

1.4.2 Inside-out signalling

In order to be able to engage the ECM an integrin must first be rendered competent to bind ligand. This can be achieved via signalling pathways from within the cell that operate to activate the integrin: a process termed inside out signalling. There are a number of ways in which this can be achieved: firstly, the integrin must be delivered appropriately to the plasma membrane, secondly, its affinity must be sufficient to bind monovalent ligand and thirdly, the integrin can be clustered thus increasing its avidity for multivalent ligands such as ECM proteins.

I have recently revealed that PDGF stimulates a Rab4-dependent recycling of αvβ3 integrin from early endosomes to the plasma membrane and that Rab4 is essential for spreading on vitronectin (Roberts et al., 2001 and chapter 3) indicating that recycling via this pathway may contribute to an inside-out activation mechanism for αvβ3 integrin.

1.4.2.1 Affinity modulation

The affinity state of integrins refers to the modulation of receptor affinity through conformational changes in the αβ heterodimers. Activation of platelets by external stimuli at the site of vascular injury induces inside out signalling to the αIIbβ3 integrin, resulting in conformational change and leading to an increased affinity for its ligand fibrinogen. Leisner et al., (1999) have demonstrated that binding of the ECM induces a conformational change in the transmembrane region of the integrin and exposes an epitope that is recognised by the anti ligand induced-binding site monoclonal antibody (LIBS). Site-directed mutagenesis of the identified LIBS epitope resulted in increased
ligand binding affinity of αIIbβ3, indicating that the extracellular ligand binding site and the LIBS epitope are functionally coupled (Leisner et al., 1999). The development of ligand mimetic antibodies that recognise activated forms of αIIbβ3 such as the PAC-1 antibody (Puzon-McLaughlin et al., 2000) and the anti-αvβ3 WOW-1 antibody, which is monovalent and therefore insensitive to integrin clustering have enabled precise examination of the affinity states of integrin (Pampori et al., 1999).

Several factors are reported to be involved in integrin activation and these include mechanotransduction and intracellular signalling pathways. For instance, in response to blood flow, Tzima et al. (2001) show that shear stress activates αvβ3 integrin in Bovine aortic endothelial cells and this is accompanied by an increase in ECM binding (Tzima et al., 2001). In this study, αvβ3-activation was as a result of affinity modulation as cells show an increased binding of WOW-1, whereas binding of a control anti-αvβ3 antibody was unchanged.

The Ras family of small GTPases and their effectors has been implicated in regulating integrin affinity. In CHO cells suppressors of integrin activation were identified and it was found that activated H-Ras and Raf-1 inhibit integrin activation (Hughes et al., 1997). R-Ras has high homology to H-Ras and was also was identified as an integrin affinity modulator, however, this protein was found to stimulate integrin ligand binding (Zhang et al., 1996). Consistent with this, microinjection of R-Ras into PC-12 cells resulted in extensive cell spreading and R-Ras has been shown to activate the αMβ2 integrin in a macrophage cell line via the small GTPase Rap-1 (Caron et al., 2000 and Self et al., 2001).

Several studies have identified a role for PI(3)K in the inside–out activation of integrins. Wortmannin, an inhibitor of PI(3)K is able to block the activation of αIIbβ3 integrin in platelets as measured by PAC-1 binding (Kovacsovics et al., 1995 and Zhang et al., 1996). It is possible that the effect of PI(3)K on integrin activation occurs via activation of downstream proteins which then subsequently facilitate heterodimer activation. The PI(3)K product PIP3 binds to certain PH domains leading to the recruitment of the PH domain containing proteins to the plasma membrane. Cytohesin-1 contains such a domain and interacts with the cytoplasmic tail of β2 integrin leading to the activation of αLβ2 (Kolanus et al., 1996). Another possibility is that PI(3)K activates integrins via another of its downstream target proteins, such as PKB/Akt. Indeed PKB/Akt has been shown to be involved in the inside-out activation of αvβ3, α5β1, αvβ5
and α2β1 integrins (Byzova et al., 2000) although the precise mechanism by which this occurs is still unknown.

The involvement of the receptor for activated protein kinase C (RACK1) in integrin activation is controversial. RACK1 consists of seven WD repeats with the and the C-terminal three repeats are responsible for its interaction with the membrane proximal region of β cytoplasmic tails (Liliental & Chang, 1998). RACK1 has been shown to bind to activated PKC and this promoted cell spreading and adhesion, suggesting that RACK1 links PKC to integrins and that this is necessary for integrin function (Liliental & Chang, 1998). Another study, however, suggests that RACK1 acts to bind, localize and stabilize PKC isoforms that are involved in downstream outside-in signalling pathways. In this study overexpression of RACK-1 did not affect ligand binding and inhibited cell migration and the overexpression of a RACK1 variant with alanine substitutions in the putative PKC binding site in its third WD domain, did not affect cell migration (Buensuceso et al., 2001).

1.4.2.2 Avidity modulation

The avidity state of integrins refers to the avidity of an integrin for a multivalent matrix ligand that may be increased by regulating the clustering of active heterodimers. The protease calpain has been reported to markedly facilitate the spreading of NIH-3T3 fibroblasts (Potter et al., 1998), and calpain is implicated in the clustering of αvβ3 integrin prior to its incorporation into focal complexes upstream of integrin-mediated Rac activation (Balikowska et al., 2000). The β1 and β3 integrin binding protein skelemin, co-localises with integrin in these calpain-induced clusters and cells transiently expressing skelemin C2 motifs, which contain the integrin binding site, fail to form integrin clusters or to spread on substrates for β1 and β3 integrins, indicating a role for this protein in integrin activation (Reddy et al., 2001). Additionally calpain is able to cleave the integrin- and actin-binding protein, talin into a 47 kDa globular head domain and a 190 kDa C-terminal rod (Yan et al., 2001). The talin head has a much higher affinity than the intact protein for the β3 cytoplasmic domain, and this association leads to integrin clustering and activation (Yan et al., 2001 and Calderwood et al., 1999). The proteolytic cleavage of talin may, therefore, explain the increase in integrin clustering and activation in response to this protease.
1.5 FOCAL ADHESION /COMPLEX ASSEMBLY

Rho proteins are members of the Ras superfamily of small GTP-binding proteins; the best characterised of these being RhoA, Rac and Cdc42. Like many small GTPases, Rho family members cycle between a cytoplasmic GDP bound, inactive state and a membrane associated GTP bound active form and the transition between the states is controlled by interacting proteins such as nucleotide exchange factors (GEFS), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Rho GTPases are activated by growth factor and hormone stimulation (i.e. PDGF/EGF and insulin can activate Rac (Nobes et al., 1995)) by increasing guanine nucleotide exchange and inhibiting the GTPase activity (Hawkins et al., 1995). Integrin engagement can also activate Rho subfamily members (i.e. the engagement of integrin with fibronectin activates Cdc42 and Rac and subsequently PAK (Price et al., 1998) and the activation of Rac1 by integrin-mediated adhesion may occur through the tyrosine phosphorylation of the exchange factor Vav (Yron et al., 1999).

Rho family members have large numbers of effectors (reviewed in Bishop & Hall, 2000) and it is through these proteins they are able to exert their effects on both focal adhesion/complex assembly and actin cytoskeleton remodelling.

1.5.1 Rho induced focal adhesion assembly and actin organisation

Rho is able to drive the formation of large focal adhesions by activating a Rho kinase (ROCK), which is able to phosphorylate the myosin-binding subunit of myosin light chain phosphatase leading to its inhibition (Kawano et al., 1999). This causes an accumulation of phosphorylated MLC, which allows the actomyosin complex to exert tension and consequently form stress fibres, thereby concentrating proteins into focal adhesions. Rho also activates mDia1, whose activity induces the formation of thin stress fibres, which are disorganised in the absence of ROCK (Watanabe et al., 1999). Furthermore, mDia1 has been shown to transform ROCK-induced condensed stress fibres into those reminiscent of Rho- induced stress fibres, therefore, mDia and ROCK work in concert to induce stress fibres of various thickness and density (Watanabe et al., 1999). Additionally, Rho binds to and activates PIP2, a lipid messenger strongly implicated regulation of actin dynamics and focal adhesion assembly. For instance, PIP2 binds to vinculin enabling it to interact with talin and actin (Gilmore & Burridge, 1996) and PIP2 is also involved in
the release of actin monomers from profilin and gelsolin complexes and this enhances actin polymerisation (Lassing & Lindberg, 1985 and Janmey et al., 1987) (see Fig. 1.9).

1.5.2 Rac and Cdc42 induced focal complex assembly and actin reorganisation

Rac and Cdc42 stimulate the assembly of integrin containing focal complexes. Less is known about how Rac and Cdc42 exert their effects on focal complex assembly although some of the mechanisms whereby they regulate actin polymerisation are now becoming clear. Rac activates a PIP4(5) kinase which catalyses PIP2 production enabling the removal capping proteins from the barbed ends of actin filaments to facilitate actin polymerisation (Tolias et al., 2000). Rac and Cdc42 are also able to influence actin reorganisation via activation of the downstream effector p21-activated serine/threonine kinase (PAK). Several PAK family members have been identified and these proteins possess an N-terminal regulatory domain, which contains the GTPase binding domain also known as CRIB (Cdc42 and Rac interacting domain), and a C-terminal kinase domain. PAK influences actin polymerisation via phosphorylation and activation of LIM kinase, which in turn phosphorylates and inactivates the actin depolymerising proteins ADF/cofilin (Dan et al., 2001). Activated PAK also phosphorylates the myosin light chain kinase (MLCK) resulting in its inhibition (Sanders et al., 1999). Additionally Rac activates the arp2/3 complex and this can occur via IRSp53, which then interacts with WAVE/SCAR (Miki et al., 2000), or via WASP (Rohatgi et al., 2000 and Higgs & Pollard, 2000). The arp2/3 complex promotes actin nucleation that enables filament growth at barbed ends, and additionally facilitates filament branching (Condeelis, 2001) (see Fig. 1.10).

1.5.3 ARFs and Focal complex formation

Although the Rho family GTPases are primarily responsible for the formation of focal adhesions and complexes, there is evidence to suggest that they act in concert with ADP ribosylation factors (ARF) GTPases to form paxillin rich focal adhesions. ARF1 has been shown to regulate the recruitment of paxillin from a perinuclear compartment to focal adhesions independently of vinculin recruitment and the Rho-dependent assembly of actin stress fibres (Norman et al., 1998). Paxillin associated ARF-GAP protein (PAG1), which acts as an ARF1-GAP (Premont et al., 2000), has been shown to be involved in the intracellular dynamics of paxillin, the organisation of the golgi and the
Rho kinase

MLCK

MLCP

MLC

PI(4,5)K

PIP

PIP2

GTP

LPA

mDia

Actin stress fibres

Rho

Actomyosin Contraction

Stress fibre and Focal adhesion formation

Release of monomers from Gelsolin/ profilin complexes

Actin polymerisation

Fig. 1.9 Rho induced focal adhesion assembly. Rho activates a Rho kinase (ROCK), which is able to phosphorylate the myosin-binding subunit of myosin light chain phosphatase. This causes an accumulation of phosphorylated MLC, which results in actomyosin contraction. Rho also activates mDia1, whose activity also induces the formation of thin stress fibres. Additionally, Rho binds to and activates PI(4,5)K leading to the production of PIP2, which can interact with vinculin resulting in an open conformation enabling it to bind to talin and actin. dynamics and focal adhesion assembly. PIP2 is also involved in the release of actin monomers from profilin and gelsolin complexes and this enhances actin polymerisation. Abbreviations: MLCK-myosin light chain kinase, MLCP-myosin light chain phosphatase.
Fig. 1.10 Rac and Cdc42 and actin reorganisation. Rac activates a PIP4(5) kinase which catalyses PIP2 production enabling the removal capping proteins from the barbed ends of actin filaments. Rac and Cdc42 activate PAK which phosphorylates and activates LIM kinase, which in turn phosphorylates and inactivates the actin depolymerising proteins ADF/cofilin. Activated PAK also phosphorylated the myosin light chain kinase (MLCK) resulting in its inhibition. Additionally Rac activates the arp2/3 complex and this can occur via IRSp53, which then interacts with WAVE/SCAR, or via WASP. The arp2/3 complex promotes actin nucleation that enables filament growth at barbed ends, and additionally facilitates branching.
formation of stress fibres (Mazaki et al., 2001). Overexpression of a shortened isoform of PAG1 (Git2 short) causes a loss of paxillin from focal complexes and stimulates cell motility (Mazaki et al., 2001). In addition to this it is now been shown that the overexpression ARNO, which is most likely to be a GEF for ARF1 and not ARF6 (Macia et al., 2001), strongly promotes lamellar ruffles and focal complex assembly (Santy & Casanova, 2001). Furthermore, the ARF6 GAP protein, PAG3, which binds directly to paxillin, and is found (like PAG1) to associate with paxillin in the cytoplasm and in regions at the cell periphery is also involved in paxillin recruitment to focal complexes (Kondo et al., 2000).

1.5.4 Focal adhesion turnover

Adhesion disassembly is observed at both the cell rear, where it is involved in tail retraction during cell migration, and at the cell front, where it accompanies the formation of new protrusions and this is referred to as turnover. The molecular mechanisms that govern focal complex turnover are not yet fully understood, however, many proteins have been implicated and these include both tyrosine kinases and tyrosine phosphatases. Fibroblasts from both FAK and Src knockout mice exhibited reduced spreading and motility and moreover, cells lacking FAK have an increase in number and size of peripheral focal adhesions, as do cells overexpressing kinase defective forms of Src (Ilic et al., 1995, Klinghoffer et al., 1999 and Fincham & Frame, 1998). Furthermore, the catalytic activity of Src at cell-matrix attachment sites is was found to be necessary for remodelling of RhoA-dependent focal adhesions into smaller focal complexes associated with Rac1-induced lamellodopodia (or Cdc42-induced filopodia) (Timpson et al., 2001). Fibroblasts deficient in the phosphatases SHP-2 and PTP-PEST also exhibit reduced spreading and motility and have increased numbers of focal adhesions (Yu et al., 1998, Angers-Loustau et al., 1999), suggesting that the balance of phosphotyrosine in focal adhesions is critical for focal adhesion dynamics.

1.6 CELL MIGRATION

1.6.1 Actin cytoskeleton

Cell migration is a complex cellular behaviour that involves protrusion and adhesion at the cell front, and contraction and detachment at the rear. In motile cells, Cdc42 produces a polarization signal defining the leading edge of the cell and this is
followed by Rac-induced lamellopodia and focal complex formation. Cdc42 stimulates actin polymerization via its interaction with the ARP2/3 complex (Ma et al., 1998), or by its interaction with PIX, which subsequently activates Rac (Obermeier et al., 1998). Rac can then bind to and activate PAK, which in turn activates LIM kinase1 (Dan et al., 2001), which can inhibit ADP/cofilin-induced actin depolymerisation at the leading edge as I have previously discussed. The actin cytoskeleton at the leading edge is thought to be a loose, low-tension scaffold and this is achieved via several mechanisms. Engagement of integrin to the ECM initially inactivates RhoA in a Src-dependent fashion via the tyrosine phosphorylation of a p190RhoGAP (Arthur et al., 2000), and this could limit acto-myosin contraction. Furthermore, Rac activation of PAK also results in inactivation of MLCK, thereby inhibiting MLC phosphorylation and the formation of acto-myosin bundles. In addition to driving actin polymerisation, a recent study has shown that Rac can selectively recruit high affinity active αvβ3 integrin to the leading edge of cells, and this may promote the formation of focal complexes in this region (Kiosses et al., 2001).

As the cell moves forward, the release of inhibition of RhoA enables the low-density integrin containing focal complexes at the lateral edge of the lamellopodia to transform into high-density integrin containing focal adhesions, and the acto-myosin contractile machinery drives this (Ballestrem et al., 2001) (see Fig. 1.11). Release of adhesions and retraction at the cell rear completes the cell migration cycle allowing net translocation of the cell in the forward direction.

1.6.2 Membrane dynamics

Vesicular trafficking events are thought to be important in cell migration. To enable the advancement of the cell, new material is required at the leading edge for adhesive structures to form. It is thought that this new material is, in part, membrane recycled from the back of the cell and that focal adhesions can be formed by integrins recycling from the rear of the cell to the leading edge (Bretscher, 1996) (see Fig. 1.12). Cell migration proceeds in cells even when protein synthesis has been inhibited (Bretscher, 1996b), indicating re-use of integrins by exocytosis rather than denovo synthesis. Further evidence to support this comes from the work of Hopkins et al. (1994) who showed using fluorescent labelled transferrin that exocytosis of recycling membrane can occur at the tips of lamellopodia in chick fibroblasts (Hopkins et al., 1994).
Fig. 1.11 Model of cell adhesion and actin cytoskeleton during cell migration. Cell migration is driven by Rac1 and Cdc42 dependent actin polymerisation in the leading lamellipodium. ανβ3 is incorporated in the lamellipodium in low density focal complexes and these adhesion remain stationary with respect to the substratum. As the cell moves forward low density focal complexes transform into high density focal adhesions at the lateral edges of the lamellipodium directed by the acto-myosin contraction as lamellipodia actin filament are transformed into stress fibres.
Fig. 1.12 Integrin trafficking and the formation of focal adhesions. It is thought that the new material at the leading edge is, in part, membrane recycled from the back of the cell and that focal adhesions can be formed by integrins undergoing endocytosis at the cell rear followed by directional recycling to the leading edge.
1.7 ENDOCYTOSIS

In mammalian cells, cell surface receptors can undergo endocytosis via clathrin coated pits, caveolae, and other non-clathrin dependent pathways. Clathrin-coated pits are the most extensively studied route of internalisation. Briefly, a clathrin lattice builds upon the cytofacial membrane, whilst AP2 adaptors of the adaptin complex (comprising of 100 kDa α and β2 adaptins together with smaller μ2(50 kDa) and σ2 (17 kDa) subunits) and arrestin bind to their selected receptors, with the β subunits of AP2 contacting the clathrin N-terminal domains (Shih et al., 1995). Amphiphysin recruits the GTPase dynamin, which then spontaneously self assembles into supramolecular structures consisting of single rings and spirals (Hinshaw & Schmid, 1995). The GTPase activity of dynamin is activated by an intramolecular GAP encoded within its own effector domain (aa 658-750). This becomes activated upon self assembly (Muhlberg et al., 1997 and Sever et al., 1999) and is needed to constrict invaginated coated pits and this enables them to pinch off and form coated vesicles (Sever et al., 2000). Surface receptors can also be internalised by caveolae, which are flask shaped, non-coated plasma membrane invaginations characterised by their association with caveolins that function to maintain these structures. Several membrane receptors including integrins and signalling molecules have been localised to caveolae and this has led to the hypothesis that caveolae are centers for signalling activity (reviewed in Simons & Toomre, 2000). Some studies have also revealed an additional method of receptor internalisation, termed “non clathrin dependent pathways,” but this is poorly characterised (Lamaze et al., 2001).

The first structures labelled by incoming cargo are the early endosomes. Early endosomes are sorting organelles wherein material entering the compartment is appropriately directed to other parts of the intracellular trafficking pathway (see Fig. 1.13). Early endosomes are made up of a network of tubules and vesicles that are dispersed throughout the cytoplasm. The early endosome has an acidic pH (pH6.0) (Yamashiro & Maxfield, 1987), which is thought to be involved in the dissociation of pH sensitive ligand/receptors complexes, and once receptors are free of ligand they can be directed back to the plasma membrane and this is termed ‘recycling’. Studies on the transferrin receptor have shown that it has two routes of recycling; one fast recycling loop where it returns to the plasma membrane directly from the early endosome, and an indirect route where it first passes through an additional compartment situated in the perinuclear region (Daro et al., 1996 and Sheff et al., 1999). These studies led to the
Fig. 1.13 Vesicular trafficking pathways and intracellular compartments. Clathrin coated pits from the cell surface pinch off to form clathrin coated vesicles. These cargo-carrying vesicles fuse with the early endosome. From this compartment, cargo can be recycled directly back to the plasma membrane or directed to the late endosome (or multivesicular body) for degradation in the lysosomes. Alternatively, cargo can be trafficked via the peri-nuclear recycling compartment back to the plasma membrane. Bi-directional transport between the TGN and early endosome has also been proposed.
definition of the 'recycling compartment', which is situated close to the microtubuleorganising center and this has a less acidic luminal pH (pH 6.5) than the early endosome (Gagescu et al., 2000). Cargo within the early endosome can also be directed to late endosomes (sometimes referred to as multi-vesicular bodies by virtue of their internal membrane structure) from which it can be targeted to lysosomes for degradation. The endosomal system can also send and receive cargo from the transgolgi network (TGN) and this is how some things are proposed to reach lysosomes without being exposed at the plasma membrane (Traub & Kornfield, 1997).

Some cells have adaptations of the endocytic pathway that allow them to perform specialised physiological functions. For instance, in neurons and neuroendocrine cells synaptic vesicles can bud from early endosomes. These vesicles lack typical constitutive recycling receptors such as the transferrin receptor, and undergo regulated exocytosis and fusion with the pre-synaptic membrane (Schmidt et al., 1997). Additionally, the GLUT4 transporter is found in a distinct endosome vesicle population in adipocytes and muscle cells, and these are targeted to the plasma membrane in response to insulin (Hashiramoto & James, 2000 and Ramm et al., 2000). Another example of an adaptation of the endocytic pathway is found in melanocytes. These contain endo/exocytic organelles known as melanosomes, and these are responsible for the transportation of the pigment melanin to the cell surface (Marks & Seabra, 2001).

1.7.1 Rabs in endo/exocytic cycling

The Rab family of GTPases control endo/exocytic pathways and over 40 family members have been identified in mammalian cells. Several Rab proteins are associated with endocytosis and recycling and these are shown in Fig. 1.14. Initially Rabs are recruited to donor membranes where they are important in vesicle budding. They then act to facilitate vesicular movement via interactions with motor proteins, and finally participate in docking and fusion. This functional diversity is thought to be achieved by recruitment of a number of Rab effectors which help to regulate protein:protein interactions and fulfill tethering functions, although the precise mechanisms of how Rab effectors function is unclear. It has been suggested that Rabs and their effectors may regulate membrane fusion by activating specific SNARE complexes, which are necessary for vesicle docking and fusion (Lian et al., 1994 and Lupashin & Waters, 1997). For example, Rab4 has been shown to interact with syntaxin4 in a direct and specific manner,
<table>
<thead>
<tr>
<th>Rab</th>
<th>Intracellular location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab4</td>
<td>Early and recycling endosomes</td>
<td>Endocytic recycling to plasma membrane</td>
</tr>
<tr>
<td>Rab5</td>
<td>Clathrin coated vesicles and early endosomes</td>
<td>Endocytic internalisation and early endosome fusion</td>
</tr>
<tr>
<td>Rab7</td>
<td>Late endosomes</td>
<td>Transport from early to late endosomes</td>
</tr>
<tr>
<td>Rab9</td>
<td>Late endosomes</td>
<td>Transport from late endosomes to trans-Golgi</td>
</tr>
<tr>
<td>Rab11</td>
<td>Recycling endosomes and TGN</td>
<td>Apical and basolateral endocytic recycling, export from TGN</td>
</tr>
<tr>
<td>Rab15</td>
<td>Early and recycling endosomes</td>
<td>Inhibitor of endocytic internalisation</td>
</tr>
<tr>
<td>Rab17</td>
<td>Epithelial cell specific, apical recycling endosome</td>
<td>Transport through apical recycling endosome</td>
</tr>
<tr>
<td>Rab18</td>
<td>Epithelial cell specific kidney dense apical tubules and basolateral domain of intestine</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td>Rab20</td>
<td>Epithelial cell specific, kidney dense apical tubules</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td>Rab22</td>
<td>Endosomes and plasma membrane</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td>Rab24</td>
<td>Endoplasmic reticulum, golgi and late endosomes</td>
<td>Uncharacterised</td>
</tr>
<tr>
<td>Rab25</td>
<td>Epithelial cell specific, apical recycling endosome</td>
<td>Transport through apical recycling endosomes</td>
</tr>
<tr>
<td>Rab27</td>
<td>Melanosomes</td>
<td>Transport of melanosomes to plasma membrane</td>
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**Fig. 1.14 Endosomal Rab proteins**—adapted from and references within Rodman & Wandinger-Ness 2000, see text for additional references.
and the interaction is regulated by the guanine nucleotide status of Rab4 as well as by the conformational status of syntaxin 4 (Li et al., 2001).

Rab proteins are subject to regulation by accessory proteins on several different levels. They are post translationally modified by the addition of a geranyl-geranyl moiety to two C-terminal cysteines (Casey & Seabra, 1996), catalysed by a heterodimeric enzyme Rab geranylgeranyl transferase (GGTase). Rab escort protein (REP) binds to mono, di- and unprenylated Rab proteins (Shen & Seabra, 1996) ensuring that proteins remain bound to REP until both cysteines are modified. Two REPs have been identified, REP1 and REP2 and defects in REP1 lead to an X-linked condition called chorioderemia, which presents retinal degeneration where Rab27 is unprenylated in the retinal pigment epithelium (Seabra et al., 1995). Inhibition of Rab GGTases by analogues of biphosphonate drugs causes drastic alteration in osteoclast morphology involving the formation of large intracellular vacuoles consistent with a disruption of membrane trafficking (Coxon et al., 2001).

Rab proteins are tightly controlled by accessory proteins, which regulate Rab nucleotide binding and hydrolysis, and membrane association. Switching of all GTPases from the GDP bound form to the GTP bound form is mediated by nucleotide exchange activity stimulated by GEFs. To date, two Rab GEFS have been identified and these are Rabex 5 that acts on Rab5 (Horiuchi et al., 1997) and Rab3 GEP, which acts a Rab3 GEF (Wada et al., 1997). Cycling back to the GDP bound form by endogenous GTP hydrolysis is increased by GAPs, and RN-Tre has been identified as an example of a Rab5 GAP (Lanzetti et al., 2000). Two other factors GDI (GDP-dissociation factor inhibitor) and GDF (GDI-displacement factor) regulate the cycling of Rab between membranes and also affect Rab nucleotide cycling. GDI can act to remove GDP-bound Rab from membranes and recycle them to their donor compartment, and GDF recruits Rab to specific donor membranes (see Fig. 1.15). GDIs are well characterised (see Luan et al., 1999), yet less is known about Rab GDFs, however, Rabip5 is a candidate GDF and this binds to GDP-Rab5 (Hoffenberg et al., 2000).

1.7.1.1 Rab5

Several isoforms of Rab 5 have been identified (Bucci et al., 1995) and these are Rab5a, Rab5b and Rab5c, and of these Rab5a is the best characterised. The first step of internalisation is a sequestration of ligands into clathrin-coated vesicles, and Rab 5 found
Fig. 1.15 The Rab cycle. The Rab cycle is based on a model where nucleotide exchange activity stimulated by GEFs results in the membrane association of Rab-GTP proteins on donor compartments enabling budding of transport vesicles. Rab GTP carrying vesicles move to the acceptor compartment where docking is mediated by Rab, Rab effector and SNARE proteins. Two other factors GDI (GDP-dissociation factor inhibitor) and GDF (GDI-displacement factor) regulate the cycling of rab between membranes and also affect rab nucleotide cycling. GDI extracts GDP-bound Rab from membranes to recycle them to their donor compartment, keeping Rab in the inactive GDP-bound state, and GDF recruits Rab to specific donor membrane.
to be important for both sequestration and the subsequent fusion of vesicles with early endosomes (see Mills et al., 1999 and Woodman, 2000 for reviews). Studies using dominant negative mutants of Rab5 were found to inhibit early endosomal fusion (Stenmark et al., 1994), whereas overexpression of wild type Rab5a and a GTP hydrolysis mutant Rab5aQ79L, gave rise to enlarged early endosomes indicating that Rab5a is involved in the fusion of clathrin-coated vesicles with early endosomes (Stenmark et al., 1994).

Rab5 mediates endosome fusion via interaction with several effector proteins and the identification of over 20 polypeptides that can bind to active Rab5 has indicated that endosomal fusion is a complex reaction (Christoforidis et al., 2000). Rab5-GTP is unstable on the early endosomal membrane as a result of the GAP activity of RN-Tre. The recruitment of the Radaptin5/ Rabex5 complex to the membrane, acts to reduce the intrinsic GTPase activity of Rab5 and stimulate nucleotide exchange, thus ensuring a stable active form of Rab5 is maintained on the vesicle membrane (Lippe et al., 2001). The membrane localised active Rab5 then brings in hVPS34-p150 PI(3)K that acts to make PI(3)P. The concomitant presence of Rab5 and PI(3)P promotes recruitment of early endosomal antigen 1 (EEA1) via association of its zinc finger FYVE domain to PI(3)P (Lawe et al., 2000). EEA1 has been shown to interact directly with the t-SNARE syntaxin 13 and this interaction then initiates fusion (McBride et al., 1999).

An additional Rab5 effector has been identified, Rabenosyn5, which like EEA1 contains a FYVE finger domain, is recruited to PI(3)P regions of the early membrane, and has been shown to interact with a sec1 like protein hVPS45 (Nielsen et al., 2000). Rabenosyn5 and EEA1 are both required for endosomal fusion but over-expression of Rabenosyn5 has been found to inhibit cathespin D processing, suggesting that Rabenosyn5 may function in the trafficking of newly synthesized protein through the early endosomal system (Nielson et al., 2000).

1.7.1.2 Rab4

Rab proteins are also associated with exocytosis and recycling of cell surface proteins (Rodman & Wandinger-Ness 2000), and Rab4 in particular is associated with receptor recycling (Daro et al., 1996 and van der Sluijs et al., 1992). Two isoforms of Rab4 have been cloned and named Rab4a and Rab4b, with Rab4a being the most extensively studied. Rab4a is primarily located to microdomains on early endosomes,
distinct from those containing Rab5, and to a lesser extent to the perinuclear recycling compartment (van der Sluijs et al., 1992 and Sonnichsen et al., 2000). Studies to investigate the function of Rab4 in the endocytic pathway have shown it is involved in transferrin receptor (TFN-R) recycling. Cells overexpressing Rab4 exhibit normal kinetics of TFN-R endocytosis but display an accumulation of TFN-R on the cell surface (van der Sluijs et al., 1992), indicating that this receptor is targeted directly back to the plasma membrane from the early endosome.

In addition to the direct recycling from early endosomes to the plasma membrane, Rab4 has also been implicated in regulating a transport step from early endosomes to the perinuclear recycling compartment. The expression of dominant negative Rab4, N121IRab4, which does not bind GTP (Cormont et al., 1996), redistributed transferrin to a population of vesicles which were derived from early endosomes, but prevented it from reaching the perinuclear recycling compartment (van der Sluijs et al., 1992). Furthermore, studies using the GTP hydrolysis deficient Q67LRab4, which is thought to be stabilised in its association with the target membrane, has been found to be associated on the perinuclear recycling compartment (Nagelkerken et al., 2000) indicative of this being a target organelle for Rab4.

Additional studies have implied that Rab4 may also regulate exit from the early endosome to a degradative pathway. The dominant negative Rab4 mutant, S22NRab4, has been shown to reduce EGF and LDL receptor degradation suggesting that Rab4 does indeed have a sorting function in the early endosome (McCaffrey et al., 2001). Rab4 has been shown to regulate the recycling of other cell surface receptors. Rab4 is involved in GLUT4 recycling in response to insulin and is found associated with GLUT4 containing vesicles (Cormont et al., 1996, Vollenweider et al., 1997 and Cormont et al., 2001), and the recycling of the β2-adrenergic receptor has also been shown to be recycled via a Rab4 dependent mechanism (Seachrist et al., 2000).

To date, two Rab4 effectors have been identified and these are Rabaptin4 that exhibits sequence homology to Rabaptin5, and Rabip4. Rabaptin4 has been shown to bind to Rab4-GTP by both yeast two hybrid screening and co-immunoprecipitation (Nagelkerken et al., 2000). Rabaptin4 is primarily a cytosolic protein, however, when cells are co-transfected with Rabaptin4 and the GTP active Rab4Q67L, Rabaptin4 redistributes to the perinuclear recycling compartment suggesting a functional interaction of Rabaptin4 and Rab4GTP (Nagelkerken et al., 2000). Furthermore, Rabaptin4 was
found to reduce the intrinsic GTPase activity of Rab4 thus it is suggested that Radaptin 4 may act to stabilise Rab4-GTP on its target compartment (Nagelkerken et al., 2000).

Rabip4 is a Rab4GTP effector that possesses a RUN domain, which defines its subcellular localization, two coiled-coil domains, a Rab4-binding and a FYVE finger domain, allowing the recruitment of the protein to PI(3)P on the early endosome (Mari et al., 2000 and Cormont et al., 2001). Rabip4 co-localises with EEA1 (with which it has 40% homology), and with the actin cytoskeleton, but is not co-localised with Rab11 (Mari et al., 2001). Studies have shown that co-expression of Rabip4 and active Rab4 lead to an enlargement of early endosomes, whereas, the expression of a Rabip4 mutant, which lacks the Rab4 binding domain, or co-expression of Rabip4 with dominant negative Rab4 (N121IRab4) had no effect on endosome size (Cormont et al., 2001). Furthermore, these workers found that the expression of Rabip4 resulted in the intracellular retention of the glucose transporter Glut 1 (Cormont et al., 2001). This suggests that Rabip4/Rab4 interactions have an inhibitory function in the endocytic pathway, although it is unclear if this is due to retention of cargo in the early endosome, or from the stimulation of a "backward" transport step from the recycling compartment to early endosomes (Cormont et al., 2001).

During mitosis, Rab4 is subject to additional regulation via phosphorylation. The carboxy terminus of Rab4a, but not Rab4b, contains a consensus sequence for phosphorylation by p34cdc kinase (van der Sluijs et al., 1992). This phosphorylation prevents the association of Rab4 with endosomes and results in the accumulation of in the cytoplasm (Ayad et al., 1997). Membrane transport is inhibited from both the biosynthetic and endocytic pathways during mitosis and it is possible that mitotic phosphorylation of Rab4 may form part of the mechanism whereby endosomal trafficking during is shut off during cell division.

1.7.1.3 Rab11

In addition to Rab5 and Rab4, a third Rab protein, Rab 11, is associated with endocytic organelles. Rab 11 has been localised to both the trans-golgi network and the perinuclear recycling endosome and it is implicated in trafficking from both of these compartments (Chen et al., 1998 and Ren et al., 1998). Rab11 is concentrated on the perinuclear recycling compartment where it co-localises with internalised transferrin (Ullrich et al., 1996 and Ren et al., 1998) and mediates recycling of the transferrin
receptor from the perinuclear recycling endosome to the plasma membrane (Ullrich et al., 1996 and Chen et al., 1998). Dominant negative Rab11 (Rab11S25N) has been found to inhibit transferrin recycling from the perinuclear recycling compartment, and causes fragmentation of this compartment into the cytoplasm (Ullrich et al., 1996). Ren et al, (1998) who used a low temperature method to inhibit transport between the early endosome and the recycling compartment, have been able to show that Rab11S25N inhibits transferrin recycling, whereas Rab11Q70L and wild type Rab 11 did not, also suggesting that Rab11GTP is required for transferrin receptor recycling (Ren et al., 1998).

As discussed previously, Rab proteins are able to exert their effects on endocytic trafficking via interactions with specific Rab effector proteins and to date, four Rab11 effectors have been identified. These are Rabphilin11, Rip11, Rab11-Family Interacting Protein (Rab11 FIPs) and myosinV. The first Rab11 effector identified was Rabphilin11, which was isolated from rat and bovine brain and found to interact with Rab11aGTP (Mammoto et al., 1999). Both GTP-Rab11 and Rabphilin-11 were co-localised to the perinuclear recycling compartment in MDCK cells, and along microtubules, oriented towards membrane lamellopodia in Hela cells plated on fibronectin (Mammoto et al., 1999). Overexpression of the C-terminal fragment of Rabphilin-11 (aa 607-730), which lacks the GTP-Rab11 binding domain, was found to reduce accumulation of transferrin in perinuclear recycling endosomes and inhibited cell migration (Mammoto et al., 1999), suggestive of the Rab11GTP and Rabphilin11 interaction being necessary for the trafficking of transferrin and perhaps receptors involved in cell migration such as integrins.

Rip11 is a novel Rab11 effector and is found enriched in polarized epithelial cells where, like Rab11, it is localized to apical recycling endosomes (ARE) and the apical plasma membrane (Prekeris et al., 2000). Rip11 is recruited to ARE by binding to Rab11 as well as through a Mg$^{2+}$-dependent interaction of its C2 domain with neutral phospholipids and via regulation of a phosphorylation and dephosphorylation cycle (Prekeris et al., 2000). It has been suggested that the association of Rip11 with Rab11 GTP on apical recycling vesicles facilitates their targeting to the apical membrane.

Other Rab 11 effectors have been identified and these include a family of three Rab11-interacting proteins: Rab11-Family Interacting Protein 1 (Rab11-FIP1), Rab11-Family Interacting Protein 2 (Rab11-FIP2) and Rab11-Family Interacting Protein 3 (Rab11-FIP3). All four of these interacting proteins associate with Rab11 and to the closely related Rab 25, and Rab11-FIP2 also interacts with dominant negative Rab11a
Rabl laS25N) and the tail of myosin Vb (Hales et al., 2001). The binding of Rab11-FIPs to Rab 11 is dependent upon a conserved carboxyl-terminal amphipathic alpha helix. Rab11-FIP1, Rab11-FIP2, and Rip11 were found to co-localise with Rab11a in plasma membrane recycling systems in both non-polarized HeLa cells and polarized Madin-Darby canine kidney cells, and GFP-Rab11-FIP3 also co-localised with Rab11a in HeLa cells (Hales et al., 2001). It is as yet unknown how these effectors contribute to Rab11 mediated recycling events. Interestingly, Rab11a has also been shown to bind to the tail of myosinVb by yeast 2 hybrid, and Rab11a and myosinVb co-localise by immunofluorescence on vesicles containing the transferrin receptor (Lapierre et al., 2001). This suggests that Rab11a may play a role in docking the motor protein to vesicles suggestive of a mechanism by which vesicles could move along actin tracks.

1.7.1.4 Rab 27

The most convincing evidence for the involvement of motor protein/Rab protein interactions in vesicle movement comes from work on the myosinVa and Rab27 interactions. Several papers indicate a role for Rab27-myosinVa interactions in organelle movement in pigment cells of mice and humans (Menasche et al., 2000, Wilson et al., 2000, Bahadoran et al., 2001 and Hume et al., 2001). In mammals, the pigment melanin is produced in melanocytes and deposited in the skin and hair by exocytosis and then is endocytosed by adjacent cells. Pigment mutations have been extensively studied in mice and have led to the identification of several genes responsible for pigmentation. Genes identified include 'dilute' which was found to code for the myosinVa protein (Mercer et al., 1991) and the ashen gene which codes for Rab27 (Wilson et al., 2000). In humans Griscelli syndrome, an autosomal recessive disorder that causes pigment defects and neurological disorders in humans, has also been mapped to the Rab27 gene (Menasche et al., 2000). It is suggested that Rab27a works as part of the pigment transportation machinery that uses myosinV as a motor. Evidence to support this comes from work where transfection of dominant negative Rab27 resulted in the perinuclear accumulation of melanosomes similar to that seen in the ashen and dilute phenotypes (Wilson et al., 2000, Hume et al., 2001 and Wu et al., 2001). Additionally, transfection of wild-type Rab 27 into cells cultured from a patient with Giscelli syndrome, or into ashen mouse model melanocytes restored the wild-type distribution of the melanosomes to the cell periphery (Bahadoran et al., 2001 and Wu et al., 2001). Indeed, in wild-type
melanocytes, Rab27 and myosinV are localised to the cytoplasmic face of melanosomes, yet in ashen melanocytes, myosinV fails to associate with melanosomes (Hume et al., 2001 Wu et al., 2001). This indicates that Rab27 is actually needed to localise myosinV to the melanosome. In support of this, an interaction of myosinV and Rab 27 by immunoprecipitation has been reported (Hume et al., 2001) and may indicate that Rab 27 provides the binding site on the melanosome for the motor protein to dock. The Rab27/myosinV interaction, and the possible interactions of other effectors, may thus allow the transport of the organelle to the cell surface.

1.7.2 The ADP-Ribosylation factors (ARFs)

In addition to Rab GTPases it is well documented that ARFs regulate endosomal budding and fusion. The ARFs can be subdivided into three classes with ARF1, ARF2 and ARF3 belonging to Class1, ARF4 and 5 are class II ARFs, and ARF5 and ARF 6 are class III ARFS. ARFs are post translationally modified by myristoylation at their N terminus and this modification is essential for their function (Franco et al., 1996). As for all Ras-like proteins, spontaneous GDP release from ARF is slow and must be catalysed by GEFs. In the case of ARF 1, the GDP to GTP transition induces a conformational change that allows the amino-terminal myristoylated amphipathic helix of ARF1 to interact with the lipid bilayer (Paris et al., 1997).

ARFs are implicated in protein coat assembly /disassembly in the endocytic/biosynthetic pathway. The best characterised ARF is ARF1 and it is localised to the Golgi complex (Stearns et al., 1990). It is required for the binding of coat proteins, COPI (Teal et al., 1994) and the AP1 clathrin complex (Traub et al., 1993) to the Golgi membrane and for vesicle trafficking along the biosynthetic pathway. ARF 6, the least conserved ARF protein, is suggested to play a role in plasma membrane recycling and cytoskeletal organisation. ARF6 is localised to the cell periphery and cycles between the plasma membrane and an intracellular endosomal compartment, depending on its nucleotide status (D’Souza-Schorey et al., 1995). Over-expression of a mutant defective in GTP hydrolysis (Q67LARF6) has been shown to reside exclusively at the plasma membrane (D’Souza-Schorey et al., 1995). Conversely, the dominant negative GDP bound mutant of ARF6 (T27NARF6) is localised to intracellular compartments (D’Souza Schory et al., 1995) and its expression inhibits transferrin receptor recycling (D’Souza-Schorey et al., 1998). In addition to its effects on intracellular trafficking ARF6 is also
involved in remodeling the actin cytoskeleton and stimulates cortical actin reorganisation
(Radhanakrishna et al., 1996), and therefore it has been postulated that ARF6 may link
membrane traffic with the organisation of the actin cytoskeleton. Interestingly, ARF6
appears to be connected to Rac1 as ARF6 and Rac1 co-localise in the perinuclear
endosomal compartment and the trafficking of Rac1 to the plasma membrane may require
ARF6 activity (Radhakrishna et al., 1999).

Upon activation of cells with some agonists, ARF GEFS are targeted to the
plasma membrane via binding of their PH domain to PIP3 that is generated by PI(3)K
activity. ARF nucleotide binding site opener (ARNO) is an ARF GEF that potentially
acts on both ARF1 and ARF6 (Macia et al., 2001), however, EFA6 (exchange factor for
ARF6) a protein contains both a PH domain and Sec7 domains, has been shown to
stimulate GTP/GDP exchange onto ARF6 but not onto ARF1 (Franco et al., 1999 and
Macia et al., 2001). Overexpression studies have shown that EFA6 is associated with the
plasma membrane and regulates endosomal recycling (Franco et al., 1999). Furthermore,
expression of EFA6 induces membrane ruffles that are inhibited by co-expression of
dominant negative ARF6 and Rac1 (Franco et al., 1999), suggesting that that EFA6 also
regulates Rac1 activation.

1.7.3 The Rho subfamily

Rho GTPases are involved in phagocytosis, a specialised form of endocytosis
practiced by macrophages and neutrophils to engulf large particles or bacteria.
Phagocytosis involves actin-dependent internalisation and two distinct mechanisms exist
for this to occur. Type I phagocytosis involves the extension of plasma membrane
protrusions to engulf the particle and then drag it into the cell, and this is mediated by
Cdc42 and Rac (Caron & Hall, 1998). In type II phagocytosis, particles sink into actin
lined invaginations in the plasma membrane and the internalisation is dependent on Rho
A (Caron & Hall, 1988). Many pathogenic bacteria such as listeria and salmonella have
developed ways to manipulate Rho family members in order to gain entry to a host cell or
to protect themselves against phagocytosis e.g. the YopH protein from Y
psuedotuberculosis is a protein tyrosine phosphatase that dephosphorylates Cas (Hamid et
al., 1999), thereby preventing actin rearrangements and therefore ingestion.

Rho proteins have also been implicated in the regulation of endo/exocytosis,
independently from their effects on the actin cytoskeleton. Several lines of evidence link
the Rho subfamily to clathrin-mediated and clathrin-independent endocytosis and endocytic trafficking. The primary step in endocytosis is the budding of membrane vesicles from the plasma membrane. Activated Rho and Rac have been shown to inhibit transferrin receptor mediated endocytosis (Lamaze et al., 1996) and activated Rac inhibits endocytosis from both the basolateral and apical membrane in polarized epithelial cells (Jou et al., 2000). Furthermore Rac1 has been shown to interact with synaptojanin 2, a protein implicated in the uncoating of endocytic vesicles (Malecz et al., 2000). It is possible therefore that Rac is able to exert its effects on endocytosis by targeting synaptojanin to the plasma membrane and thereby reduce the formation of coated pits. Inhibition of clathrin-mediated endocytosis can stimulate clathrin-independent endocytosis (Damke et al., 1995) and therefore it may be significant that activated forms of RhoA and Rac have been localised to caveolin (Michaely et al., 1999).

RhoB and RhoD have been localised to the cytoplasmic face of endocytic vesicles, implying that they are involved in the regulation of endocytic trafficking. RhoB action is thought to be mediated by the activation of PRK kinases, which are members of the protein kinase C family (Mellor et al., 1998). RhoB activates PRK1 on endosomes and thereby slows the trafficking of the epidermal growth factor (EGF) receptor from endosomes to pre-lysosomal compartments (Gampel et al., 1999). Mitogeneic signals from the EGF receptor terminate in the lysosomes and the slowing of EGF trafficking by RhoB may act to prolong EGF signalling and provides an example of how signalling may be regulated by intracellular trafficking.

RhoD also localises to endosomes and appears to regulate transport rates along cytoskeletal tracks by motor proteins (Murphy et al., 1996). Although the nature of these cytoskeletal tracks is undetermined, activated RhoD shows a remarkably similar arrangement to actin filaments (Murphy et al., 1996). RhoD also induces loss of stress fibres (Murphy et al., 1996) and this has also been observed for another Rho family member rnd (Nobes et al., 1998), however it is not known if this itself may modulate endosomal trafficking.

The Rho subfamily has also been shown to regulate exocytic pathways. Cdc42 has been localized to the golgi by immunocytochemical and fractionation approaches (Erickson et al., 1996) and it had been proposed that Cdc42 (via WASP) may induce actin propulsion of vesicles, similar to the propulsion of pathogenic bacteria such as *Listeria* and *Shigella* (Frischknecht & Way, 2001). This may occur within the golgi or as a mechanism to propel vesicles from the golgi to the plasma membrane, and in support of
this Cdc42 has been found to regulate the targeting of vesicles to the basolateral membrane in MDCK cells (Kroschewski et al., 1999). The directional targeting of vesicles to the basolateral membrane by Cdc42 also suggests that Cdc42 plays a role in cell polarity. Indeed, the activation of Cdc42 by integrin engagement at the front of a migrating cell results in the polarised recruitment of Cdc42, which in turn recruits the par6/ PKCζ complex, which together with the motor protein dynein is found to be necessary for establishment of cell polarity. (Etienne-Manneville & Hall, 2001). It is possible that the transport of vesicles along microtubules by dynein may contribute to this effect.

RhoA and Rac1 have been also been reported to regulate exocytosis in endocrine cells, neuronal cells and mast cells (Gasman et al., 1998, Komuro et al., 1996 and Price et al., 1995). For example, constitutively active mutants of RhoA and Rac1 have been found to enhance regulated secretion in mast cells in response to calcium, whilst dominant negative Rac1 (N17Rac1) and C3 transferase reduce mast cell secretion (Price et al., 1995). Therefore it is possible that the ability of Rac1 to enhance exocytosis may facilitate lamellopodia extension, by the directing the incorporation of new membrane into ruffles and lamellopodia (Bretscher & Aguado-Velasco, 1998).

1.8 Regulation of Integrin Trafficking

The trafficking of some proteins is highly regulated e.g. receptor tyrosine kinases by their autophosphorylation (Sorkin et al., 1992) and the G-protein coupled receptors by β arrestin (Lin et al., 1997). The GLUT 4 transporter is highly regulated and is sequestered into a special internal compartment from which it is recycled to the membrane following insulin stimulation (Morris et al., 1996). The recycling of GLUT4 to the plasma membrane is regulated by Rab4 (Cormont et al., 1996, Vollenweider et al., 1997 and Cormont et al., 2001) and it is possible that the recycling of integrins may be regulated in a similar fashion.

Certain integrins (α5β1, α6β4 and αMβ2) have been shown to take part in both endocytosis and recycling back to the plasma membrane (Bretscher, 1992). Integrin recycling has been observed in neutrophils (Lawson & Maxfield 1995). The density of αvβ3 integrins in the adherent membrane of neutrophils migrating on vitronectin is much higher at the leading edge than at the rear. The release of αvβ3 from its ligand relies on intracellular calcium transients and when internal calcium is buffered, αvβ3 becomes
depleted from both endocytic vesicles and the recycling compartment and become concentrated in clusters at the rear of the adherent cells, indicating that integrins are normally recycled by way of endocytosis and intracellular trafficking during cell migration (Lawson & Maxfield, 1995). α5β1 trafficking has been examined in lung endothelial cells in response to tumour necrosis factor α (TNFα), a factor that is known to change lung cell matrix interactions and increase endothelial barrier permeability. TNFα was able to drive α5β1 internalisation into structures reminiscent of endocytic vesicles, and also increased the rate of α5β1 recycling back to the plasma membrane (Gao et al., 2000).

Integrins cytoplasmic tails contain tyrosine-based motifs (NPXY), and these sequences are found in receptors that undergo regulated endocytosis into clathrin coated pits such as the LDL receptor. Disruption of this motif in β1 and β3 integrins has been shown to affect cell spreading (Ylanne et al., 1995 and Kaappa et al., 1999) and this motif is highly conserved in integrin cytoplasmic domains indicating an essential function. Several reports indicate that this motif is essential for the recruitment of integrin binding proteins (Kaappa et al., 1999 and Chang et al., 1997), integrin affinity modulation (O'Toole et al., 1995), and targeting of integrin to adhesion sites (Vignoud et al., 1997). However, this motif is not necessary for integrin endocytosis (Vignoud et al., 1994) and it is not known whether this motif is necessary for integrin recycling. Some integrins expressed on leukocytes, such as β2 and β7, have additional tyrosine based signals YXXφ (where X is any amino acid and φ is an amino acid with a bulky hydrophobic residue). Fabbri et al. (1999) have shown the YXXφ motif in β2 is important for recycling of this integrin back to the cell surface after internalisation. In this study a non-conservative mutation at Tyr735 in αLβ2 (Y735A), resulted in diversion of αLβ2 integrin from the recycling pathway to a degradative pathway, and this mutation did not impair the binding of cytoskeletal proteins to the integrin. Disruption of this motif did however inhibit cell motility on ligands for this integrin (Fabbri et al., 1999) thus indicating a requirement for integrin recycling in cell migration.

Several studies have investigated aspects of β1 integrin trafficking and adhesion. Pulse-chase approaches reveal that β1 traffics through a similar Rab11-positive endocytic recycling compartment to that of the transferrin receptor in MCF7 cells (Ng et al., 1999). The significance of this co-localisation of β1 with Rab11 is unknown but is perhaps suggestive of the involvement of Rab GTPases in integrin recycling.
Classical protein kinase C (PKC) has been implicated as a key component in integrin mediated cell spreading and adhesion and treatment with 12-O-tetradecanoyl phorbolester 13-acetate (TPA), a PKC activator, stimulates α2β1 mediated adhesion to laminin and collagen1 (Rosfjord et al., 1999). Further evidence for the involvement of PKC in β1 integrin dynamics has revealed that expression of PKCα upregulated β1 integrin on the surface of MCF7 cells (Ng et al., 1999). This integrin initially trafficked to an endosomal compartment in a Ca(2+)/PI 3-kinase/dynamin I-dependent manner, and subsequently entered an endocytic recycling pathway (Ng et al., 1999). Furthermore, the ERM protein, ezrin, is found to be downstream of PKC dependent β1 trafficking and the association of ezrin with β1 was necessary for migration on substrates for this integrin (Ng et al., 2001).

Taken together these studies indicate that the regulation of integrin vesicular transport plays an important role in cell migration, and highlighted the need for a more precise description of the pathways involved in integrin internalisation and recycling.
Chapter Two: Materials and Methods
Chapter 2 Materials and Methods

2.1 ANTIBODIES AND REAGENTS

Monoclonal rat anti-mouse α5 integrin (clone 5H10-27 (MFR5)), hamster anti-mouse β3 integrin (clone 2C9.G2), mouse anti-human β3 integrin (clone VI-PL2), and mouse anti-hamster immunoglobulin were purchased from Pharmingen (San Diego, CA). Monoclonal mouse anti-ERK1/2 (13-6200) from Nymed (San Francisco, CA) was used for immunofluorescence, and polyclonal rabbit anti-ERK1/2 (sc-93) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and monoclonal anti-Thr202/Tyr204 phospho-p44/42 ERK (clone E10) from New England Biolabs (Beverly, MA) were used for western blotting. Peroxidase-conjugated anti-phosphotyrosine (clone PY-20H) was from BD Transduction Laboratories (San Diego, CA). Monoclonal mouse anti-phosphothreonine (clone 14B3) and PD98059 (513000) were from Calbiochem (La Jolla, CA). FITC-conjugated goat anti-mouse and anti-rat immunoglobulins were from Southern Biotechnology (Birmingham, AL). Horseradish peroxidase-conjugated streptavidin was from Amersham Pharmacia Biotech (Chalfont, Bucks, UK). Texas Red-conjugated phalloidin was purchased from Molecular Probes (Leiden, NL). Magnetic sheep anti-mouse IgG1 Dynabeads from Dynal (Oslo) and bovine serum albumin (BSA) from First Link (U.K) Ltd. EZ-link sulfo-NHS-SS-Biotin, sulfo-NHS-Biotin, enhanced chemiluminescence reagents (ECL) and a Coomassie blue protein assay kit were from Pierce and Warriner Ltd (Chester, Cheshire, UK). PDGF-BB (100-14B) and VEGF165 were from PreProTech Inc (Rocky Hill, NJ) and recombinant Human VEGFD and PIGF were from R&D systems (Minneapolis, MN). Fugene 6 transfection reagent was from Roche Diagnostics GmbH (Mannheim, Germany). A rabbit antibody against rab4 was described before (Bottger, 1996). All other reagents were purchased from Sigma Chemical Co (Poole, Dorset).

2.2 MOLECULAR BIOLOGY

2.2.1 Plasmids

Rab expression constructs were a generous gift from P van der Sluijs (University of Utrecht). Rab4 constructs were generated by excising cDNAs with EcoRI from yeast two hybrid plasmids (Nagelkerken et al., 2000) and ligating them in pcDNA3
Rab11 cDNA was generated by RT-PCR from HeLa cell mRNA and ligated in the BamHI site of pcDNA3 and Rab11 mutants were made by overlap extension PCR. PKB constructs were provided by Dario Alessi (University of Dundee) and these have been described before (Wang et al., 1999, Hajduch et al., 1998). β-galactosidase expressing construct, pPGKbgeopA, used in cell spreading assays has been described previously (Friedrich & Soriano, 1991). Integrin expressing constructs were provided by Simon Barry (Astra Zeneca, Macclesfield U.K.). β3 and αv clones were generated by RT-PCR from DX3 RNA prepared using the Promega RNAagents total RNA isolation system. The β3 cDNA was identical to the published sequence, M35999, and was cloned as a HindIII/XbaI fragment into pcDNA-3 (Invitrogen) using restrictions sites incorporated into the PCR primer proximal to the initiation codon and distal to the termination codon respectively. The αv cDNA was identical to the published sequence, M14648 and was cloned as a BamH1, EcoR1 fragment into pCDNA3 again using restriction sites incorporated at the 5' and 3' ends of the cDNA. The β1 and α5 integrins were cloned by RT-PCR from K562 RNA prepared using the Promega RNAagents total RNA isolation system. The β1 cDNA was identical to the published sequence, X07979, except that 4 silent changes were introduced to create a HindIII site, tca to agc (Ser263), atg to ttg (Leu264). The β1 cDNA was cloned into pcDNA3 as a BamH1 NotI fragment. The α5 sequence was identical to the published sequence, X06256 except that 4 silent changes were introduced to create a BamH1 site, agc to tgc (Ser 170) gac to tag (Asp171), and 3 silent changes introduced to create a Xba1 site, tcc to tct (Ser 611) and ttg to cta (Leu 612). The α5 cDNA was cloned into pCDNA3 as a Not-1 fragment.

2.2.2 Bacterial Transformation of Plasmid DNA

Plasmids were transformed into competent *E. Coli* DH5α (prepared according to Sambrooks *et al.*, 1989). Briefly 1μg of plasmid DNA was added to 4μl of competent DH5α on ice and then heat shocked at 42°C for 2 minutes and 200μl of Luria Broth (LB 1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl, pH 7.0) was added. αv constructs were transformed into SoloPack® Gold competent cells (Stratagene La Jolla CA) according to manufacturers instructions. Transformation mixtures were plated onto LB ampicillin plates (1.5% bacto-agar, 100μg/ml ampicillin) and incubated at 37°C overnight.
2.2.3 Large scale preparation of plasmid DNA

Individual colonies from LB amp plates were picked into LB amp and incubated shaking (200rpm) at 37°C overnight. 1.5 mls of the overnight culture was diluted into 400mls LB amp (or NZY broth (1% NZ amine (caesin hydrolysate), 0.5% yeast extract, 0.5% NaCl, with 0.0125 M MgCl₂, 0.0125 M MgSO₄ and 0.01 M glucose, pH 7.5 for αv constructs). The overnight culture was centrifuged at 6000rpm (Sorvell GSA) for 10 min and the supernatant removed and resuspended in 10 mls of 50mM glucose, 50mM Tris-HCl pH 8.0, 10 mM EDTA. Cells were lysed in 20 mls of 200mM NaOH, 1% SDS and then neutralised with 15 mls 3M potassium acetate pH 5.5. The precipitate was removed by centrifugation at 9000rpm for 15 mins and the supernatant strained and added to 50 mls of ice-cold isopropanol followed by centrifugation at 9000rpm for 15 mins. Pellets were left to dry for then resuspended in 5.5 mls of TE buffer with 550μl ethidium bromide (5mg/ml) and 6g of caesium chloride. This solution was then clarified by a 5min centrifugation step at 4000 rpm and the density of the solution was adjusted to be between 1.55 and 1.56 g/cm³ and then transferred to Beckman quickseal centrifuge tubes and spun at 20 °C overnight at 100 000 rpm. Plasmid bands were removed using a 19G needle and the ethidium bromide extracted by with equal volumes of water saturated isopropanol. The DNA was then precipitated with 2 volumes of water and 2 volumes of 100% ethanol and centrifuged at 4°C for 15 mins 10 000 rpm. Pellets were washed in 70% ethanol and resuspended in sterile H₂O. DNA concentrations were measured by optical density at 260 nm assuming that 1 absorbance unit = 50μg/ml.

2.3 CELL CULTURE

2.3.1 NIH and Swiss 3T3 cells

Swiss and NIH 3T3 mouse fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life technologies Inc.) with 10% foetal calf serum (Globeepharm, Esher, Surrey) and 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Life Technologies, Inc) at 37 °C with 10 % CO₂.

2.3.2 Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECS isolated according to methods within Marin et al. (2001) were generously provided by Jo Carter (University of Leicester). Cells between passages 2
and 9 were plated on gelatin coated plastic in Glutamax media 199 (Life technologies Inc.) and supplemented with 20% FCS, 5 units/ml Heparin and 50 µg/ml Endothelial cell growth supplement (ECGS) (Totam biological, Northampton, U.K.) and grown at 37°C with 5% CO₂.

2.3.3 Transient transfection

For transient transfection experiments, NIH 3T3 fibroblasts were grown to 50% confluence, fed with fresh DMEM containing 10% foetal calf serum and transfected with integrin, Rab, ARF6 and PKB constructs using Fugene 6 according to the manufacturer’s instructions. 8.3 µl of Fugene 6 was used per 10 cm plate in biochemical assays and for immunofluorescence studies, 5µl of Fugene 6 was used per glass coverslip. The ratio of Fugene 6 to DNA was maintained at 3 µl Fugene 6:1 µg DNA and experiments were carried out exactly 24 hours post-transfection.

2.4 PROTEIN ANALYSIS

2.4.1 Immunoprecipitations and western blotting

Magnetic beads conjugated to sheep anti-mouse IgG1 were blocked in PBS containing 0.1% BSA, and then bound to mouse anti-rat IgG2α followed by rat anti-α5 integrin or to mouse anti-hamster followed by hamster anti-β3 integrin. Unbound antibodies were removed by washing in lysis buffer (150 mM NaCl, 50 mM Tris, 10 mM NaF, 1 mM Na3VO4, 5 mM EDTA and 5 mM EGTA, 1% Triton X-100, 0.5 % Igepal CA-630) and the beads were incubated with lysates overnight at 4 °C with constant rotation. Beads were washed 6 times with lysis buffer and immunoisolated material eluted by boiling for 10 min in non-reducing Laemmli sample buffer followed by western blotting.

2.4.2 Western blotting

Integrin chains were resolved by 6 % SDS-PAGE and transferred to PVDF membrane for 1 h at 0.6mA/cm² using a BioRad semi-dry transfer cell. Excess protein sites were blocked with 1 % BSA in TBS (20 mM Tris-HCl pH 7.4, 137 mM NaCl) with 0.1% Tween 20. The membrane was incubated with streptavidin conjugated to
horseradish peroxidase in TBS-Tween containing 0.1 % BSA for 45 min to detect the biotinylated integrins for 60 mins, or with primary antibodies at 1:1000 overnight at 4° C, followed by incubation with HRP-conjugated secondary antibodies at 1:3000 dilution. Peroxidase reaction was developed using enhanced chemiluminescence.

2.4.3 Immunofluorescence

2.4.3.1 Surface only integrin labelling

Cells plated onto glass coverslips were washed in PBS and fixed in 2 % paraformaldehyde in PBS for 20 min at room temperature. Non-specific binding sites were blocked with PBS containing 1 % BSA (PBS-BSA) for 1 hr and cells incubated with the integrin primary antibodies; at 5 µg/ml in PBS-BSA, at room temperature for 30 min. Following this, cells were permeabilised with 0.2 % Triton X-100 in PBS for 5 min and then re-blocked in PBS-BSA overnight at 4 °C. Detection was by FITC-conjugated secondary antibodies. The actin cytoskeleton was visualised by counterstaining with texas red-conjugated phalloidin in PBS for 10 min at room temperature. Coverslips were mounted in Profade antifade mountant (Molecular Probes) and viewed on a Leica confocal laser scanning microscope.

2.4.3.2 Tracking of internal integrin

For tracking the internalisation of αvβ3 integrin, NIH 3T3 fibroblasts were transfected with hαv and hβ3 integrin in combination with wtRab4 or wtRab11. Following serum-starvation, surface αvβ3 was tagged by incubation with mouse anti-hβ3 monoclonal antibody for 30 min at 4°C in PBS containing 1% BSA. Surface bound antibody was allowed to internalise for 15 min at 22°C or 30 min at 37 °C and the cells rapidly cooled to 4°C. Antibody remaining at the cell surface was removed by incubation in acid-PBS (corrected to pH 4.0 by addition of HCl) at 4°C for 6 min. The cells were then fixed in 2% paraformaldehyde and detergent permeabilised. Internalised antibody was visualised using FITC-conjugated anti-mouse and the cells were counterstained with rabbit anti-Rab4 and rabbit anti-Rab11 followed by detection with Texas Red-conjugated anti-rabbit antibody.
2.4.3.3 ERK staining

Cells were washed in PBS and fixed in 2 % paraformaldehyde in PBS for 20 min at room temperature followed by permeabilisation with 0.2 % Triton X-100 for 4 mins. The cells were stained for ERK kinase using mouse anti-ERK1/2 monoclonal antibodies followed by a Texas Red-conjugated goat anti-mouse secondary antibody. Where appropriate, the actin cytoskeleton was counterstained with Texas red-conjugated phalloidin in PBS for 10 min at room temperature.

2.4.4 Capture ELISA

Maxisorb 96 well plates (Life Technologies Inc.) were coated overnight with 5 µg/ml anti-mouse α5, anti-mouse β3 or anti-human β3 integrin antibodies in 0.05 M Na2CO3 pH 9.6 at 4 °C. Non-specific binding sites were blocked in PBS containing 0.05 % Tween-20 (PBS-T) with 5 % BSA for 1 hour at room temperature. Integrins were captured by overnight incubation of 50 µl of cell lysate at 4°C. Unbound material was removed by extensive washing with PBS-T and wells were incubated with streptavidin-conjugated horseradish peroxidase in PBS-T containing 1 % BSA for 1 hr at 4 °C. Following further washing, biotinylated integrins were detected by chromogenic reaction with 0.56 mg/ml ortho-phenylenediamine in a buffer containing 25.4 mM Na2HPO4, 12.3 mM citric acid, pH 5.4 with 0.003 % H2O2 at room temperature for 10 min. The reaction was stopped with 8 M H2SO4 and absorbance read at 490 nm.

2.5 CELL BASED ASSAYS

2.5.1 Total surface expression, internalisation and recycling assays

Cells were plated onto 10 cm diameter dishes and grown to 70-90 % confluency over 3 to 5 days. On the day of experimentation the cells were fed with fresh DMEM containing 10 % FCS. 20 mins following feeding, the medium was changed for serum-free DMEM (or serum free Glutamax 199 media for HUVECs) and the cells serum-starved for 30 min. This has previously been determined to be the minimum time necessary for an early phase of disassembly of stress fibres and focal adhesions and complexes in Swiss 3T3 and BALB-C fibroblasts (Jim Norman, personal communication). Internalisation and recycling of integrins was measured using a modification of the method described in Bretscher & Lutter, 1988.
2.5.1.1 Total surface expression

Serum-starved Swiss 3T3 fibroblasts were treated with 10 ng/ml PDGF-BB, 1 μg/ml LPA, 30 ng/ml EGF or 0.6 μM Primaquine (PMQ) for 10 min. Cells were then washed and surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C and lysed in a buffer containing Triton X-100 and NP-40. Supernatants were corrected to equivalent protein concentration and levels of biotinylated integrin determined by capture-ELISA.

2.5.1.2 Internalisation

Serum-starved cells were transferred to ice and washed twice in cold PBS and surface-labelled at 4 °C with 0.2 mg/ml NHS-SS-biotin in PBS for 30 min. Labelled cells were washed twice in ice-cold PBS and transferred immediately to DMEM (or Glutamax 199 media for HUVECs) at 37 °C in the absence or presence of 0.6μM PMQ and 10 ng/ml PDGF-BB for fibroblasts, or 50 ng/ml VEGF165, 25 ng/ml PlGF or 150ng/ml VEGFD for HUVECs, to allow internalisation. At the indicated times the medium was aspirated, the dishes rapidly transferred to ice and washed twice with ice-cold PBS. Biotin was removed from proteins remaining at the cell surface by reduction with the membrane impermeant reducing agent, Sodium 2-mercaptopoethanesulphonate (MesNa) according to the method of Schmid & Smythe (1991). Briefly, a solution of 20mM MesNa in 50 mM Tris, 100 mM NaCl was adjusted to pH 8.6 with 10 N NaOH and immediately added to the monolayers. Reduction was allowed to proceed for 15 min on ice with gentle rocking. MesNa was quenched by addition of 20 mM iodoacetamide (IAA) for 10 min. Cells were lysed in 100 μl of a buffer containing 200 mM NaCl, 75 mM Tris, 15 mM NaF, 1.5 mM Na3VO4, 7.5 mM EDTA and 7.5 mM EGTA, 1.5% Triton X-100, 0.75 % Igepal CA-630, 50 μg/ml leupeptin, 50 μg/ml aprotinin and 1mM 4-(2-Aminoethyl)benzynesulphonyl fluoride (AEBSF) and scraped from the dish with a rubber policeman. Lysates were passed three times through a 27G needle and clarified by centrifugation at 10,000g for 10 min. Supernatants were corrected to equivalent protein concentration and levels of biotinylated integrin determined by capture-ELISA or integrins isolated by immunoprecipitation and analysed by SDS-PAGE. A sample of cells, which had been surface, labelled but not exposed to...
MesNa was included to demonstrate total surface integrin. Internalisation assay data was expressed as a percentage of the value obtained from cells that had not been reduced; this was taken to be the ‘total’.

2.5.1.3 Recycling

Serum starved cells were surface labelled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4°C as before. For measurement of recycling from the ‘recycling endosome’, labelled cells were washed twice in ice-cold PBS and transferred to serum-free DMEM at 37 °C for 30 min to allow internalisation. For measurement of recycling from the early endosome in Swiss 3T3 and NIH 3T3 cells the following internalisation regime was enforced: Following surface labelling, 10 ml of serum-free DMEM pre-adjusted to 12 °C was added to the cells. The cells were then immediately placed in an incubator at 37 °C and internalisation allowed to proceed for precisely 15 min. During this time the temperature of the medium rises to 22 °C allowing internalisation to proceed largely unchecked, but slowing the transport through the endosomal pathway and ensuring that the majority of the label is present in the early ‘sorting’ endosomes (Ren et al, 1998; Le et al, 1999; Czekay, 1997). For HUVECs this internalisation period was adjusted to 20 minutes due to a slower rate of internalisation. Cells were then transferred to ice, washed twice with ice-cold PBS and biotin removed from proteins remaining at the cell surface by reduction with MesNa. The internalised fraction was then chased from the cells by returning them to 37 °C in serum-free DMEM in the absence or presence of 10 ng/ml PDGF-BB, and in the case of HUVECs, in the absence and presence of 50 ng/ml VEGF165, 25ng/ml PIGF, 20 ng/ml FGF or 150 ng/ml VEGFD. At the indicated times, cells were returned to ice and biotin removed from recycled proteins by a second reduction with MesNa. Unreacted MesNa was quenched with 20 mM IAA for 10 min and cells lysed as before. Levels of biotinylated integrin were determined by capture-ELISA and expressed as a proportion of the levels found in cells that had not been warmed to 37 °C during the chase period; this represents the internal pool from which the integrins recycle.
2.5.2 Cell adhesion and spreading assays

12 well tissue culture plates (Life technologies Inc) were coated with fibronectin (Sigma, F-1141) or vitronectin (Sigma, V-8379) at concentrations of 20 µg/ml overnight at 4°C. The wells were rinsed with PBS and subsequently blocked with 2 % BSA for 2 h at room temperature. NIH 3T3 fibroblasts were transfected with wt or dominant negative Rab4 in conjunction with the β-galactosidase expressing construct, pPGKbgeopA (Friedrich & Soriano, 1991). 24 hours following transfection, cells were harvested by trypsinisation and collected by centrifugation in the presence of 20 µg/ml soyabean trypsin inhibitor. The cell suspensions were then added immediately to ligand coated wells in serum free DMEM containing 10 ng/ml PDGF-BB in the presence and absence of 12 µM PD98059. Cells were allowed to attach for 60 min, and non-adherent cells were removed by washing 6 times with PBS. Attached cells were fixed for 1 min in 0.2 % glutaraldehyde containing 5 mM EGTA and β-galactosidase expressing cells were visualised by incubation with 5 mM potassium ferricyanide and 1 mg/ml X-gal overnight at 37 °C. For cell adhesion assays, the numbers of stained cells per well was counted and expressed as a proportion of total transfected cells for each condition. To obtain an index of cell spreading, adherent cells were photographed with a digital camera and the area of those expressing β-galactosidase determined by delineation of the cell envelope and calculation of the encompassed area using the 'NIH image’ software. Each assay was performed three times in triplicate wells. For immunofluorescence analysis, spread cells were washed in PBS and fixed and stained as above.
Chapter Three: Integrin Trafficking in Fibroblasts
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3.1 INTRODUCTION

In order for cells to spread on or migrate across flat surfaces, integrin-containing focal complexes must form rapidly at the peripheral or leading lamellae. Formation of these focal complexes requires integrin engagement, but is also driven by growth factors such as PDGF (Hotchin & Hall, 1995). Many surface receptors, including integrins, participate in an endo/exocytic cycle (Bretscher, 1992). They are internalised, delivered to endosomes and then recycled to the plasma membrane, for reutilisation. It has been suggested that this cycle may facilitate focal complex assembly by internalising ECM receptors at the rear of the cell and transporting them forwards within vesicles for exocytosis at the leading lamellae (Bretscher, 1996).

Previous studies have shown that the integrin endo/exocytic cycle is important, notably the study by Fabbri et al. (1999), in which a YXX\(\phi\) motif in the cytoplasmic tail of \(\beta_2\) integrin was shown to be essential for recycling to the plasma membrane following internalisation, and disruption of this motif inhibited \(\beta_2\) dependent cell migration. Furthermore, Ng et al., 1999 have show that \(\beta_1\) integrins are internalised via a dynamin-dependent step and that inhibition of \(\beta_1\) integrin internalisation by expression of dominant negative dynamin also reduced cell motility. It is unclear, however, whether the recycling of these integrins is regulated by cell signalling pathways that that coordinate cell motility.

Transferrin receptor recycling operates in two distinct time-domains; a short circuit recycling pathway directly from early ‘sorting’ endosomes to the plasma membrane (Daro et al., 1996) and an indirect route involving transit through the perinuclear or ‘recycling’ endosome (Hopkins et al., 1994). The Rab family of small GTPases control key targeting events in these recycling pathways. Rab 11 localises to the perinuclear recycling compartment and has been shown to control recycling from this compartment, as well as transport to the trans Golgi network (Ullrich et al., 1996, Ren et al., 1998 and Wilcke et al., 2000). Rab4, on the other hand is localised predominantly to early endosomes (Daro et al., 1996) and to a lesser extent on recycling endosomes where it co-localises with Rab11 (Sheff et al., 1999 and Sonnichsen et al., 2000) and is thought to be involved in recycling from both these compartments. ARF6 now has an established role in the regulation of an endosomal recycling pathway distinct from those regulated by
Rab GTPases (Radhakrishna & Donaldson, 1997). It is possible, therefore, that integrins are recycled via this pathway and thus mediate some of the effects of ARF6 on cell spreading and motility (Radhakrishna et al., 1999). Although some studies have shown that β1 integrins co-localise with Rab11 and the transferrin receptor (Ng et al., 1999) it is unclear, however, which Rab- or ARF-dependent steps are involved in the endo/exocytic cycle of integrins.

Following serum-starvation, Swiss 3T3 fibroblasts rapidly lose integrin-containing focal complexes and actin-rich lamellae. Following re-addition of serum, or a serum-factor such as PDGF, they rapidly assemble lamellae and put down focal complexes (Nobes et al., 1995). Such tight regulation of assembly and disassembly of these structures makes Swiss 3T3 fibroblasts an ideal choice for investigating the control of integrin endo/exocytic rates. Here I show that PDGF regulates the recycling rate for αvβ3, but not α5β1. This process involves Rab4 and does not require Rab11 or ARF6, and thus defines a mechanism whereby growth factors can regulate integrins during cell adhesion and spreading.
3.2 Results

3.2.1 Surface expression of ανβ3 and α5β1 integrins in Swiss 3T3 Fibroblasts

In order to measure cell-surface expression of α5β1 and ανβ3 integrins a membrane impermeant biotinylation reagent (NHS-S-S-Biotin) was used followed by a capture-ELISA assay to determine the degree of integrin labelling. Briefly, cells were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4°C and lysed. Integrins were captured by overnight incubation in microtitre wells coated with anti-α5 and anti-β3 integrin monoclonal antibodies. Biotinylated integrin was then detected using peroxidase-conjugated streptavidin followed by chromogenic reaction with o-phenylenediamine. The assay was established to respond linearly to a range of lysate concentrations (data not shown) and samples were diluted appropriately to lie within this range. Using this approach, I determined surface expression of α5β1 and ανβ3 integrins following treatment of serum-starved Swiss 3T3 fibroblasts with serum factors known to modify the actin cytoskeleton and/or stimulate cell migration. LPA, for instance, produces focal adhesions and stress fibres via activation of Rho. However, minimal change in ανβ3 surface expression was detected in response to LPA treatment of serum-starved cells (Fig. 3.1A). Greater changes were driven by PDGF and EGF, factors known to cause appearance of membrane ruffles via the activation of Rac. Notably, PDGF was able to drive a large (≈2 fold) increase in ανβ3 surface levels. These increases were only detected for ανβ3 and there was minimal change in α5β1 integrin surface expression following addition of PDGF, EGF, or LPA (Fig. 3.1B) or even 10% serum (not shown). Inhibition of receptor recycling by a 10 min exposure to primaquine (Reid & Watts, 1990) reduced the surface levels of α5β1 and ανβ3 indicating that, even following serum-starvation, these integrins participate in endo/exocytic cycling. To confirm the integrity of immunoisolated material, lysates from serum-starved and PDGF-stimulated cells were immunoprecipitated with anti-α5 and anti-β3 monoclonal antibodies. Immunoprecipitates were analysed using 6% SDS-PAGE under non-reducing conditions followed by western blotting and detection with peroxidase-conjugated streptavidin and enhanced chemiluminescence. Consistent with the capture-ELISA data, the surface expression of ανβ3 was increased by PDGF treatment whilst that for α5β1 remained unchanged (Fig. 3.1C).
Fig. 3.1 Effect of various agents on surface expression of α5β1 and αvβ3 integrins.
Serum-starved Swiss 3T3 fibroblasts were treated with 10 ng/ml PDGF-BB, 1 μg/ml LPA, 30 ng/ml EGF or 0.6 μM PMQ for 10 min. Cells were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C and lysed in a buffer containing Triton X-100 and NP-40. Integrins were captured by overnight incubation in microtitre wells coated with anti-β3 (A) and anti-α5 (B) integrin monoclonals. Biotinylated integrin was then detected using peroxidase-conjugated streptavidin followed by chromogenic reaction with o-phenylenediamine. Values are expressed as % of control. (Mean ± s.e.m. from 5 separate experiments. *P<0.0001 compared with serum-starved control cells). To confirm the integrity of immunoisolated material and antibody specificity, lysates from serum-starved and PDGF-BB-stimulated cells were immunoprecipitated (I.P.) with anti-β3 and anti-α5 monoclonals. Immobilised material was then analysed by 6% SDS-PAGE under non-reducing conditions followed by western blotting and detection with peroxidase-conjugated streptavidin and enhanced chemiluminescence (C). The positions of α5, αv, β1 and β3 integrin chains are indicated.
3.2.2 Integrin internalisation

I developed assays for integrin endocytosis and recycling to determine whether PDGF-regulation of these processes could account for this change in $\alpha\nu\beta3$ surface levels. Integrin internalisation was determined by surface-labelling Swiss 3T3 fibroblasts with NHS-SS-biotin at 4°C followed by incubation at 37°C for various times. Biotin was removed from proteins remaining on the cell surface by exposure to MesNa at 4 °C and internalised integrin assessed by immunoprecipitation followed by western blotting with streptavidin. Both $\alpha\nu\beta3$ and $\alpha5\beta1$ heterodimers were internalised with similar kinetics, their internal pools reaching a steady level by 10 min (Fig. 3.2 A,B). Using capture-ELISA to quantify biotinylated integrins, I found that addition of the receptor recycling inhibitor, primaquine (PMQ), increased the measured internalisation rate of $\alpha\nu\beta3$ and $\alpha5\beta1$ integrin (Fig. 3.2C, D), indicating that both integrins recycle back to the plasma membrane very shortly following internalisation. Therefore, to measure the effect of PDGF on the endocytic rate of integrins I performed internalisation assays in the presence of PMQ. This analysis revealed that PDGF did not affect the endocytic rate for either $\alpha\nu\beta3$ or $\alpha5\beta1$ integrin (Fig. 3.2E,F).

3.2.3 Integrin endosomal compartments

Recycling receptors do not normally accumulate in early endosomes, but pass rapidly through them and proceed to the perinuclear recycling compartment where they accumulate, before making the last step in recycling to the plasma membrane. Incubation times of 30-60 min at 37°C have been routinely used to load the perinuclear recycling compartment with internalised tracer (Radhakrishna et al., 1999). Additionally, internalised receptor may be concentrated in the early endosomes by reducing the temperature to slow transport through the endosomal system (Faundez et al., 1998 and Lim et al., 2001).

To monitor the trafficking of integrin through endosomal compartments, cell-surface $\alpha\nu\beta3$ was tagged using anti-\beta3 monoclonal antibody at 4°C. Internalisation was initiated by raising the temperature to either 22°C for 15 min or to 37°C for 30 min. Following this, antibody remaining at the cell surface was removed by a low pH wash at 4°C, and $\alpha\nu\beta3$ integrin and Rab proteins visualised by immunofluorescence. Following the shorter internalisation period, $\alpha\nu\beta3$ integrin became closely co-localised with Rab4 in endocytic vesicles distributed relatively evenly about the cytoplasm (Fig. 3.3A-C).
Fig. 3.2 Internalisation of integrins.

(A-B) Serum-starved Swiss 3T3 fibroblasts were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C and warmed to 37 °C for the times indicated. Biotin was released from proteins remaining at the cell surface by MesNa-treatment at 4 °C, the cells lysed and integrins immunoprecipitated with anti-β3 (A) or anti-α5 (B) monoclonal antibodies. Immobilised material was then analysed by 6 % SDS-PAGE followed by western blotting with peroxidase-conjugated streptavidin. The positions of α5, αv, β1 and β3 integrin chains are indicated. (C-D) Surface labelled cells were warmed to 37°C in the absence (△) or presence (▲) of 0.6 μM PMQ for the times indicated. Biotin was released from proteins remaining at the cell surface and biotinylated integrin determined by capture-ELISA using microtitre wells coated with anti-β3 (C) or anti-α5 (D) integrin monoclonal antibodies. (E-F) Surface-labelled cells were warmed to 37 °C in the presence of 0.6 μM PMQ in the absence (○) and presence (■) of 10 ng/ml PDGF-BB for the times indicated. Internalised integrin was determined as for (C) and (D) using microtitre wells coated with anti-β3 (E) or anti-α5 (F) integrin monoclonal antibodies. (Mean ± s.e.m. from 3 separate experiments)
During this time, αvβ3 did not reach the perinuclear recycling compartment and showed little co-localisation with Rab11 (Fig. 3.3D-F). Longer internalisation times resulted in the integrin being transported out of the Rab4-positive compartment such that following 30 min at 37°C, αvβ3 was observed to focus in the perinuclear region and co-localise with Rab11 (Fig. 3.3G-I). Similar results were obtained for α5β1 integrin (data not shown).

3.2.4 Recycling of integrins

I proceeded to monitor the recycling of integrins from early endosomes and the perinuclear recycling compartment using a pulse-chase approach. Cells were surface-labelled and internalisation allowed to proceed for 15 min at 22°C to allow integrin to accumulate in Rab4-positive early endosomes. Biotin was removed from proteins remaining on the cell surface by exposure to MesNa at 4°C, and internalised integrin chased from the cells at 37°C for various times in the presence or absence of PDGF. At each time the cells were re-exposed to MesNa to remove biotin from integrins that had recycled back to the cell surface and the level of biotinylated integrin remaining within the cell assayed by capture-ELISA. PDGF stimulated the rate of αvβ3 recycling from the early endosomes by approximately 2-fold, such that in the presence of the growth factor, all internalised integrin had returned to the plasma membrane within 10 min (Fig. 3.4A). A limited amount of α5β1 recycled from this compartment, but this was unaffected by PDGF (Fig. 3.4B).

To monitor recycling of tracer accumulated in the perinuclear recycling compartment, cells were surface labelled and internalisation allowed to proceed for 30 min at 37°C. Integrin recycling was then determined as for Fig. 3.4A,B. The recycling rates were similar for both α5β1 and αvβ3 and were unaffected by PDGF (Fig. 3.4C,D), indicating that αvβ3 is subject to PDGF-regulated recycling only when present in the Rab4-positive early endosomes and that recycling from endocytic compartments distal to this is refractory to this kind of regulation.

3.2.5 Rab4-dependence of αvβ3 recycling

Rab4 is known to regulate the recycling of receptors from early endosomes to the plasma membrane (van der Sluijs et al., 1992). To investigate the potential involvement
NIH 3T3 fibroblasts were transfected with αvβ3 and hβ3 integrin in combination with wtRab4 (A-C) or wt Rab11 (D-I). Surface αvβ3 was tagged by incubation with mouse anti-hβ3 monoclonal antibody for 30 min at 4°C. Surface bound antibody was allowed to internalise for 15 min at 22°C (A-F) or 30 min at 37°C (G-I) and the cells rapidly cooled to 4°C. Antibody remaining at the cell surface was removed by a low pH wash and the cells fixed and detergent permeabilised. Internalised antibody was visualised using FITC-conjugated anti-mouse (A,D,G; shown in green) and the cells were counterstained with Rabbit anti-Rab4 (B) and rabbit anti-Rab11 (E,H) followed by detection with Texas Red-conjugated anti-rabbit antibody (shown in red). Yellow indicates colocalisation of the two fluorophores. Bar 16 μm.
Fig. 3.4 Recycling of α5β1 and αvβ3 integrins.

Cells were surface-labelled and internalisation allowed to proceed for 15 min at 22 °C (A,B) or 30 min at 37 °C (C,D) and biotin removed from receptors remaining at the cell surface by treatment with MesNa at 4 °C. Cells were then re-warmed to 37 °C for the times indicated in the absence (○) or presence of 10 ng/ml PDGF-BB (■) to allow recycling to the plasma membrane, followed by a second reduction with MesNa. Cells were lysed and integrin-biotinylation determined by capture-ELISA using microtitre wells coated with anti-β3 (A,C) or anti-α5 (B,D) integrin monoclonal antibodies (C-F). The proportion of integrin recycled to the plasma membrane is expressed as % of the pool of integrin labelled during the internalisation period (Values are mean ± s.e.m. from 5 separate experiments).
of Rab proteins in integrin recycling I employed NIH 3T3 fibroblasts which are similar to Swiss 3T3s but can be transfected to high efficiency. To measure recycling from transfected cells, I transiently expressed human integrins and detected using human integrin-specific antibodies. The antibodies used were highly selective for hανβ3 and hα5β1 and did not cross-react with mouse integrins (Fig. 3.5A). Additionally, when cells were co-transfected with Rab4 and human integrins, both receptor and GTPase were expressed in the same cells (Fig. 3.5 B, C).

Treatment with PDGF increased recycling of hανβ3 from early endosomes and expression of wtRab4 increased both the basal and PDGF-stimulated rates of hανβ3 recycling (Fig. 3.5D). hα5β1, however, did not recycle directly from these endosomes (Fig. 3.5E).

I employed two dominant negative mutants to assess the Rab4-dependence of ανβ3 recycling; S22NRab4, which binds GDP poorly but is unable to bind GTP (Gerez et al., 2000) and N121IRab4 which is unable to bind guanine nucleotide (Cormont et al., 1996). S22NRab4 significantly reduced, and N121IRab4 completely abolished PDGF-stimulated recycling of ανβ3 (Fig. 3.5D). These data indicate an absolute requirement for Rab4 in this process. The data previously shown in Fig. 3.4 suggest that ανβ3 must be present in Rab4-positive early endosomes to be amenable to PDGF-regulated recycling. Transport through the recycling compartment is inhibited by dominant negative mutants of Rab11 (Ren et al., 1998; Ullrich et al., 1996). Dominant negative N124IRab11 has no effect on PDGF-induced recycling of ανβ3 (Fig. 3.5D), indicating that the growth factor regulates recycling directly to the plasma membrane without involving passage of the integrin through the perinuclear recycling compartment.

Experiments employing a 30 min internalisation period indicated that both hανβ3 and hα5β1 integrin recycled at similar rates from the perinuclear recycling compartment, such that during 30 min of chase, ≈60% of integrin had returned to the plasma membrane (Fig. 3.5F,G); a value similar to that observed in Swiss 3T3s (previously shown in Fig. 3.4C,D). In complete contrast to PDGF-regulated recycling of hανβ3 from the early sorting endosomes, recycling of hανβ3 and hα5β1 from the perinuclear compartment was unaffected by N121IRab4, but strongly inhibited by dominant negative N124IRab11 (Fig. 3.5F,G).
Fig 3.5 Rab4-dependence of ανβ3 recycling.

(A) NIH 3T3 fibroblasts were transfected with human αν and β3 (ανβ3) or human α5 and β1 (α5β1) integrins, or empty vector control (mock). Cells were surface labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C and lysed. Lysates were immunoprecipitated (I.P.) with anti-human β3 (hβ3) or anti-human α5 (hα5) monoclonal antibodies. Immobilised material was then analysed by 6 % SDS-PAGE followed by western blotting with peroxidase-conjugated streptavidin. The migration positions of human αν, β3, α5 and β1 integrin chains are indicated. (B&C) Cells were transfected with human αν and β3 integrins in combination with wtRab4. 24hr post-transfection the cells were fixed, permeabilised and co-stained for human β3 integrin (B) and Rab4 (C) as for Fig. 2. Bar, 150μm. (D-G) NIH 3T3 fibroblasts were transfected with human ανβ3 (D,F) or α5β1 (E,G) integrins either alone (Control), or in combination with wtRab4, S22NRab4, N121IRab4, N124IRab11 and T27NARF6 as indicated. Cells were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C, and internalisation allowed to proceed, for 15 min at 22°C (D,E) or 30 min at 37°C (F,G). Cells were exposed to MesNa at 4°C and internalised integrin was chased back to the cell surface at 37°C for 10 min in the absence (open bars; Basal) and presence (shaded bars) of 10 ng/ml PDGF-BB (D,E) or for 30 min at 37°C in the absence of PDGF (F,G). Cells were then re-exposed to MesNa and biotinylated integrin determined by capture-ELISA using microtitre wells coated with anti-human β3 (D,F) or anti-human α5 (E,G) monoclonal antibodies. Values are mean ± s.e.m. from at least 3 separate experiments.
A  

**IP:** anti-hβ3  anti-hα5  

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B  

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D  

**hαβ3:** 15min intern  

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<td>Control</td>
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E  

**hα5β1:** 15min intern  

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**hαβ3:** 30min intern  

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G  

**hα5β1:** 30min intern  

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Endosomal recycling can be regulated by the ARF subfamily of GTPases, notably ARF6. I therefore determined the effect of dominant negative T27NARF6 on PDGF-dependent αvβ3 recycling. Consistent with previous observations that ARF6 regulates a membrane recycling pathway distinct from that controlled by Rab4 (Radhakrishna & Donaldson, 1997) T27NARF6 did not inhibit αvβ3 recycling (Fig. 3.5D).

3.2.6 Visualisation of PDGF-dependent αvβ3 recycling

Treatment with PDGF for 10 min increased surface staining for αvβ3 and resulted in its localisation to numerous small puncta, distributed over the cell surface, but sometimes enriched in lamellar ruffles (Fig. 3.6C,D). Examination of optical slices from the confocal image indicate that these puncta were present primarily on the dorsal surface of the cell (not shown). Following longer treatments with PDGF, αvβ3 was seen to be incorporated into larger complexes in a peripheral distribution characteristic of integrin-containing focal complexes (Fig. 3.6E,F) (Hotchin & Hall, 1995). The time course of appearance of αvβ3-rich puncta parallels that measured for PDGF-dependent integrin recycling from early endosomes, suggesting that recycling integrin may be targeted to these puncta. Integrist recycling was visualised by tagging αvβ3 with a NHS-SS-Biotin-labelled antibody which was allowed to internalise and recycle as for Fig. 3.5D. PDGF stimulated the recycling of αvβ3-bound antibody (Fig. 3.6I) and this was targeted to numerous small punctate complexes, distributed over the cell surface (Fig. 3.6K).

Given that PDGF-regulated recycling is dependent on Rab4 and that early endosomal recycling of αvβ3 is directed to cell surface puncta, I determined whether Rab4 inhibition would affect the appearance of these puncta. S22NRab4 blocked assembly of αvβ3-containing puncta (Fig. 3.6G), but membrane ruffling was uncompromised (Fig. 3.6H). Taken together, these data indicate that recycling of αvβ3 to the plasma membrane, via a Rab4-dependent pathway, directs this integrin to punctate plasma membrane complexes. These puncta then subsequently organise into the more familiar integrin-containing focal complexes.

3.2.7 Involvement of Rab4 in cell adhesion and spreading

To investigate the possibility that Rab4-dependent αvβ3 recycling was necessary for cell adhesion or spreading, cells transfected with wt or dominant negative Rabs were adhered to either vitronectin (VN), a good ligand for αvβ3, or fibronectin (FN), a ligand
Fig. 3.6 Visualisation of PDGF-dependent recycling of αvβ3 integrin.

(A-H) NIH 3T3 fibroblasts were transfected with hαvβ3 integrin either alone (A-F) or in combination with S22NRab4 (G,H). 24hr post-transfection the cells were serum-starved for 30 min and then challenged with 10 ng/ml PDGF-BB for 10 min (C,D,G,H) or 30 min (E,F) or allowed to remain quiescent (A,B). Cells were fixed in 2% paraformaldehyde and surface αvβ3 visualised by indirect immunofluorescence and F-actin counterstained with Texas Red-conjugated phalloidin. Surface-only integrin staining was obtained by addition of the primary antibody prior to the detergent permeabilisation step. Bar, 20μm. (I) Anti-β3 antibody was biotinylated with NHS-SS-Biotin and bound to the surface of the cells at 4°C. Biotinylated antibody was allowed to internalise for 15min at 22°C and the cells exposed to MesNa at 4°C to remove biotin from antibody remaining on the plasma membrane. Internalised antibody was chased back to the cell surface at 37°C for 10 min in the absence (Basal) and presence of 10 ng/ml PDGF-BB. Cells were then re-exposed to MesNa and biotinylated antibody determined by capture-ELISA using microtitre wells coated with anti-hamster monoclonal antibody. (J,K) Biotinylated antibody was bound, internalised and recycled in the absence (J: Basal) and presence (K) of PDGF as for (I). Following this the cells were fixed, and recycled antibody visualised using FITC-conjugated streptavidin (surface-only labelling ensured by omission of a detergent permeabilisation step). Bar 16 μm.
for both α5β1 and αvβ3, in the presence of PDGF. N121IRab4, which abrogated early endosomal recycling of αvβ3, inhibited the adhesion of transfected cells to VN (Fig. 3.7A). Adhesion to FN was unaffected by N121IRab4, indicating that the inhibition was specific for αvβ3 mediated events. S22NRab4, however, did not affect adhesion to VN (Fig. 3.7A), implying that the partial blockade of αvβ3 recycling with this construct was insufficient to block adhesion. However, partial inhibition of αvβ3 recycling by S22NRab4 compromised spreading of cells following attachment to VN. Within 1 hr of plating, cells transfected with wtRab4 spread better than their untransfected neighbours (Fig. 3.8A,B). In contrast, cells transfected with S22NRab4 did not spread efficiently on VN and seemed unable to properly organise their actin cytoskeleton (Fig. 3.8C,D). Spreading on FN was similar irrespective of transfection with wtRab4 (Fig. 3.8E; arrowed) or S22NRab4 (Fig. 3.8F; arrowed), consistent with the observation that α5β1 recycling is unaffected by dominant negative Rab4. Quantification of cell area indicated that S22NRab4 reduced spreading on VN by approximately 50% (Fig. 3.7B), a value consistent with the reduction of αvβ3 recycling by this construct.

In contrast to dominant negative Rab4s, N124IRab11 did not inhibit the adhesion of cells to either matrix protein (Fig. 3.7A) and even increased spreading onto VN (Fig. 3.7B). Therefore, direct recycling of integrin from early endosomes to the plasma membrane, and not that from the perinuclear recycling compartment, is critical for αvβ3 function.
Fig 3.7 Effect of dominant negative Rab4s on cell adhesion and spreading.

NIH 3T3 cells were transfected with wtRab4, S22NRab4, N121IRab4 or N124IRab11 in combination with a β-galactosidase transfection marker. The cells were then briefly trypsinised and allowed to adhere to either vitronectin (VN), or fibronectin (FN), in the presence of 10 ng/ml PDGF-BB for 1 hr. Following this, the unattached cells were washed-off with ice-cold PBS and attached cells fixed and stained for β-galactosidase expression. The number of β-galactosidase expressing cells adherent to the VN or FN matrices were expressed as a proportion of those adherent to poly-L-lysine in the same experiment (A). Adherent cells were then photographed with a digital camera and the area of transfected cells determined by delineation of the cell envelope using the ‘NIH image’ software (B). The data are expressed as a % of the cell area of wt Rab4 expressing cells following spreading on VN. (Values are mean ± s.e.m)
NIH 3T3 fibroblasts were transfected with human αv and β3 integrins in combination with wtRab4 (A,B,E) or S22Rab4 (C,D,F). The cells were then briefly trypsinised and allowed to adhere to either vitronectin (A-D), or fibronectin (E,F), in the presence of 10 ng/ml PDGF-BB for 1 hr. Cells were fixed in 2% paraformaldehyde and double stained for F-actin (B,D,E,F) and cell-surface human β3 integrin (A,C). The arrows in E,F indicate transfected cells. Bar, 40 μm.
3.3 DISCUSSION

The Rab family of GTPases regulate key targeting events in the endo/exocytic pathway and here I reveal that they are involved in integrin trafficking events. In serum-starved cells internalised αvβ3 and α5β1 integrins were transported through Rab4-positive, early endosomes and arrived at the perinuclear recycling compartment approximately 30 minutes after endocytosis. From the recycling compartment, integrin is recycled in a Rab11-dependent manner in what I have termed a 'long loop' recycling event (Fig. 3.9A). Treatment of serum-starved fibroblasts with PDGF rapidly stimulated the rate of αvβ3 recycling from the early endosome, but not α5β1, in a process that was dependent on Rab4, and this is referred to as 'short loop recycling' in Fig. 3.9B.

Inhibition of αvβ3 early endosomal recycling using dominant negative Rab4 mutants impaired cell spreading onto vitronectin (a ligand for αvβ3) but not on fibronectin (a ligand for both α5β1 and αvβ3). This indicates that Rab4-dependent recycling events are necessary for the function of αvβ3 integrin.

3.3.1 Regulation of recycling from early endosomes

GLUT4 (Rea & James, 1997) the Aquaporin-2 water channel (Brown et al., 1998) and more recently, E-cadherin (Le et al., 1999) are all sequestered within endosomes under basal conditions and recycled to the plasma membrane in response to the appropriate stimuli. E-cadherin has been shown to recycle in this fashion from an early, Rab5-positive endosome and this was regulated by cell-cell contact (Le et al., 1999).

Insulin stimulation of GLUT4 recycling has been shown to be dependent on Rab4 (Vollenweider et al., 1997) mooring the early endosome and this GTPase as a target for regulation of receptor recycling by growth factors. Rab4 is proposed to be necessary for the formation of vesicles involved in recycling proteins from the early endosome to the cell surface. Overexpression of wtRab4 reduces the ability of the TFN-R to reach acidic endosomes, presumably by rapidly mistargeting internalised TFN-R directly to the plasma membrane (van der Sluijs et al., 1992). Consistent with this, I found that overexpression of wtRab4 enhanced PDGF-dependent rapid recycling of αvβ3, while dominant negative Rab4 mutants opposed this. The pulse-chase experiments presented in Fig. 3.2 indicate that in order to be amenable to PDGF-regulated recycling, αvβ3 must be present in early endosomes at the time of growth factor addition.
A  ‘Long-loop’ recycling of αvβ3 and α5β1 (serum-starvation)

B  ‘Short-loop’ recycling of αvβ3 (PDGF/VEGF-addition)

Fig. 3.9 Integrin recycling pathways

Under serum starvation conditions αvβ3 and α5β1 recycle to the plasma membrane from the perinuclear recycling compartment via a Rab11 dependent mechanism. Upon addition of PDGF, αvβ3 recycles from the early endosome to the plasma membrane via a Rab4 dependent pathway.
Following addition of PDGF, αvβ3 may take two pathways to the plasma membrane, either directly or via recycling endosomes. Previous studies showed that transport through, and exit from, recycling endosomes requires the function of Rab11 (Ren et al., 1998; Ullrich et al., 1996). The fact that N124IRab11 has no effect on the ability of PDGF to trigger αvβ3 recycling, suggested that the direct route to the plasma membrane is the one regulated by the growth factor. Possibly, PDGF regulation of Rab4 occurs via activation of PI 3-kinases (Kamohara et al., 1995) that have been shown to regulate guanine nucleotide exchange on Rab4 (Shibata et al., 1997). I found that inhibition of PI(3)K with wortmannin, albeit unable to affect αvβ3 endocytosis, potently inhibited its recycling (see chapter 6). Additionally certain activated cell signalling pathways have been shown to impinge upon Rab function in the early endosome. For instance, PKB/Akt, a kinase activated by PDGF-stimulation of PI 3-kinase, regulates Rab function in macrophages (Barbieri et al., 1998) and in B cells, receptor signalling triggers Rab recruitment to endosomal compartments (Xu et al, 1996).

3.3.2 αvβ3 recycling and the regulation of cell adhesion and motility

Experiments with bound antibodies showed that PDGF induced recycling of αvβ3 is distributed relatively evenly over the cell surface and not concentrated into particular region of the cell, for instance the ruffling cell periphery or the leading lamella in cells that are migrating. This is consistent with the fact that, unlike the Rab11-positive recycling endosome, which has a distinct location in some cells, Rab4 positive early endosomes are distributed more evenly about the cytoplasm of fibroblasts and consequently one would not expect the recycling from them to be abjectly polarised. This indicated to us that early endosomal recycling may have a general role in integrin mediated events. Indeed, inhibition of PDGF-regulated αvβ3 recycling using dominant negative Rab4 mutants does impair cell adhesion and non-polarised spreading, suggesting that this aspect of regulated integrin vesicular transport may have a general role in integrin activation, rather than a specialised one such as vectoral delivery to the leading edge of a migrating cell.

It has been suggested that factors such as PDGF promote cell spreading by modulating the affinity of the integrins for their ligands, a mechanism that has been termed inside-out signalling (Hughes & Pfaff, 1998). The precise and rapid regulation of the affinity of integrins for their ligands is a feature of many members of this family. A
classic example of this is the activation of the platelet receptor αIIbβ3 following thrombin stimulation. Upon stimulation, αIIbβ3 undergoes a conformational change rendering it competent to bind ligand, and the total levels of active receptor exposed at the cell surface are increased. The affinity of both α4β1 (Chan, 1991) and the β2 integrins αLβ2 and αMβ2 (Geiger, 2000) for their ligands is also regulated by exposure of cells to various stimuli. Although certain signalling pathways, including PI 3-kinase activation, have been implicated in mediating inside-out activation, the mechanism through which this is achieved remains unclear. Here I have found that the rapid recycling of αvβ3 to the cell surface is dependent on Rab4 and that this can be regulated by PDGF, a growth factor that promotes cell spreading and motility. This rapid recycling pathway must be necessary for integrin function as dominant negative Rab4 mutants blocked αvβ3 mediated cell adhesion and spreading on vitronectin. Therefore, modulating the flux of integrin to and from the cell surface is one mechanism through which the cell can achieve the inside-out activation of integrins. It will be interesting to determine whether the same mechanism can regulate other integrins.

I have shown that PDGF stimulates Rab4-dependent recycling of αvβ3 integrin from early endosomes to the cell surface. Inhibition of this process using dominant negative Rab4 mutants impaired cell adhesion and spreading on ligands for αvβ3. These data describe for the first time the regulated recycling of an integrin by a growth factor, and the dependence of integrin-mediated adhesion and cell spreading on this process.
Chapter Four: ERK1 associates with αvβ3 integrin and regulates cell spreading on vitronectin.
Chapter 4: ERK1 associates with αvβ3 integrin and regulates cell spreading on vitronectin.

4.1 INTRODUCTION

The integrins have received a great deal of attention in the literature over the last few years, not only for their ability to bind and model the extracellular matrix (ECM), but also owing to their ability to activate a number of cell signalling cascades that influence a range of biological processes including cell growth, differentiation, migration and apoptosis (Schwartz & Baron, 1999). The distal stretches of many of these signalling pathways are reasonably well defined. For instance ligation of integrins is well established to both activate the MEK/ERK signalling axis and to prolong the activation of the pathway in response to growth factors and thereby confer anchorage-dependent growth (Roovers & Assoian, 2000). However, precise descriptions of the upstream events in integrin signalling remain elusive.

Much of the evidence that kinases associate physically with integrins is controversial. The integrin-linked kinase (ILK) was identified as a ligand for the cytodomain of β1 integrin by yeast-2-hybrid analysis, and engagement of β1 integrins has been shown to activate ILK (Delcommenne et al., 1998). However, recent work in Drosophila suggests that the main function of ILK is that of an adaptor rather than a kinase (Zervas et al., 2001). On the other hand pp125FAK is well established to be activated by ligation of integrins and to be a key activator of numerous downstream signalling cascades (Schlaepfer et al., 1999), but it is unclear whether FAK associates physically with integrins. Recently, however, good data has been obtained that the tyrosine kinase, Syk, associates with β3 integrin cytodomains, and activation of Syk by clustering of αIIbβ3 integrin is likely to be a key upstream event in the activation of platelets and locomotion of haemopoietic cells (Woodside et al., 2001).

The transmembrane and extracellular domains of integrins also form so-called ‘lateral associations’ and these are proposed to couple integrins to key signalling kinases. The α5β1, αvβ3 and α1β1 heterodimers associate with caveolin (Giancotti & Ruoslahti, 1999), most probably via their transmembrane stretches. There is no evidence that this interaction is direct, but it is likely to be important in linking integrins to the tyrosine kinase, Fyn, and the adaptor protein, Shc. The recruitment of Shc may be a critical link between integrins and the Ras/Raf/MEK/ERK signalling cascade and the regulation of...
anchorage-dependent growth (Giancotti & Ruoslahti, 1999). Integrins are now well established to form stable complexes with proteins of the transmembrane 4 superfamily (Hemler, 1998). These tetraspanins have been shown to bind tyrosine kinases (Skubitz et al., 1996), phosphatidylinositol 4-kinase (Berditchevski et al., 1997) and conventional protein kinase C (Zhang et al., 2001), and the observed modulatory effects of integrin-tetraspannin complexes on adhesion-dependent signalling may involve these associations.

Prior to engagement with the ECM, integrins must be rendered competent to bind ligand via a process termed 'inside-out' signalling (Shattil, 1999). There are several ways in which the availability of ligand-competent integrin may be increased. Growth factors have recently been shown to regulate delivery of αvβ3 from endosomal compartments to the plasma membrane, and this process is necessary for efficient integrin function (Roberts et al., 2001). Once upon the cell surface, conformational changes in an individual heterodimer may increase its affinity for monovalent ligand (Shattil et al., 1999). This is well documented to be a key event in the activation of αIIbβ3 following treatment of platelets with thrombin. In addition to affinity modulation, the avidity of an integrin for a multivalent matrix ligand may be increased by regulating the clustering of active heterodimers (Bazzoni & Hemler, 1998). For example, clusters of αvβ3 integrin form at early stages during cell spreading and clearly prior to the assembly of focal complexes (Bialkowska et al., 2000). Several growth factor-activated signalling pathways have been implicated in the activation of integrins prior to focal complex assembly. For instance the small GTPase, Rab4 regulates delivery of αvβ3 to the plasma membrane (Roberts et al., 2001), PI 3-kinase (Nagel et al., 1998) and H-Ras (Kinashi et al., 2000) are clearly involved in integrin affinity modulation, and the calcium-dependent protease, calpain, has recently been shown to mediate integrin clustering (Bialkowska et al., 2000). However the overall picture is far from complete.

Clearly any kinase or other signalling protein found to associate with an integrin shortly following cell activation, but prior to its incorporation into focal complexes would be potentially of interest as a possible regulator of integrin function. To identify candidates for this job, I immunoprecipitated integrins from fibroblasts shortly following activation with PDGF, and screened them for associated proteins that were rich in phosphotyrosine and phosphothreonine. I report that following PDGF addition, active
ERK1 is an abundant component of αvβ3 integrin immunoprecipitates. The association of ERK with αvβ3 forms in plasma membrane puncta prior to delivery of integrin to focal complexes. Moreover, I find that recruitment of active ERK1 to αvβ3 integrin is necessary for cells to spread effectively on vitronectin and thus may define a mechanism whereby a growth factor activated signalling pathway can directly influence integrin function.
4.2 RESULTS

4.2.1 Active ERK1 is associated with αvβ3 integrin

Swiss 3T3 fibroblasts were serum starved for 30 mins and then stimulated with 10 ng/ml PDGF for 10 mins, or allowed to remain quiescent. Cells were immediately cooled to 4°C and surface-labelled with NHS-SS-Biotin. Labelled cells were lysed in a buffer containing 0.5 % Triton X-100 and 0.25% Igepal, and α5β1 and αvβ3 integrin heterodimers immunoprecipitated from lysates using monoclonal antibodies to either the mouse α5 or mouse β3 integrin chains respectively. PDGF increased the surface expression of αvβ3 by approximately 2-fold (Fig. 4.1A), and this is consistent with my previous work documenting the rapid stimulation of αvβ3 integrin recycling following growth factor addition (Roberts et al., 2001 and chapter 3).

To gain insight into key biochemical events that occur during the early stages of focal complex assembly, I was interested in the complement of active cell-signalling proteins that may coimmunoprecipitate with integrins shortly following treatment of cells with growth factor. Therefore, integrin immunoprecipitates were analysed by western blotting with antibodies recognising phosphotyrosine and phosphothreonine. In serum-starved cells, low levels of phosphotyrosine- or phosphothreonine-containing proteins coimmunoprecipitated with either α5β1 or αvβ3 integrins (Fig. 4.1B&C). However following addition of PDGF, a 44kDa protein (p44) that was rich in both phosphotyrosine and phosphothreonine was particularly abundant in immunoprecipitates of αvβ3 integrin (Fig. 4.1B&C). p44 was only present at low levels in α5β1 immunoprecipitates and, moreover, there was no phosphotyrosine signal at 120 kDa or 70 kDa, indicating that FAK and paxillin were not associated with either integrin. I was, however, able to coimmunoprecipitate small quantities of a phosphotyrosine-containing protein of 190 kDa with αvβ3 and this is likely to represent association of the PDGF receptor with the integrin (Borges et al., 2000).

A previous report has documented the presence of ERK kinases in newly forming focal complexes (Fincham et al., 2000). Both p44 ERK1 and p42 ERK2 would be expected to be rich in phosphotyrosine and phosphothreonine following cell activation, so I tested for the presence of these kinases in the integrin immunoprecipitates. Western blotting with an antibody that recognises both p44 ERK1 and p42 ERK2 revealed that p44 indeed represented ERK1, and this was associated with αvβ3 only following PDGF treatment (Fig. 4.1D). A small quantity of ERK1 was found to be associated with α5β1
integrin, however this was not increased by addition of PDGF (Fig. 4.1D). Interestingly, no detectable p42 ERK2 was associated with αvβ3, indicating that recruitment of ERK1 was completely isoform specific. ERK1 must be phosphorylated on both threonine 183 and tyrosine 185 to be active, and the presence of signals for phosphotyrosine and phosphothreonine in p44 implied that it was indeed active ERK1. This was confirmed by western blotting using a phosphospecific antibody that recognised ERK kinase only when phosphorylated at both of these positions (Fig. 4.1D). The phosphotyrosine-containing protein at 60 kDa (p60; Fig. 4.1B) is likely to represent a Src-family kinase. We are currently investigating the identity of p60 and the significance of its association with αvβ3 integrin.

To determine whether ERK1 was also able to associate with human αvβ3, and to confirm that its presence in mouse αvβ3 immunoprecipitates was not an artifactual characteristic of the antibody employed, human integrins were transfected into NIH 3T3 fibroblasts and αvβ3 was immunoprecipitated with a monoclonal antibody that was specific for the human β3 integrin chain (hβ3). Surface labelling indicated that this antibody is unable to precipitate mouse αvβ3 (Fig. 4.2A), and accordingly I was unable to detect any phosphotyrosine/phosphothreonine-containing proteins nor ERK kinases associated with anti-human β3 monoclonal antibody-coated beads when they were incubated with lysates prepared from mock transfected cells that had been treated with PDGF (Fig. 4.2 B-E). However, following transfection of NIH 3T3 fibroblasts with the human αv and β3 integrin chains, surface labelled proteins corresponding to the αvβ3 heterodimer were prominent in the immunoprecipitates and these displayed increased surface expression following PDGF addition (Fig. 4.2 A). Moreover, in transfected cells a profile of phosphotyrosine/phosphothreonine-containing proteins, similar to that found associated with the mouse integrin, were coimmunoprecipitated with human αvβ3 following PDGF addition (Fig. 4.2 B&C), and western blotting revealed that the 44 kD band (p44) represented active ERK1 (Fig. 4.2 D).

4.2.2 Recruitment of αvβ3 and ERK to plasma membrane puncta

I have previously shown that αvβ3 integrin is incorporated into punctate complexes at the dorsal plasma membrane immediately following Rab4-dependent recycling, and these are subsequently redistributed into peripheral focal complexes (Roberts et al., 2001 and Chapter 3). Given that ERK1 association with αvβ3 was
Fig. 4.1 Active ERK1 is associated with endogenous mouse αvβ3 integrin in Swiss 3T3 fibroblasts
Swiss 3T3 fibroblasts were serum-starved for 30 min and then treated with 10 ng/ml PDGF-BB for 10 min or allowed to remain quiescent. Cells were surface-labelled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4 °C and lysed in a buffer containing 0.5% (v/v) Triton X-100 and 0.25% (v/v) Igepal CA-630. Lysates were immunoprecipitated (IP) with monoclonal antibodies against mouse α5 (mα5) and β3 (mβ3) integrins. Immunoprecipitated material was then analysed by western blotting with peroxidase-conjugated streptavidin (A; SA-HRP), anti-phosphotyrosine (B; PY), anti-phosphothreonine (C; PT), anti-ERK1/2 and anti-phospho-ERK1/2 (D). The positions of α5, αv, β1 and β3 integrin chains, immunoglobulin heavy chain (IgG HC) and the ERK1/2 kinases are indicated.
Fig. 4.2 Active ERK1 is associated with human αβ3 integrin expressed in NIH 3T3 fibroblasts

NIH 3T3 fibroblasts were transfected with human αβ3 integrin (hαβ3) or empty vector control (mock), then serum-starved and challenged with PDGF-BB as for Fig. 1. Following surface labelling, cells were lysed and immunoprecipitated (IP) with monoclonal antibodies against human β3 (hβ3) integrin. Immobilised material was analysed by western blotting with peroxidase-conjugated streptavidin or anti-human β3 integrin (A; SA-HRP & hβ3), anti-phosphotyrosine (B; PY), anti-phosphothreonine (C; PT), anti-ERK1/2 and anti-phospho-ERK1/2 (D). The positions of αv and β3 integrin chains, immunoglobulin heavy chain (IgG HC) and the ERK1/2 kinases are indicated.
established within 10 min of PDGF addition, I wished to determine whether the kinase was also recruited to plasma membrane puncta. Cells were serum starved for 30 min, treated with PDGF for 10 min and surface αvβ3 and ERK kinase visualised by immunofluorescence. In serum-starved cells, immunoreactive ERK was seen to focus in the perinuclear region, perhaps suggesting sequestration of the kinase upon an endomembrane compartment or even at the microtubule organising centre (Fig. 4.3C). Upon addition of PDGF, ERK rapidly redistributed such that it could now be seen in the nucleus and also dispersed into a punctate array across the cell surface (Fig. 4.3G). This resembled the distribution assumed by αvβ3 (Fig. 4.3E), and examination of the higher magnification micrograph shown in Fig. 4.4, revealed a close co-localisation of ERK and αvβ3 integrin in these small punctate structures. These puncta did not contain caveolin1 so are distinct from caveolin-containing membrane islands, nor did they contain paxillin or other markers of focal adhesions and complexes (data not shown). Following a longer (30 min) exposure to PDGF, ERK can still be clearly seen in the nucleus, but the surface puncta are no longer prominent and the kinase is present as a fine array of focal complexes in the peripheral lamellae that paralleled the distribution of αvβ3 integrin (Fig. 4.3 I-L). Taken together, these immunofluorescence and biochemical data suggest that shortly following growth factor addition, ERK kinase and αvβ3 form a physical association within small punctate complexes in the plasma membrane, these then subsequently redistribute to form focal complexes in the peripheral lamellae.

4.2.3 Association of ERK1 with αvβ3 integrin requires the activity of MEK

ERK1 is activated by phosphorylation on threonine 183 and tyrosine 185 by the dual specificity kinase, MEK. I investigated whether treatment of cells with the MEK inhibitor, PD98059 (Dudley et al., 1995), affected the association of αvβ3 with ERK1 and the recruitment of ERK1 to αvβ3-containing puncta. Serum-starved cells were treated with 12 μM PD98059 for 10 min, following which they were challenged with PDGF and lysed for immunoprecipitation or fixed for immunofluorescence. This concentration of PD98059 completely ablated the association of ERK1 with immunoprecipitates of αvβ3 (Fig. 4.5A) and, in accordance with this, markedly reduced the appearance of ERK in integrin-rich plasma membrane puncta (Fig. 4.5B-G). These
Fig. 4.3 Recruitment of αvβ3 integrin and ERK to focal complexes

Swiss 3T3 fibroblasts were serum-starved for 30 min and then challenged with 10 ng/ml PDGF-BB for 10 min (E-H), or 30 min (I-L), or allowed to remain quiescent (A-D). Following fixation in 2% paraformaldehyde, surface αvβ3 (A, E, I; green) or cellular ERK kinase (C, G, K; green) were visualised by indirect immunofluorescence and cells were counterstained with phalloidin (B, D, F, H, J, L; red). Surface-only αvβ3-staining was obtained by addition of the primary antibody prior to detergent permeabilisation. Bar, 10μm.
Swiss 3T3 fibroblasts were serum-starved for 30 min, challenged with 10 ng/ml PDGF-BB for 10 min and then fixed in 2% paraformaldehyde. Surface αvβ3 integrin was visualised by indirect immunofluorescence (A, B; green). Following this, cells were detergent-permeabilised and counterstained for cellular ERK kinase (C, D; red). Colocalisation of the two fluorophores is shown in yellow (E, F). Bars, 10μm (A, C, E; left-hand panels) and 2.5μm (B, D, F; right-hand panels).
data show that ERK1 must be phosphorylated by MEK in order to be recruited to αvβ3 integrin.

It is important to note that the concentration of PD98059 employed for these experiments (12 μM), albeit sufficient to completely negate association of ERK1 with integrin, had only partial effects on the PDGF-induced recruitment of ERK to the cell nucleus (Fig. 4.5F). This suggests the presence of two pools of ERK in fibroblasts; one whose activation by MEK is particularly sensitive to PD98059 and which is available upon activation for incorporation into integrin complexes, and another pool whose phosphorylation is more resistant to the inhibition of MEK by PD98059 and which is transported to the nucleus following activation.

4.2.4 Association of ERK1 with αvβ3 is not necessary for integrin recycling.

I have previously shown that PDGF increases the recycling of αvβ3 from early endosomes to plasma membrane via a Rab4-dependent mechanism (Roberts et al., 2001 and chapter 3). To investigate the possibility that recruitment of ERK1 to αvβ3 is necessary for recycling I studied the effect of PD98059 on αvβ3 recycling. Recycling of αvβ3 from early endosomes to the plasma membrane was assayed using the method outlined in chapter 3 and PD98059 had no effect on the ability of PDGF to elicit the recycling of αvβ3 from early endosomes (Fig. 4.6). These data are consistent with the images presented in Fig. 4.5E-G where, albeit PD98059 suppressed ERK recruitment to αvβ3-rich structures, recruitment of the integrin itself to the plasma membrane was largely unaffected by MEK inhibition. It is interesting to note, however, that the integrin-containing puncta were smaller and more numerous in the presence of PD98059, indicating that ERK may have a role in the clustering of αvβ3.

4.2.5 Recycling of αvβ3 is not a prerequisite for association of ERK1 with αvβ3

Having shown that delivery of αvβ3 to the plasma membrane was independent of ERK1 recruitment to the integrin, I wished to determine whether the recycling of αvβ3 integrin was a prerequisite for its association with ERK1. Recycling of αvβ3 from early endosomes to the plasma membrane was powerfully stimulated by PDGF and, consistent with my previous studies this component of integrin vesicular transport was completely ablated by expression of the dominant negative Rab4 construct, N121IRab4 (Fig. 4.7A). However, the association of ERK1 with αvβ3 remained undiminished (and was possibly
Fig. 4.5 PD98059 inhibits association of ERK with αvβ3 integrin

(A) Serum-starved Swiss 3T3 fibroblasts were incubated for 10 min in the absence or presence of 12μM PD98059 and then challenged with 10 ng/ml PDGF-BB or allowed to remain quiescent. Cells were lysed and αvβ3 integrin immunoprecipitated (IP) from the lysates with monoclonal antibodies against the mouse β3 integrin chain. Immobilised material was then analysed by western blotting with an antibody against ERK1/2. The migration positions of the ERK1/2 kinases are indicated. (B-G) Serum-starved Swiss 3T3 fibroblasts were incubated for 10 min in the absence (B-D) or presence (E-F) of 12μM PD98059, challenged with 10 ng/ml PDGF-BB and then fixed in 2% paraformaldehyde. Surface αvβ3 integrin was visualised by indirect immunofluorescence (B, E; green). Following this, cells were detergent-permeabilised and counterstained for cellular ERK (C, F; red). Colocalisation of the two fluorophores is shown in yellow (D, G). Bar, 10μm.
Fig. 4.6 PDGF-stimulated recycling of αvβ3 does not require the activity of MEK

Serum-starved Swiss 3T3 fibroblasts were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C and internalisation allowed to proceed for 15 min at 22 °C in the presence and absence of 12 μM PD98059. Biotin was removed from receptors remaining at the cell surface by treatment with MesNa at 4 °C and cells were rewarmed to 37 °C for 10 min in the absence or presence of 10 ng/ml PDGF-BB to allow recycling to the plasma membrane, followed by a second reduction with MesNa. Cells were lysed and integrin-biotinylation determined by capture-ELISA using microtitre wells coated with anti-mouse β3 integrin monoclonal antibodies. The proportion of integrin recycled to the plasma membrane is expressed as % of the pool of integrin labelled during the internalisation period (Values are mean ± s.e.m. from 3 separate experiments).
even increased) (Fig. 4.7B), despite the complete blockade of ανβ3 recycling imposed by this dominant negative construct. These data indicate that active ERK1 can associate with ανβ3 irrespective of whether the integrin is present in the early endosome or at the plasma membrane.

4.2.6 Recycling of ανβ3 and active ERK are required for cell spreading on vitronectin

To investigate the role of ERK1 in an ανβ3-mediated events, cells were allowed to spread on vitronectin, a good ligand for ανβ3 but not α5β1, in the presence and absence of 12μM PD98059. PD98059 inhibited cell spreading on vitronectin by approximately 50 %, indicating a requirement for active ERK in this process (Fig. 4.8). In contrast, PD98059 did not inhibit spreading on fibronectin. This matrix dependence of PD98059 action indicates that ERK activity is required for the function of a vitronectin-binding integrin, and is consistent with our observation that ERK1 is found associated with ανβ3 and not α5β1. Inhibition of ανβ3 recycling by expression of dominant negative S22NRab4, also inhibited cell spreading on vitronectin to similar extent as did PD98059 (Fig. 4.8), indicating that the activities of Rab4 and ERK are both required for the function of ανβ3, but not α5β1 integrin. Having demonstrated that recruitment of active ERK1 to ανβ3 and the Rab4-dependent recycling of the integrin can be evoked independently from one another, I was interested in determining the effect of inhibiting both these events simultaneously. The inhibitory effects of PD98059 and S22NRab4 were not additive indicating that they indeed are affecting the function of the same integrin, and that both efficient recycling and recruitment of active ERK1 to the integrin are necessary for ανβ3 function.
Fig. 4.7 Dominant negative Rab4 does not block association of ERK1 with αvβ3 integrin. NIH 3T3 fibroblasts were transfected with human αvβ3 integrin in combination with wild-type Rab4 (wtRab4), or N121IRab4 as indicated. (A) Transfected cells were serum-starved, surface-labelled and integrin recycling performed in the presence and absence of 10 ng/ml PDGF-BB as for Fig. 6. Biotinylated integrin was determined by capture-ELISA using microtitre wells coated with anti-human β3 monoclonal antibodies. Values are mean ± s.e.m. from 3 separate experiments. (B) Transfected cells were serum-starved and then challenged with 10 ng/ml PDGF-BB or allowed to remain quiescent. Cells were lysed and αvβ3 integrin immunoprecipitated (IP) from the lysates with monoclonal antibodies against the human β3 integrin chain (anti-hβ3) as for Fig. 2. Immobilised material was then analysed by western blotting with an antibody against ERK1/2. The migration positions of the ERK1/2 kinases are indicated.
Fig. 4.8 Cell spreading on vitronectin requires the activity of MEK and Rab4

NIH 3T3 cells were transfected with wild-type Rab4 or S22NRab4 in combination with a β-galactosidase transfection marker. Following trypsinisation, cells were allowed to adhere to either vitronectin (VN) or fibronectin (FN) in the presence of 10 ng/ml PDGF-BB for 1 hr, with or without 12 μM PD98059. The attached cells were fixed and stained for β-galactosidase expression, photographed with a digital camera and the area of transfected cells determined by delineation of the cell envelope using the ‘NIH image’ software. The data are expressed as a % of the cell area of wild-type Rab4-expressing cells following spreading on VN. (Values are mean ± s.e.m)
4.3 DISCUSSION

4.3.1 Summary

Here I show that following addition of PDGF to serum-starved fibroblasts, a 44kDa protein that is rich in phosphotyrosine and phosphothreonine coimmunoprecipitates with \( \alpha v \beta 3 \) integrin. Western blotting with phosphospecific antibodies revealed that this protein was active p44 ERK1. Immediately following PDGF addition, ERK was seen to co-localise with \( \alpha v \beta 3 \) in numerous small puncta distributed evenly over the cell surface and only later did these redistribute to focal complexes in the peripheral lamellae. These events are summarised schematically in Fig. 4.9. The association of ERK1 with \( \alpha v \beta 3 \) was particularly sensitive to treatment of the cells with the MEK inhibitor, PD98059, however, this compound had no effect on the Rab4-dependent flux of integrin from early endosomes to the plasma membrane. Correspondingly, inhibition of Rab4 had no effect on the recruitment of ERK1 to \( \alpha v \beta 3 \) integrin, indicating that integrin recycling and the recruitment of active ERK1 are not interdependent. PD98059 significantly reduced cell spreading on vitronectin, and did so to the same degree as did dominant negative Rab4. This indicates that \( \alpha v \beta 3 \) must recycle to the plasma membrane via the Rab4 pathway and recruit ERK1 in order to function efficiently (see Fig. 4.10).

4.3.2 Role of integrins in ERK translocation

In resting cells, ERK is retained in the cytoplasm in tight association with the microtubular cytoskeleton (Reszka et al., 1995) and it is likely, therefore, that the perinuclear accumulation of ERK that is observed in serum-starved fibroblasts indicates an association with the microtubule organising centre. Upon stimulation, ERK translocates from the cytoplasm to the nucleus, where it influences gene expression by phosphorylating the transcription factors, Elk1 and Sap-1a. This enhances expression of a number of early response elements, such as c-fos (Gille et al., 1992), and ultimately leads to the induction of cyclin D1 and progression through the G1 phase of the cell cycle (Lavoie et al., 1996). The engagement of integrin is known to profoundly enhance ERK activation in response to growth factor addition, and this provides a rationale for the much-studied phenomenon of anchorage-dependent growth (Renshaw et al., 1997). Enhancement of ERK signalling is thought to be mediated by a diverse array of integrin-activated signalling pathways, most of which also lead to reorganisation of the actin.
Fig. 4.9 Scheme for association of active ERK with αvβ3 integrin in plasma membrane complexes.

In this model PDGF addition leads to phosphorylation of cytoplasmic ERK via the Ras/Raf/MEK signalling axis. Active ERK1 is translocated to the nucleus and also recruited to a plasma membrane complex containing αvβ3 integrin. This initiates the clustering of unligated αvβ3 into punctate complexes on the surface of the plasma membrane. When these engage with the ECM at the cell periphery they are incorporated into focal complexes.
Fig. 4.10 Pathways involved in modulating integrin function. The recycling of αvβ3 via a Rab4–dependent pathway and the formation of ERK/integrin clusters are both necessary for integrin function.
cytoskeleton. Indeed, a recent study has shown that integrin mediated adhesion is necessary for efficient nuclear translocation of ERK via a mechanism that clearly requires an intact actin cytoskeleton (Aplin et al., 2001). This may be mediated by association of ERK with the focal adhesion machinery, and it is possible that incorporation of ERK1 into an integrin-containing complex is part of a sequence of molecular events leading ultimately to its delivery to the nucleus in adherent cells. Two aspects of my data, however, argue against this possibility. Firstly, ERK1 recruitment to αvβ3 begins to occur at around 5 min following PDGF addition. However, the translocation of ERK to the nucleus is, if anything, faster than this, arguing against a sequence of events whereby ERK is obliged to associate with αvβ3 and passage through focal complexes in order to reach the nucleus. Secondly, the concentration of PD98059 employed in the present study was found to completely ablate association of ERK1 with αvβ3, but had no effect on nuclear accumulation of ERK. This implies that different pools of cytoplasmic ERK are destined for transport to the nucleus and the plasma membrane following growth factor addition. The activation of the former being less sensitive to treatment of cells with PD98059 than the latter.

4.3.3 A role for ERK at the plasma membrane

A number of recent studies have shown that ERK has an important role in the cytoplasm and that this is likely to be distinct from its activity in the nucleus. The sea star oocyte homologue of ERK1 directly phosphorylates myosin light chain kinase (MLCK) (Morrison et al., 1996), and more recently activation of ERKs with a constitutively active MEK has been shown to enhance cell migration via phosphorylation of MLCK (Klemke et al., 1997). A more recent study has demonstrated that active ERK is recruited to focal adhesions, and controls their assembly by virtue of its ability to phosphorylate and activate MLCK (Fincham et al., 2000). Thus if phospho-ERK levels are lowered using U0126 (a more potent MEK inhibitor than PD98059), the assembly of focal complexes is inhibited and consequently the ability of cells to spread on the ECM is compromised. I am able to confirm that ERK is indeed targeted to focal complexes and, furthermore show that this is likely to be achieved by its association with an ECM receptor, αvβ3 integrin.

My data indicate that the association of αvβ3 with ERK occurs rapidly following PDGF addition and that the resulting complex localises to punctate clusters in the plasma
membrane, prior to its incorporation into focal complexes. Hitherto, many studies have focussed on the role of integrins in focal adhesions and complexes and it is generally accepted that integrins are brought into close proximity with the various signalling molecules that mediate focal adhesion signalling, as a consequence of the activity of the Rho subfamily GTPases, such as RhoA and Rac (Nobes & Hall, 1995). However it is now becoming clear that certain pathways promote the association of integrins with other signalling components upstream of focal complex assembly. A recent study has highlighted a novel integrin complex, referred to as an integrin cluster, that forms upstream of Rac activation and focal complex assembly (Bialkowska et al., 2000). These workers reported that integrin clusters differ from focal complexes in both their distribution and molecular composition. The αvβ3/ERK-containing complexes described in this study also differ in their composition from focal complexes. For instance, they do not stain for established focal adhesion markers, such as vinculin and paxillin nor do any of these proteins coimmunoprecipitate with αvβ3 following PDGF addition. It is interesting to speculate what kind of cellular structure these αvβ3/ERK-rich complexes may be. Labelling experiments with [32P]-orthophosphate have indicated that, even following extensive treatment with non-ionic detergent (0.5% (v/v) Triton X-100 and 0.25% (v/v) Igepal), labelled phospholipids are tightly associated with αvβ3 immunoprecipitates. Moreover, the quantity of coimmunoprecipitating phospholipid increased dramatically in response to addition of PDGF, and this was opposed by PD98059 (Jim Norman, personal communication). Reorganisation of lipid rafts and other plasma membrane lipid subdomains has been reported to occur following activation of a number of signalling pathways (Janes et al., 1999). It is possible, therefore, that active ERK kinase can act at the plasma membrane to induce clustering of integrin into large detergent-resistant raft like membrane microdomains. Integrins have been reported to associate with many other types of transmembrane and other proteins, which in principle may coalesce to form an extensive network (Claas et al., 2001). Indeed αvβ3 integrin is known to associate with CD47, or integrin-associated protein (IAP), in a plasma membrane lipid raft (Green et al., 1999). Also the integrin-associated tetraspannin, CD81, has been shown to localise to such a membrane domain (Claas et al., 2001), and it is interesting in this regard that CD81 has been observed to co-localise with αvβ3 in puncta following PDGF treatment (Jim Norman, personal communication).
Following expression of dominant negative Rab4, αvβ3 would be expected to accumulate in early endosomes. This construct, however, does not reduce the recruitment of ERK1 to αvβ3 immunoprecipitates, and indicates that the association of active kinase to the integrin may be established either on the surface of endosomes or at the plasma membrane. Indeed, large integrin-tetraspannin complexes have been detected on intracellular vesicles (Berditchevski et al., 1997), and this implies that associations made between integrins and other signalling molecules and membrane proteins may persist whilst the integrin engages in endo/exocytic cycling. There is mounting evidence that the incorporation of membrane proteins into raft-like domains is a key sorting event in the secretory pathway (Muniz & Riezman, 2000). However it is unlikely that recruitment of active ERK to αvβ3 at the endosome is necessary to direct its recycling, as the rate of delivery of early endosomal αvβ3 to the plasma membrane was clearly unaffected by PD98059.

The observation that PD98059 compromises cell spreading on vitronectin, but not on fibronectin, implicates the activity of ERK in the activation of αvβ3 integrin. Many integrins, including αvβ3, can assume different states with respect to ligand binding and engagement and there are many examples of the transition between these states being controlled by signalling pathways within the cell; a phenomenon termed inside-out signalling (Shattil et al., 1999). The affinity of an individual integrin heterodimer for its ligand may be increased, and changes in the lateral mobility and clustering of integrins can also affect the avidity of integrin binding to multivalent ligands. Regulation of either the affinity or avidity of an integrin for its ligand will have profound influence on the ability of cells to spread on the extracellular matrix. The ability of the platelet integrin αIIbβ3 to bind fibrinogen has been shown to be inhibited by dominant negative mutants of Raf-1 or MEK1 (Li et al., 2001). This implicates the MEK/ERK signalling axis in inside-out signalling to β3 integrins, and this could by achieved by influencing either the affinity state or the clustering of integrin. I favour the explanation that the recruitment of ERK leads to integrin clustering, as I find that integrin-containing puncta were clearly smaller and more numerous in the presence of PD98059.

It is interesting to speculate how active ERK may influence the clustering of αvβ3 integrin. The activation of calpain, a calcium-dependent neutral protease, has been shown to be mediated by an ERK kinase signalling pathway (Glading et al., 2000). Moreover, a recent study from the same group has elegantly demonstrated that ERK
tethered to the plasma membrane by a CAAX, Ras farnesylation sequence, is able to activate calpain at this location (Glading et al., 2001). In this study, the activation of calpain is associated with de-adhesion and the disassembly of focal complexes. However this crucially may depend on the cell type and particular system employed. Calpain has been reported to markedly facilitate the spreading of NIH-3T3 fibroblasts, and the work of Fox and co-workers clearly implicates calpain in the clustering of \( \alpha \nu \beta 3 \) integrin prior to its incorporation into focal complexes (Bialkowska et al., 2000 and Reddy et al., 2001). Indeed, there is a genetic precedent for the role of calpain in the activation of \( \beta 3 \) integrins, as disruption of the \( \mu \)-calpain gene in mice causes a significant reduction in platelet aggregation and clot retraction (Azam et al., 2001). These are both functions that depend on inside-out activation of \( \alpha IIb\beta 3 \) integrin. A recent study has provided a potential molecular explanation for the effects of calpain on integrin clustering and activation. Calpain is able to cleave the integrin- and actin-binding protein, talin into a 47 kDa globular head domain and a 190 kDa C-terminal rod (Yan et al., 2001). The talin head has a much higher affinity for the \( \beta 3 \)-integrin cytodomain than the intact protein, and this association leads to integrin activation (Yan et al., 2001 and Calderwood et al., 1999). In principle, these events could be assembled into a signalling loop in which active ERK associates with \( \alpha \nu \beta 3 \) integrin in the plasma membrane where it promotes local activation of calpain. The resulting calpain-cleavage product of talin could associate with the integrins, in turn leading to its activation and clustering. This could continue until the complex achieves sufficient size and, therefore, avidity to engage the ECM and incorporate into focal complexes.

In summary, my studies identify a novel association of active ERK1 kinase with \( \alpha \nu \beta 3 \) integrin which is established in advance of the incorporation of the integrin into focal complexes. Formation of this complex was not necessary for the trafficking of \( \alpha \nu \beta 3 \) through the Rab4-dependent recycling pathway, nor was the activity of Rab4 required for association of \( \alpha \nu \beta 3 \) with ERK. However the activity of ERK and Rab4 are clearly required for cells to spread on vitronectin. I suggest that recruitment of ERK to \( \alpha \nu \beta 3 \) and the Rab4-dependent recycling are parallel growth factor-activated events that are necessary for integrin function.
Chapter Five: Vascular endothelial growth factors (VEGFs) regulate \( \alpha v \beta 3 \) recycling in Human Umbilical Vein Endothelial Cells (HUVECs)
Chapter 5: Vascular endothelial growth factors (VEGFs) regulate αvβ3 Recycling in Human Umbilical Vein Endothelial Cells (HUVECs)

5.1 INTRODUCTION

5.1.1 Integrins and angiogenesis

At least six integrins heterodimers (αvβ3, αvβ5, α5β1, α2β1, α1β1 and αvβ1) have been linked to the control of angiogenesis (Hynes et al., 1999); the process by which new blood vessels are formed. Mounting evidence in the literature suggests αv integrin, particularly αvβ3, is pivotal to this process. Central to this argument is the fact that αvβ3 is preferentially expressed on angiogenic blood vessels (Brooks et al., 1994) and that blockade of this integrin with either antibodies (vitaxin), peptides and small molecule antagonists have been shown to inhibit blood vessel formation in a number of in vivo and in vitro systems (Guthiel et al., 2000, Posey et al., 2001, Brooks et al., 1994, Kumar et al., 2001 and Westlin, 2001). Direct approaches using knockout mouse models indicate that deletion of the αv gene is lethal and results in blood vessel haemorrhaging in a subset of organs (Bader et al., 1998). Despite this, vascular development remains normal in β3 and β5 knockout mice (Hodivala-Dilke et al., 1999, Huang et al., 2000) and this maybe explained by functional redundancy by αvβ3 and αvβ5 in this process.

5.1.2 Angiogenic growth factors

Vascular endothelial growth factors (VEGFs) are the principal growth factors involved in cell proliferation, migration and secretion of proteases in endothelial cells during the process of angiogenesis. VEGF encompasses a family of structurally related proteins that includes placental growth factor (PIGF), VEGFA, VEGFB, VEGFC and VEGFD (Tischer et al., 1991). VEGFs bind to receptors of the VEGF receptor (VEGFR) family that include Flt1 (VEGFR1), KDR (VEGFR2) and Flt4 (VEGFR3), and these belong to the PDGF receptor tyrosine kinases which are characterised by the presence of seven extracellular immunoglobulin-like domains and a split tyrosine kinase intracellular domain (Karkkainen & Petrova, 2000) (see Fig. 5.1). Neuropilin-1 has also been identified as a VEGF binding receptor but this non-tyrosine kinase receptor has a short cytoplasmic tail with no known signalling function and is thought to act as a docking co-receptor (Soker et al., 1998). VEGF165 is the most abundant of the five
VEGF receptors and their ligands. VEGF tyrosine kinase receptors possess an extracellular domain made up of seven immunoglobulin-like repeats, a single transmembrane region and a large intracellular domain which contains a catalytic domain (CAT) interrupted by a kinase insert domain (KI). Flt1 and KDR are present on vascular endothelial cell (ECs) and Flt1 is uniquely expressed on monocytes whilst Flt4 is preferentially expressed on lymphatic endothelium. Neuropilin1 is a transmembrane receptor with a very short intracellular domain which is not known to have any signalling function. The ligand VEGF165 can bind to and activate both the KDR and the Flt1 receptors, whereas, PIGF has specificity for Flt1. VEGFD can bind to and activate KDR and Flt4, whereas, VEGFC is specific for Flt4.
isoforms of VEGF and is able to bind to both the Flt1 and KDR receptors to activate intracellular signalling pathways (Waltenberger et al., 1994 and Mustonen & Alitalo, 1995). However several members of the VEGF family exhibit receptor specific binding, for instance, PI GF has a high affinity for the Flt1 receptor but is unable to interact with the KDR receptor (Park et al., 1994), whereas VEGFD acts as a ligand for the KDR receptor and the Flt4 receptor, but does not activate Flt1 (Achen et al., 1998) (see Fig. 5.1).

5.1.3 VEGF receptor signalling

Many investigators have made use of in vitro systems to study angiogenic signalling events activated by VEGFs. Human Umbilical Vein Endothelial cells (HUVECs) provide a common model cell system for signalling investigations. In addition to this KDR- and Flt1-expressing NIH 3T3 fibroblasts, and both bovine and porcine endothelial cells have also been employed for some studies, but these have yielded mixed results. For example, PLCγ has been reported to bind to and be activated by Flt1 in Flt1-expressing NIH 3T3 cells (Sawano et al., 1997). On the other hand Flt1-expressing porcine aortic endothelial (PAE) cells have no PLCγ activity in response to PI GF, although these cells show an increase in ERK activation in response to this growth factor (Landgren et al., 1998). It is contentious as to whether Flt1 elicits biologically meaningful signals as it has been difficult to demonstrate VEGF-induced autophosphorylation of Flt1. However, Flt1 has been shown to interact with the p85 subunit of PI(3)K in yeast two hybrid assays (Cunningham et al., 1995) and by in vitro phosphopeptide studies, where interaction was dependent on phosphorylation of tyr1213 in a 15 residue peptide corresponding to the amino acids 1206-1219 of the Flt1 cytoplasmic domain (Yu et al., 2001) and so is likely to signal through the PI(3)K axis. The best-characterised responses mediated by Flt1 are the increased migration of monocytes in response to VEGF and PI GF. Northern blot analysis has shown that human monocytes do not express KDR, indicating this response is likely to occur via the Flt1 receptor (Barleon et al., 1996 and Clauss et al., 1996). There is also evidence to suggest that Flt1 acts as a negative regulator of KDR signalling, for example, domain swapping experiments have revealed that an intracellular juxtamembrane domain of Flt1 can suppress KDR-mediated signalling and cell migration (Gille et al., 2000).
It is currently thought that the biological activities of VEGFs are primarily mediated by the KDR receptor. KDR is activated upon ligand-stimulated dimerisation and autophosphorylation of tyrosine residues in the cytoplasmic kinase domain. Several SH2 domain-containing proteins are recruited to the receptor including Grb2, NCK, Shc and Sck and the protein tyrosine phosphatases SHP-1 and SHP-2 (Guo et al., 1995, Warner et al., 2000 and Kroll & Waltenberger 1997). KDR signalling also activates the PI3K/PKB signalling axis and this has been shown to be involved in the inhibition of apoptosis in HUVECs (Gerber et al., 1998 and Thakker et al., 1998). VEGF stimulates KDR induced DNA synthesis and proliferation in a variety of endothelial cells by stimulating signalling pathways leading to the activation of the extracellular related kinases ERK1 and ERK2 and the activation of JNK (Wu et al., 2000). VEGF treatment can also tyrosine phosphorylate and activate PLCγ which in turn can lead to the activation of PKCs and it has been shown that PKCα and PKCζ isoforms play crucial roles in VEGF induced signalling (Xia et al., 1996).

5.1.4 VEGF and migration

It is thought that endothelial cell migration is critical to angiogenesis. VEGF treatment has been shown to result in the phosphorylation of the focal adhesion proteins FAK and paxillin, and drives the formation of FAK and paxillin-containing focal adhesions resulting in increased cell motility (Abedi & Zachary, 1997; Rousseau et al., 2000). Using specific blocking antibodies to KDR and Flt1 it was determined that focal adhesion formation was driven by KDR activated signalling (Kanno et al., 2000). In addition to this the Flt1 receptor has been shown to activate the p38/SAPK pathway and this pathway has also been suggested to result in actin reorganisation and to promote cell migration (Rousseau et al., 2000 and Kanno et al., 2000). Additionally degradation of the basement membrane is required for endothelial cell migration and is a key step in the initiation of pathological angiogenesis. VEGF induces the expression of matrix-degrading metalloproteinases (MMPs) (Lamoreaux et al., 1998) and αvβ3 integrin has been reported to associate with matrix metalloproteinase 2 (MMP2), and this complex facilitates vascular invasion (Brooks et al., 1996).

It is becoming clear that there is considerable interplay between integrins and angiogenic growth factor signalling. The KDR receptor has been reported to bind to αvβ3 (Soldi et al., 1999) in an association mediated by the extracellular portion of the
integrin and that requires the presence of the \( \alpha_v \) subunit (Borges et al., 2000). Furthermore, KDR phosphorylation and mitogenicity induced by VEGF\(_{165} \) is enhanced when cells are plated on vitronectin, a ligand for \( \alpha_v\beta3 \) (Soldi et al., 1999).

### 5.1.5 VEGFs and integrin activation

VEGF\(_{165} \) has been shown to activate \( \alpha_v\beta3 \) via signalling pathways involving PI3 kinase and PKB/AKT and this enhances cell adhesion and migration (Byzova et al., 2000). PI3 kinases and PKB/Akt are also required for normal embryonic angiogenesis (Jiang et al., 2000) and are known to be activated downstream of both integrin and VEGF receptor signalling (Gerber et al., 1998, Suzuma et al., 2000, Guinebault et al., 1995, Shaw et al., 1997 and King et al., 1997). This biochemical co-ordination of VEGFRs and integrin signalling pathways suggest that angiogenic factors may exert their effects by modulating integrin function and one possible way to achieve this is via regulation of integrin recycling pathways. Here I describe integrin recycling events in HUVECs and reveal that \( \alpha_v\beta3 \) recycling from early endosomes is stimulated by VEGF treatment and this occurs in response to activation of both the KDR and Flt1 receptors.
5.2 RESULTS

5.2.1 αvβ3 and α5β1 integrins, but not αvβ5 take part in an endo/exocytic cycle

The endo/exocytic cycles of αvβ3, α5β1 and αvβ5 integrins were examined in HUVECs. Internalisation was determined by surface-labelling cells with NHS-SS-biotin at 4°C followed by incubation at 37°C for various times. αVβ3 and α5β1 integrins were internalised by HUVECs resulting in internal pools of 10% and 20% respectively (Fig. 5.2.1A&B), similar to that determined in Swiss 3T3 and NIH fibroblasts (see chapter 3 Fig. 3.2). To determine whether this internal pool represented a steady state between endo- and exocytic rates, internalisation assays were performed in the presence of PMQ. Upon addition of PMQ a marked increase in the measured internalisation rate of αvβ3 and α5β1 integrin was apparent, indicating that both integrins recycle back to the plasma membrane very shortly following internalisation (Fig 5.2A B). In contrast, αvβ5 integrin was not internalised under these conditions even following addition of PMQ (Fig. 5.2C) indicating that this integrin resides at the plasma membrane and did not participate in an endo/exocytic pathway.

5.2.2 VEGF does not affect integrin endocytic rates

To obtain a reliable measure of the effect of VEGF165, PIGF and VEGFD, on the endocytic rate of integrins, internalisation assays were performed in the presence of PMQ. This analysis revealed that VEGF165, PIGF and VEGFD did not affect the endocytic rate for either αvβ3 or α5β1 integrins (Fig. 5.3).

5.2.3 VEGF stimulates the recycling of αvβ3 but not α5β1

In fibroblasts PDGF is able to stimulate Rab4 dependent recycling of αvβ3 from the early endosome to the plasma membrane and this event is necessary for αvβ3 function. It is possible that VEGFs may similarly promote αvβ3 recycling pathways in HUVECs. To monitor recycling from early endosomes, cells were surface-labelled and internalisation allowed to proceed for 20 min at 22°C thus allowing integrin to reach early endosomes and recycling was then determined in the presence and absence of growth factors as before. In the absence of growth factors there was little flux of αvβ3 integrin from the early endosome to the plasma membrane (Fig 5.4). Upon addition of VEGF165 αvβ3 recycling was markedly increased indicating that the trafficking of αvβ3
Fig. 5.2 Internalisation of integrins.

Serum-starved HUVECs were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C and Surface labelled cells were warmed to 37 °C in the absence (○) or presence (■) of 0.6 μM PMQ for the times indicated. Biotin was released from proteins remaining at the cell surface and biotinylated integrin determined by capture-ELISA using microtitre wells coated with anti-human β3 (A), anti human-α5 (B) or anti human αvβ5 (C) integrin monoclonal antibodies.
Fig 5.3 VEGFs do not regulate integrin internalisation

Surface-labelled cells were warmed to 37 °C in the presence of 0.6 μM PMQ in the absence (O) and presence (■) of 50 ng/ml VEGF₁₆₅ (A,B), 25ng/ml PI GF(C,D) or 150 ng/ml VEGFD (E,F) for the times indicated. Internalised integrin was determined using microtitre wells coated with anti-human β3 (A,C&E) or anti-human α5 (B,D&F) integrin monoclonal antibodies.
from early endosomes back to the plasma membrane was completely growth factor dependent. Treatment with other angiogenic factors distinct from VEGFs i.e. bFGF did not stimulate ανβ3 recycling.

VEGFs bind to and activate the tyrosine kinase receptors KDR and Flt1 to elicit cell signalling. PIGF activates Flt1 and this polypeptide stimulated the flux of ανβ3 from early endosome to the plasma membrane to a similar degree as did VEGF (Fig. 5.4A). Interestingly treatment of HUVECS with VEGFD, which activates KDR (and not Flt1) also drove ανβ3 early endosome to the plasma membrane (Fig 5.4A). Recycling of α5β1 from early endosomes did occur, but it was not reproducibly stimulated by growth factor treatment (Fig. 5.4B). These data indicate that VEGFs are able to stimulate the flux of ανβ3 from early endosomes to the plasma membrane in HUVECs via the activation of either Flt1 or KDR receptors.

5.2.4 Wortmannin inhibits VEGF-stimulated ανβ3 recycling

The fact that both KDR and Flt1 are able to drive ανβ3 recycling suggests that a common module in the downstream signalling from these receptors regulates integrin recycling. PI3-kinases have been reported to be activated downstream of KDR and Flt1 so it was of interest to determine whether these kinases contributed to signalling events controlling integrin recycling in HUVECs. WMN treatment had no effect on ανβ3 internalisation (Fig 5.5 A). However WMN significantly inhibited VEGF165-driven ανβ3 recycling (Fig. 5.5B), indicating that VEGFs act via PI3 kinases to promote the recycling of ανβ3.

5.2.5 Surface distribution of ανβ3 in HUVECs

The distribution of ανβ3 integrin on the surface of HUVECs was examined by immunofluorescence; surface-only labelling was obtained by addition of primary antibodies prior to Triton X-100 permeabilisation. HUVECs were plated onto vitronectin coated coverslips and serum starved for 30 minutes prior to experimentation. The cells were then stimulated for 30 (Fig 5.6 C&D) or 60 minutes (Fig. 5.6 E, F) with VEGF165, or left in serum free medium (Fig. 5.6 A&B), and the integrin visualised with respect to the actin cytoskeleton. Surface expression of ανβ3 on serum-starved cells was low as detected by immunofluorescence, although some focal contacts could be seen (Fig 5.6A). After a 30-minute treatment with VEGF165, the surface levels of ανβ3 integrin were
Fig. 5.4 Recycling of αvβ3 and α5β1 integrins.

Cells were surface-labelled and internalisation allowed to proceed for 20 min at 22°C and biotin removed from receptors remaining at the cell surface by treatment with MesNa at 4°C. Cells were then re-warmed to 37°C for the times indicated in the absence or presence of 50 ng/ml VEGF165, 25 ng/ml PIGF, 150 ng/ml VEGFD or 20 ng/ml FGF, to allow recycling to the plasma membrane, followed by a second reduction with MesNa. Cells were lysed and integrin-biotinylation determined by capture-ELISA using microtitre wells coated with anti-human-β3 (A) or anti-human α5 (B) integrin monoclonal antibodies. The proportion of integrin recycled to the plasma membrane is expressed as % of the pool of integrin labelled during the internalisation period.
Fig. 5.5 The Effect of Wortmannin on ανβ3 Internalisation and Recycling

(A) Internalisation: Cells were serum starved in the presence of 100 nM wortmannin (WMN) and then surface labelled with NHS SS Biotin at 4 °C. Cells were then warmed to 37 °C in the presence of 0.6 μM PMQ and 100 nM WMN in the absence (Ο) and presence (■) of 50 ng/ml VEGF<sub>165</sub> for the times indicated. Internalised integrin was determined using microtitre wells coated with anti-human β3.

(B) Recycling: Cells were surface-labelled and internalisation allowed to proceed for 20 min at 22 °C in the presence of 100 nM WMN and biotin removed from receptors remaining at the cell surface by treatment with MesNa at 4 °C. Cells were then re-warmed to 37 °C for the times indicated in the presence of 50 ng/ml VEGF<sub>165</sub> in the presence and absence of 100 nM WMN to allow recycling to the plasma membrane, followed by a second reduction with MesNa. Cells were lysed and integrin-biotinylation determined by capture-ELISA using microtitre wells coated with anti-human-β3. The proportion of integrin recycled to the plasma membrane is expressed as % of the pool of integrin labelled during the internalisation period.
increased and seen as puncta distributed evenly across the surface of the cell (Fig 5.6 C). Examination of optical slices from the confocal image indicate that these puncta were present primarily on the dorsal surface of the cell (not shown). After a 60-minute exposure to growth factor, αvβ3 integrin subsequently reorganised into/coalesced to form focal complexes, probably by tethering of integrin to the actin cytoskeleton at the cell periphery (Fig. 5.6E). Upon treatment of cells with the KDR-specific VEGFD, αvβ3 was also incorporated into puncta on the cell surface (Fig. 5.7C) and then re-distributed into focal contacts after longer growth factor treatment times (Fig. 5.7G). Similarly, visualisation of integrin on cells stimulated with PIGF also revealed initial incorporation of integrin into puncta Fig 5.7 E, I). These data indicate that both KDR and Flt1 have similar effects on the recycling of αvβ3 and its surface distribution following recycling.

Serum starved cells displayed few stress fibres and exhibited small amounts of cortical actin staining (Fig. 5.7B). Treatment with VEGFD produced actin staining reminiscent of lamellar ruffles (Fig 5.7D, H). However, PIGF did not seem to induce ruffles but the number of actin stress fibres in the cells was increased and this was particularly apparent after a 60-minute exposure to this growth factor (Fig 5.7J). Accordingly, addition of VEGF₁₆₅, which activates both Flt1 and KDR, acted to produce both stress fibres and lamellar ruffles (Fig. 5.6D, F). Thus, although KDR and Flt1 signalling elicit similar effects on integrin recycling, surface expression and focal complex formation, their effect on the actin cytoskeleton diverge; with KDR activation leading to ruffling and Flt1 activation promoting actin stress fibre assembly.
Fig. 5.6 Visualisation of αβ3 containing Puncta

Cells were plated onto coverslips coated with 20 μg/ml vitronectin. Serum-starved HUVECs were challenged with 50 ng/ml VEGF for either 30 (C, D) or 60 minutes (E, F) or allowed to remain quiescent (A, B). Cells were fixed in 2% paraformaldehyde and surface αβ3 visualised by indirect immunofluorescence (A, C, E); surface-only integrin staining was obtained by addition of the primary antibody prior to the detergent permeabilisation step. F-actin was counterstained with Texas Red-conjugated phalloidin (B, D, F).
Fig. 5.7 Visualisation of αvβ3 containing puncta in response to VEGFD and PIGF.

Cells were plated onto coverslips coated with 20 μg/ml vitronectin. Serum-starved HUVECs were challenged with 150 ng/ml VEGFD (C, D, G, H) or 25 ng/ml PIGF (E, F, I, J) for either 30 (C, D, E, F) or 60 minutes (G, H, I, J) or allowed to remain quiescent (A, B). Cells were fixed in 2% paraformaldehyde and surface αvβ3 visualised by indirect immunofluorescence (A, C, E, G, I); surface-only integrin staining was obtained by addition of the primary antibody prior to the detergent permeabilisation step. F-actin was counterstained with Texas Red-conjugated phalloidin (B, D, F, H, J).
5.3 DISCUSSION

5.3.1 Summary

Integrin trafficking in HUVECs was examined and it was discovered that \( \alpha \nu \beta 3 \) and \( \alpha 5 \beta 1 \) integrins, but not \( \alpha \nu 5 \), take part in an endo/exocytic cycle. Angiogenic factors such as VEGF\(_{165} \), PIGF and VEGFD did not affect integrin internalisation, however, VEGF\(_{165} \), PIGF and VEGFD were able to powerfully stimulate recycling of \( \alpha \nu \beta 3 \), but not \( \alpha 5 \beta 1 \) from early endosomes. This indicates that signalling events downstream of both the KDR and Flt1 receptors regulate \( \alpha \nu \beta 3 \) recycling, and moreover, experiments with wortmannin identified a requirement for PI3 kinase in this process. Upon VEGF treatment, \( \alpha \nu \beta 3 \) was seen to be incorporated into small punctate structures over the cell surface presumably as a result of regulated recycling as was previously found in fibroblasts. Following longer exposures to growth factor, \( \alpha \nu \beta 3 \) was then seen to be incorporated into focal complex structures. The effects of KDR and Flt1 agonists on \( \alpha \nu \beta 3 \) recycling and puncta formation were indistinguishable, however, the activation of KDR and Flt1 had differing effects on the actin cytoskeleton; VEGFD (via the KDR receptor) produced lamellar ruffles, whilst treatment with PIGF, (which acts via the Flt1 receptor) produced actin stress fibres. A summary scheme is outlined in Fig. 5.8.

5.3.2 VEGFs stimulate \( \alpha \nu \beta 3 \) recycling

VEGFS are able to stimulate recycling of \( \alpha \nu \beta 3 \) from early endosomes to the plasma membrane in HUVECs in a strikingly similar fashion to the way PDGF regulates rab4 dependent recycling of \( \alpha \nu \beta 3 \) in fibroblasts (Roberts et al., 2001 and chapter 3). \( \alpha \nu \beta 3 \) recycling is similarly stimulated by both VEGF\(_{165} \), and the receptor specific VEGFs, PIGF and VEGFD. At present the biological role of Flt1 remains uncertain, but these findings indicate that perhaps this receptor does indeed have important role with respect to \( \alpha \nu \beta 3 \) integrin function.

The activation of \( \alpha \nu \beta 3 \) recycling by both KDR and Flt1 indicates that a common signalling element operates downstream of these receptors. PI(3)Ks are known to be activated downstream of KDR (Gerber et al., 1998 and Suzuma et al., 2000), and have been shown to be recruited to specific tyrosine phosphorylated residues on the cytoplasmic tail of Flt1 (Yu et al., 2001) and these experiments with WMN suggest that PI(3)K activity is necessary for VEGF-regulated integrin recycling. I have previously
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Fig. 5. 8 VEGFs and actin cytoskeleton

PIGF and VEGFD are both able to stimulate an EE to PM recycling of αvβ3 integrin which subsequently results in the formation of focal contacts. VEGFD via the activation of KDR induces the formation of lamellar ruffles whilst Flt1 activation by PIGF produces stress fibres.
shown the recycling of αvβ3 is necessary for integrin function during cell adhesion and spreading onto vitronectin indicating this pathway is involved in activation (Roberts et al., 2001 and chapter 3). The PI3 kinase/PKB/Akt signalling axis is required for activation of αvβ3 (Byzova et al., 2000) and it is possible that regulated αvβ3 recycling is an integral part of this process.

5.3.3 Assembly of αvβ3 puncta and subsequent focal complex formation

Following stimulation of αvβ3 recycling with VEGFs, αvβ3 is incorporated into small puncta all over the cell surface. The integrin in these puncta is probably unengaged as indicated by its location primarily on the dorsal surface of the cell. The integrin is subsequently directed to focal complexes on the cell periphery probably by tethering of the integrin to the actin cytoskeleton and engagement of the matrix. VEGF has been previously reported to drive the formation of paxillin and FAK containing focal adhesions, to induce the formation of stress fibres in confluent HUVECs and stimulate cell migration (Abedi & Zachary 1997). Other studies have shown that phosphorylation of FAK and paxillin occurs via the KDR-activated pathway as anti-KDR antibodies completely inhibit phosphorylation of FAK and paxillin, whereas, anti-Flt-1 mAb do not (Sato et al., 2000). However, both KDR and Flt1 pathways are necessary for cell motility (Kanno et al., 2000 and Sato et al., 2000), and it is possible therefore that both Flt1 and KDR are both able to influence cell motility via regulation of integrin recycling pathways.

Endothelial cell chemotaxis is known to require Rac activity (Soga et al., 2001), presumably for initiation of actin polymerisation at the tips of lamellipodia. Direct activation of Rac by KDR has not been demonstrated, but PI3(K) is activated by KDR and it is possible that this in turn could activate Rac and thereby direct the formation of lamellar ruffles. Flt1 is known to regulate endothelial cell migration by modulating the actin cytoskeleton and my data show that this receptor may act through Rho to induce actin stress fibre assembly. Direct activation of Rho by Flt1 has not been demonstrated, but the SAPK/p38 pathway has been shown to be responsible for assembly of stress fibres in endothelial cells and so is possible that p38 acts as an intermediary between Flt1 and activation of Rho.
5.3.4 **Endo-/exocytic cycles and angiogenesis**

It is possible that the endo/exocytic cycles of membrane proteins may be important for angiogenesis. Membrane type 1 metalloprotease (MT1-MMP) is a transmembrane metalloprotease that plays a major role in angiogenesis by directly degrading extracellular matrix components, and indirectly by activating pro-MMP2 (Deryugina *et al.*, 2001). A putative interaction of MT1-MMP and αvβ3 has been proposed in endothelial and carcinoma cells (Puyraimond *et al.*, 2001, Deryugina *et al.*, 2001) and moreover, the membrane localisation of MT1-MMP is critical for cell migration and invasion (Galvez *et al.*, 2001). MT1-MMP has been shown to be internalised via a dynamin dependent mechanism (Jiang *et al.*, 2001) and also appears to recycle from endosomes to the plasma membrane (Gillian Murphy, personnel communication). It is tempting to speculate that MT1-MMP and αvβ3 integrin are co-transported to the plasma membrane in the same recycling vesicles. Such coordination of the targeting of an integrin and a transmembrane protease may greatly enhance the invasive capacity of endothelial cells during angiogenesis.

Urokinase-type plasminogen activator (uPA) binds to its receptor (uPAR) on the cell surface and facilitates cell migration during angiogenesis most probably by directing local proteolysis of the ECM. uPAR takes part in an endo/exocytic cycle and this has been proposed to facilitate the matrix engagement/disengagement cycle during cell migration (Nykjaer *et al.*, 1997). The uPAR is a GPI-linked protein and therefore lacks conventional cytoplasmic domain motifs that could direct its internalisation and/or recycling. uPAR overcomes this by undergoing clathrin-mediated endocytosis by virtue of an association with the α2-macroglobulin receptor (Nykjaer *et al.*, 1992, Conese *et al.*, 1995 and Heegaard *et al.*, 1995). Although uPAR is internalised by ‘piggy-backing’ in this way, no information is available as to the recycling route it takes. Indeed given the fact that the association of PAI1 with the uPAR (which initiates its association with the α2-macroglobulin receptor) is disrupted in the endosome (Nykjaer *et al.*, 1997), it is likely that uPAR and the α2-macroglobulin receptor recycle via different pathways. uPAR is known to associate with αvβ3 and it is interesting to speculate that it may recycle in association with the integrin via a VEGF-regulated pathway. Indeed VEGF has been found to increase uPAR surface levels by 2.8-3.5 fold (Mandriota *et al.*, 1995) suggesting that the growth-factor regulation of uPAR recycling is a distinct possibility. It will be interesting to determine the Rab4-dependence of uPAR recycling.
Mice lacking β3 integrin are viable and fertile and suffer relatively few vascular defects (although they have defects in platelet aggregation and clot retraction), perhaps suggesting that this integrin is not necessary for vascular development (Hodivala-Dilke et al., 1999). However it is well-established that blockade of αvβ3 integrin function by use of blocking antibodies or small molecule inhibitors results in profound inhibition of angiogenesis (Guthiel et al., 2000, Posey et al., 2001, Brooks et al., 1994 Kumar et al., 2001 and Westlin, 2001). A number of explanations have been offered to account for this apparent paradox. For instance, the absence of β3 could up-regulate signalling through VEGF receptors or the blockade of αvβ3 (if it is present) could suppress the function of other proteins that are essential for angiogenesis; a phenomenon termed ‘trans-dominant inhibition’ (Diaz-Gonzalez et al., 1996). It is possible that trans-dominant inhibition may be manifested at the level of the endosome. For instance, cross linking of αvβ3 into immune complexes by vitaxin may disrupt endothelial cell endosomal function and thus compromise the recycling of other angiogenic mediators such as MT1-MMP and uPAR. It will be interesting therefore to determine the effect of Vitaxin and other blocks of αvβ3 function on the recycling of not only the integrin, but of the uPAR and MT1-MMP and other mediators of angiogenesis that may engage in regulated endo/exocytic cycling.

5.3.5 αvβ3 and cell-survival

During angiogenesis, endothelial cell survival is correlated with the degree of spreading on FN and VN, indicating that integrin engagement is important (Re et al., 1994), and moreover antagonists of αvβ3 integrin block are well-established to block growth factor and tumour-induced angiogenesis via a mechanism that triggers apoptosis (Brooks et al., 1994). The balance of proliferation and apoptosis is a major determinant in tumour angiogenesis and numerous angiogenic factors not only induce angiogenesis, but also function as endothelial cell survival factors. VEGF is linked to cell survival and integrins can function as endothelial cell survival factors by preventing anoikis by enhancing binding to the extracellular matrix (reviewed in Liu et al., 2000). It is tempting to suggest that αvβ3 may have a ‘housekeeping’ function to promote cell survival during vascular invasion and it would be interesting to investigate the role of Rab4 in maintaining cell survival.

In conclusion VEGFs are able to stimulate an early endosome to plasma membrane recycling of αvβ3 integrin but not α5β1 in HUVECs. This pathway provides
a mechanism to modulate integrin function and is likely to have implications for the process of angiogenesis and cell survival.
Chapter Six: PKB/Akt and Integrin Trafficking
Chapter 6: PKB/Akt and Integrin trafficking

6.1 INTRODUCTION

In previous chapters I have described integrin recycling events occurring in fibroblasts, in response to PDGF, and in HUVECs in response to VEGF. PDGF and VEGF both activate similar downstream signalling cascades and these are the Shc-Grb2-Sos-Ras-Raf-MEK-ERK pathway and the PI3K/PKB/Akt signalling axis. In chapter 4 I have presented evidence that active ERK1 associates with αvβ3, however treatment with the PD98059 inhibitor does not perturb αvβ3 recycling indicating that this cascade does not have a role to play in integrin recycling. I turned my attention therefore to the possibility of the involvement of PI3K/PKB/Akt in αvβ3 recycling.

The Class I p85/110 PI3Ks are activated downstream of receptor tyrosine kinases such as the PDGF and VEGF receptors (Heldin et al., 1998 and Petrova et al., 1999) and these are involved in diverse downstream signalling pathways, for instance the activation of the small GTPase Rac, phospholipase C (PLC), novel and atypical PKCs and PKB/Akt (see Cantrell, 2001 for a review). PKB/Akt is a 60 kDa protein that exists in 3 isoforms α,β and and γ or Akt 1,2,3 respectively (Coffer et al., 1998). Each isoform has an amino terminal plexstrin homology domain (PH) domain, a kinase domain and a COOH terminal regulatory domain. PKB/Akt is activated rapidly in response to growth factor treatment in a PI3K dependent manner, and this is thought to be brought about by a 2-step process; firstly the kinase is recruited to the membrane by virtue of its association with the 3' phosphoinositides PIP2 and PIP3, products of PI3K which selectively bind to the PH domain of PKB/Akt (Klippel et al., 1997). Secondly, upon association with the membrane the kinase is activated by phosphorylation. The phosphorylation of PKB/Akt occurs at specific regulatory sites the best characterised of these is the phosphorylation of Thr 308 (for PKBα) by 3-phosphoinositide dependent kinase or PDK1 (Alessi et al., 1997). Phosphorylation of Ser 473 (for PKBα) is also required for full activation of PKB/Akt although the kinase responsible for this has not been clearly identified. Alessi and co-workers suggest the model in which a proposed additional kinase, PDK2, phosphorylates the ser 473 (Alessi et al., 1997). A fragment from the PKC-related kinase termed PDK1 interacting fragment (PIF) has been shown to interact with PDK1 at its C-terminus and this interaction has been proposed to convert PDK-1 into a ser 473 kinase (Balendran et al., 1999), indicating perhaps that the elusive
PDK2 is in fact PDK1. Interestingly, integrin linked kinase (ILK) has been shown to phosphorylate ser 473 and this protein may be the hypothetical PDK2 (Persad et al., 2001). However this is controversial as the role of ILK as a kinase is disputed (Zervas et al., 2001). Additionally, Src kinases are involved in PKB/Akt activation and have been shown to phosphorylate tyrosine residues in its ATP-binding site (Chen et al., 2001).

PKB/Akt has been shown to be involved in many cellular processes such as prevention of apoptosis, metabolism and vesicular trafficking and this diverse function is brought about by phosphorylation of several target molecules including Bad, GSK-3, Caspase 9 & Raf (Lawlor & Alessi, 2001). In order to identify the substrates and physiological function of PKB/Akt many workers have made the use of dominant negative and constitutively active mutants of PKB. Only one dominant negative form of PKB has been shown to oppose growth factor induced phosphorylation of the well-characterised PKB/Akt substrate GSK-3. In this mutant the activator phosphorylation sites Thr 308 and Ser 473 as well as the lys 179 in the kinase domain, have been substituted for alanine residues and it has been termed “PKBAAA” (Wang et al., 1999). Reports that a single substitution of lys179 to aspartate produces a kinase dead PKB act as a dominant negative are mixed, with some workers reporting that this kinase dead PKB cannot oppose growth factor induced GSK-3 phosphorylation (Haddock et al., 1998). A number of workers have used several different manipulations to obtain constitutively active PKB. The first manipulation involves the substitution of alanine residues in the activation phosphorylation site residues T308 and S473, and this mutant is referred to as ‘PKB DD’ (Alessi et al., 1996). Others have attached membrane targeting sequences to the amino terminus of PKB, such as a Lck myristylation/palmitylation signal (Hajduch et al., 1998), and this mutant known as ‘mPKB’, has been shown to significantly increase protein synthesis and phosphorylate GSK3. The fusion of PKB to the viral gag protein, gagPKB, also acts as a constitutively active mutant and this has also been shown to induce glucose uptake, glycogen synthesis and protein synthesis in L6 myotubes (Ueki et al., 1998). Furthermore, a conditionally active version of PKB has been made in which PKB is fused to the oestrogen receptor and this is activated upon treatment of the cells with 4-hydroxytamoxifien (Kohn et al., 1998).
6.1.1 PI(3)K and Endosomal Trafficking

Although PI(3)Ks are now known to play a key role in pinching off or final internalisation events during phagocytosis (Vieira et al., 2001), these enzymes are not thought to be required for clathrin-mediated endocytosis. For instance, a number of studies have indicated that pharmacological blockade of PI(3)K with wortmannin or LY294002 does not affect endocytosis of the PDGF receptor and the transferrin receptor, nor that of the GLUT4 transporter protein (Shpetner et al., 1996, Shepherd et al., 1995 and Cormont et al., 1996). Furthermore, mutants of the PDGF receptor that are unable to recruit p85/p110 PI(3)K internalise normally following ligand binding (Joly et al., 1994). By contrast, there is copious evidence that PI(3)Ks regulate the post-internalisation trafficking and endosome to plasma membrane recycling of different receptor and transporters.

The role of PI(3)K in intracellular trafficking was initially mooted by the observation that VPS-34, a yeast protein essential for delivery of hydrolytic enzymes to the vacuole, had sequence homology to the p110 catalytic subunit of mammalian PI(3)K (Schu et al., 1993). VPS-34 and its human homologue hVPS-34 are class III PI(3)Ks that will act to produce PI(3)P. PI(3)P is specifically recognised by proteins containing a cysteine rich finger termed the FYVE domain. FYVE domains are found in several proteins implicated in endosomal trafficking including EEA1 and Rabenosyn 5 (Stenmark et al., 1996, Nielsen et al., 2000). Association of EEA1 with endosomes and its consequent regulation of endosome-endosome fusion requires binding to both PI(3)P and GTP-Rab5 (Lawe et al., 2000). Although they clearly have a key role in maintenance of endosomal transport and morphology, class III PI(3)Ks are not known to be regulated by growth factors. It is unlikely therefore that this class of PI(3)K are responsible for the PDGF-regulated recycling events that are described in this thesis.

The growth factor regulated Class I PI(3)Ks have also been shown to regulate post-endocytic trafficking and recycling of various receptors and transporters. Microinjection of inhibitory anti p110α antibodies inhibits TFN-receptor recycling and PDGF-receptor mutants that fail to bind p85/p110 PI(3)Ks are unable to enter the lysosomal pathway following internalisation (Siddhanta et al., 1998, Joly et al., 1994). Some of the most compelling evidence that class I PI(3)Ks regulate endosomal membrane trafficking comes from studies on the GLUT4 transporter. Under basal conditions GLUT4 is sequestered within endosomal compartments and is rapidly recycled to the
plasma membrane following insulin stimulation (Morris et al., 1996). The recruitment of class I PI(3)K to the insulin receptor via IRS-1 (αIRS-2) is necessary to elicit many of insulin’s effects on glucose metabolism and it is now established that regulated recycling of GLUT4 requires the activity of p85/p110 PI(3)K in adipocytes and muscle cells (Tsakiridis et al., 1995 and Somwar et al., 2001). As discussed above, one of the major effector pathways downstream of PI(3)K is the PDK/PKB/Akt signalling axis and it is now apparent that PKB/Akt plays a key role in regulating GLUT4. For instance, a number of reports have shown that inactivation of PKB/Akt, whether by dominant negative constructs (PKB-AAA) (Wang et al., 1999) or introduction of substrate peptides (Hill et al., 1999) opposes the ability of insulin to activate GLUT4 translocation. Furthermore, three different constitutively active mutants, PKB-DD, gag-PKB and mPKB have been shown to increase delivery of the GLUT4 to the plasma membrane in the absence of insulin stimulation (Foran et al., 1999, Ueki et al., 1998 and Hajduch et al., 1998). Further evidence for a mechanistic connection between PKB/Akt and GLUT4 is indicated by the fact that PKB-β is found associated with GLUT4 containing vesicles when purified on sucrose density gradients (Calera et al., 1998, Hill et al., 1999). Interestingly, GLUT4 has also been shown to be recycled to the plasma membrane via a Rab4-dependent pathway (Cormont et al., 1996, Vollenweider et al., 1997 and Cormont et al, 2001), similar to that found for αvβ3 integrin (Chapter 3 and Roberts et al., 2001). This may suggest that GLUT4 and αvβ3 recycling pathways have similar regulation mechanisms and raises the possibility that PKB/Akt is also involved in αvβ3 regulated recycling.

6.1.2 PKB/Akt and Integrin Trafficking

PKB/Akt has been shown to phosphorylate peptides corresponding to sequences contained in integrin cytoplasmic tails. It has recently been shown that PKB/Akt can directly phosphorylate threonine (Thr-753) of the cytoplasmic tail of the platelet β3 integrin αIIbβ3 (Kirk et al., 2000). Indeed phosphorylation of this residue compromised outside-in signalling by this receptor by inhibiting the binding of She, and possibly of other phosphotyrosine binding domain and SH2 domain-containing proteins to the integrin. These observations suggest that activation of PDK1 and/or PKB/Akt in platelets may modulate the binding activity and/or specificity of β3 for signalling molecules (Kirk et al., 2000) and thus this may influence directly the ability of the integrins to recycle.
PKB/Akt is localised to the leading edge of migrating cells and was shown to increase cell motility, which may contribute to enhanced invasiveness of malignant cells (Kim et al., 2001). Inside-out activation of $\alpha v\beta 3$, $\alpha 5\beta 1$, $\alpha v\beta 5$ and $\alpha 2\beta 1$ integrins is mediated by PKB/Akt (Byzova et al., 2000) and it is possible that this activation event involves the regulation of integrin recycling.

Here I present preliminary data for the involvement of PI(3)K and PKB/Akt in integrin trafficking and show that the recycling of $\alpha v\beta 3$ from the early endosomes, and the recycling of $\alpha v\beta 3$ and $\alpha 5\beta 1$ from the perinuclear recycling compartment to the plasma membrane involves PKB/Akt. These recycling pathways are necessary for integrin function as blockade of PI(3)K and PKB/Akt activity inhibits spreading onto vitronectin and fibronectin.
6.2 RESULTS

6.2.1 Integrin endocytosis does not require PI(3)K or PKB/Akt

Many workers have reported that the activity of PI(3)K is not necessary for the endocytosis of a number of receptors and transporters (Shpetner et al., 1996, Shepherd et al., 1995 and Cormont et al., 1996). To determine if the same was true for integrins, αvβ3 internalisation was measured following treatment of cells with 100nM wortmannin for 30 minutes. The assays were conducted in the presence of primaquine in order to negate the effect that membrane recycling has on measurements of endocytosis. αvβ3 integrin was internalised rapidly following transfer of the cells to 37°C and the rate and extent of endocytosis was completely unaffected by wortmannin (Fig. 6.1A). Similar results were obtained for α5β1 integrin.

PKB/Akt depends on PI(3)K for activity and numerous reports show that PKB/Akt activity is negligible following treatment with wortmannin (Burgering & Coffer 1995, Didichenko et al., 1996, Moule et al., 1999). Given the insensitivity of integrin internalisation to wortmannin treatment, it seemed unlikely that integrin internalisation would require PKB/Akt. To confirm this, cells were transfected with hαvβ3 integrin in combination with either wild-type PKBα or the dominant negative PKB-AAA, and integrin internalisation determined in the presence of primaquine. As expected, the dynamics of αvβ3 internalisation were the same irrespective of overexpression of wild-type or dominant negative PKB constructs (Fig 6.1B). These data indicate that integrin endocytosis does not require activity of the PI(3)K/PKB signalling axis.

6.2.2 Integrin recycling requires PI(3)K and PKB/Akt

I proceeded to determine whether integrin recycling was regulated by PI(3)K and PKB/Akt. Initially I concentrated on the PDGF regulated αvβ3 recycling that I have previously shown to be dependent on Rab4. Cells were surface labelled and internalisation allowed to proceed for 15 mins at 22°C to allow integrin to accumulate in Rab4- positive endosomes, and recycling was determined in the presence and absence of PDGF as described in chapter 3. Wortmannin treatment suppressed PDGF-stimulated αvβ3 recycling by approximately 50% (Fig. 6.2A), indicating the dependence of integrin recycling on PI(3)K. To determine whether wortmannin suppressed recycling by disabling PKB/Akt, recycling assays were performed on cells transfected with wild-type PKB.
Fig. 6.1 Integrin Internalisation is not affected by wortmannin or dominant negative PKB

(A) NIH fibroblasts were transfected with hαvβ3 integrin, and serum starved in the presence and absence of 100nM WMN for 30 minutes and then surface labelled with 0.2 mg/ml NHS SS Biotin. The internalisation of hαvβ3 was measured by warming the cells to 37 °C for the times indicated with 0.6μM PMQ, in the presence (■) or absence (○) of WMN. (B) Cells were transfected with hαvβ3 integrin together with either WT PKB (○) or PKBAAA (■). Serum starved cells were surface labelled and then warmed for the times indicated in the presence of 0.6 μM PMQ. Biotin was released from proteins remaining at the cell surface and biotinylated integrin determined by capture-ELISA using anti human αvβ3 integrin monoclonal antibody.
and dominant negative PKB-AAA. Interestingly, PKB-AAA suppressed PDGF-induced αvβ3 recycling to much the same extent as did wortmannin (Fig. 6.2A).

In chapter 3, I show that recycling of both αvβ3 and α5β1 integrin occurs from the perinuclear recycling compartment in the absence of growth factor addition. I wished, therefore, to determine the dependence of this ‘constitutive’ recycling pathway on PKB/Akt. Cells were transfected with hαvβ3 and hα5β1 integrins together with PKB-AAA or empty vector control, surface labelled and integrin allowed to internalise for 30 mins at 37°C to accumulate integrin in the perinuclear recycling compartment. Recycling was then determined in the absence of growth factor. Surprisingly, dominant negative PKB-AAA profoundly inhibited recycling of αvβ3 and α5β1 from the recycling compartment (Fig.6.2 B, C). This indicates that growth factor regulated ‘short loop’ recycling and basal ‘long loop’ recycling both depend on the activity of the PI(3)K/PKB signalling axis.

6.2.3 Membrane targeted PKB/Akt drives αvβ3 recycling

I wished to determine the effect of constitutively active membrane targeted PKB on αvβ3 integrin recycling. Initially attempts to measure this directly indicated that it was not possible to load αvβ3 integrin into early endosomes upon expression of mPKB. Indeed following expression of mPKB, αvβ3 appeared to remain on the cell surface (Fig. 6.3A). This could be owing to either the ability of mPKB to stimulate recycling from the early endosome, or conversely may represent a blockade on αvβ3 endocytosis. To distinguish these possibilities I determined internalisation of αvβ3 in mPKB transfected cells in the presence and absence of PMQ. Upon addition of PMQ, αvβ3 internalisation increased rapidly indicating that mPKB does not oppose integrin internalisation (Fig. 6.3B). Taken together these data show that following expression of mPKB, αvβ3 is returned to the plasma membrane from the early endosome without the need for growth factor stimulation, indicating that activation of PKB is necessary and sufficient to drive the recycling of αvβ3 integrin.

6.2.4 PKB/Akt is necessary for cell spreading

The blockade of αvβ3 by PKB-AAA was incomplete (Fig 6.2A) in much the same way as was seen following expression of S22NRab4 in chapter 3. This would be
Recycling from Early Endosomes:

A

<table>
<thead>
<tr>
<th>% Recycled</th>
<th>Basal</th>
<th>PDGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WMN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT PKB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKB AAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recycling from Recycling Endosome:

B

<table>
<thead>
<tr>
<th>% Recycled</th>
<th>hαvβ3</th>
<th>hα5β1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKBAAA</td>
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<td></td>
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</tbody>
</table>

C

<table>
<thead>
<tr>
<th>% Recycled</th>
<th>CON</th>
<th>PKBAAA</th>
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Fig 6.2 PKB is necessary for both αvβ3 and α5β1 recycling

Cells were transfected with hαvβ3 integrin or hα5β1 integrins in combination with wild type PKB or dominant negative PKB-AAA. Serum starved cells were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C, and internalisation allowed to proceed, for 15 min at 22°C (A) or 30 min at 37°C (B&C). Cells were exposed to MesNa at 4°C and internalised integrin was chased back to the cell surface at 37°C for 10 min in the absence (open bars; control) and presence (shaded bars) of 10 ng/ml PDGF-BB for 10 minutes (A) or for 30 min at 37°C in the absence of PDGF (B&C). Cells were then re-exposed to MesNa and biotinylated integrin determined by capture-ELISA using microtitre wells coated in with anti human β3 or anti human α5 integrin monoclonal antibodies. The proportion of integrin recycled to the plasma membrane is expressed as % of the pool of integrin labelled during the internalisation period.
expected to allow sufficient integrin to be recycled to maintain cell adhesion. Indeed cells expressing wild-type PKB, constitutively active mPKB and PKB-AAA all adhered normally to VN and FN (Fig. 6.4). In previous chapters I have shown that by use of Rab4 constructs to selectively block the recycling of αvβ3 (and not α5β1), I was able to compromise cell spreading on VN and not on FN. I hypothesised that given the fact that PKB-AAA affects the recycling of both αvβ3 and α5β1, PKB activity would be necessary for cells to spread on ligands for both these integrins. Cells were transfected with wild-type, dominant negative or constitutively active PKBs, and allowed to adhere to either VN or FN in the presence of PDGF. Cells transfected with wild-type PKB and mPKB spread normally on both VN (Fig. 6.5A) and FN (Fig 6.5B). However inhibition of PKB/Akt with PKB-AAA, or treatment with WMN, resulted in inefficient cell spreading on both matrix proteins (Fig. 6.5 A&B). The spreading responses on VN and FN were both had been reduced by approximately 80% following transfection with PKBAAA, consistent with the idea that integrin recycling is indeed necessary for cell spreading on the appropriate matrix.
Fig 6.3 Constitutively active PKB stimulates \(\alpha v\beta 3\) recycling

NIH fibroblasts were transfected with \(\alpha v\beta 3\) integrin together with constitutively active PKB (mPKB)(\(
\bullet\),\(\bigcirc\)) and dominant negative PKB-AAA(\(
\bullet\)). Serum-starved cells were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C and warmed to 37 °C for the times shown in the absence (\(\bullet\),\(\blacksquare\)) and presence (\(\bigcirc\)) of 0.6μM primaquine. Biotin was released from proteins remaining at the cell surface and biotinylated integrin determined by capture-ELISA using anti human \(\alpha v\beta 3\) integrin monoclonal antibody.
Fig 6.4 Adhesion of cells expressing mutant PKB's

NIH 3T3 cells transfected with hαvβ3 together with either wtPKB, mPKB, or PKB-AAA in combination with a β-galactosidase transfection marker. The cells were then briefly trypsinised and allowed to adhere to either vitronectin (VN), or fibronectin (FN), in the presence of 10 ng/ml PDGF-BB for 1 hr, in the presence and absence of 100 nM WMN. Following this, unattached cells were washed-off with ice-cold PBS and the remaining cells fixed and stained for β-galactosidase expression. The number of β-galactosidase expressing cells adherent to the VN or FN matrices were expressed as a proportion of those adherent to poly-L-lysine in the same experiment.
NIH 3T3 cells were transfected with hoxβ3 in combination with wtPKB, mPKB, or PKBAAA in combination with a β-galactosidase transfection marker. The cells were then briefly trypsinised and allowed to adhere to either vitronectin (A;VN), or fibronectin (B;FN), in the presence of 10 ng/ml PDGF-BB, in the presence and absence of 100nM WMN. Attached cells were fixed and stained for β-galactosidase expression and then photographed with a digital camera. The area of transfected cells was then determined by delineation of the cell envelope using the ‘NIH image’ software. (Values are mean ± s.e.m).
6.3 DISCUSSION

6.3.1 Summary

Here I describe that PI(3)K and its downstream target PKB/Akt are regulators of integrin recycling events. Wortmannin treatment and blockade of PKB/Akt using dominant negative proteins did not affect integrin internalisation, but inhibited PDGF-stimulated recycling of $\alpha v \beta 3$ from the early endosome and recycling of both $\alpha v \beta 3$ and $\alpha 5 \beta 1$ from the perinuclear recycling compartment to the plasma membrane. Additionally, constitutively active PKB stimulated the flux of $\alpha v \beta 3$ from the early endosome to the plasma membrane. Consistent with the recycling data, inhibition of PKB resulted in impaired spreading on both vitronectin and fibronectin. The data indicate that PKB is required for both Rab4- and Rab11-dependent integrin recycling and upon blockade of both of these pathways the cells were unable to spread on ligands for either integrin.

6.3.2 PKB/AKT and Integrin Recycling

It has been established that the internalisation of many receptors is independent of PI(3)K or PKB/Akt activity (Shpetner et al., 1996, Shepherd et al., 1995 and Cormont et al., 1996) and in agreement with this I find that both $\alpha v \beta 3$ and $\alpha 5 \beta 1$ internalisation is unaffected by wortmannin treatment or blockade of PKB/Akt. However, PKB/Akt has been extensively reported to be involved in the translocation of GLUT4 to the plasma membrane via a regulated recycling pathway (Wang et al., 1999, Foran et al., 1999, Ueki et al., 1998, Hajduch et al., 1998, Calera et al., 1998 and Hill et al., 1999), and here I show that PI(3)K and PKB/Akt are also involved in the recycling of both $\alpha v \beta 3$ and $\alpha 5 \beta 1$. Dominant negative PKB was able to inhibit the growth factor stimulated early endosomal recycling of $\alpha v \beta 3$ and the constitutive recycling of both $\alpha v \beta 3$ and $\alpha 5 \beta 1$ from the perinuclear recycling compartment. It is possible that dominant negative PKB acts to inhibit the endosomal trafficking of integrins via its ability to sequester inositol phospholipids. However, as my data clearly indicate that constitutively active PKB drives $\alpha v \beta 3$ early endosomal recycling this would argue against this possibility. Previously I have shown that the flux of $\alpha v \beta 3$ from early endosomes to the plasma membrane is dependent on Rab4 and this is stimulated by growth factors (Chapter 3 and Roberts et al., 2001). It is possible that PI(3)K and PKB/Akt operate downstream of growth factor receptors to regulate this recycling. Indeed, there is evidence to suggest
that PI(3)K and PKB/Akt are involved in activating Rab proteins. The possibility that Rab may be activated downstream of PI(3)K was first suggested by the observation that insulin can stimulate GTP exchange onto Rab4 in a fashion that was opposed by wortmannin (Shibata et al., 1997). Additionally, other Rabs have been shown to be activated downstream of the PI(3)K/PKB/Akt axis. For instance, in Dictyostelium following the sequential activation of PI(3)K and PKB/Akt, Rab7 is recruited to macropinosomes (Rupper et al., 2001), and Rab5-mediated internalisation events also require PKB/Akt activity (Barberli et al., 1998). More pertinently, expression of a prenylation deficient mutant of Rab4 (Rab4 ΔCT) has been recently shown to ablate GLUT4 translocation induced by both constitutively active PI(3)K and gag-PKB, clearly indicating that Rab4 indeed acts downstream of the PI(3)K/PKB/Akt axis in this particular regulated vesicular transport event (Cormont et al., 2001). My data suggest that PKB/Akt is involved in the activation of Rab4 in response to growth factor stimulation and this facilitates the early endosomal to plasma membrane recycling of αvβ3 integrin.

In addition to early endosome recycling, integrins also take part in recycling from the perinuclear compartment, a pathway that has previously been shown to involve Rab11 (Chapter 3 and Roberts et al., 2001). Here I show that PKB/Akt is also involved in recycling of both αvβ3 and α5β1 from the perinuclear recycling compartment. Perinuclear recycling of both α5β1 and αvβ3 was inhibited by expression of a dominant negative PKB indicating a role for PKB/Akt in this pathway. Therefore I propose that PKB/Akt activity is necessary for constitutive integrin recycling events and not just those elicited by growth factors.

6.3.3 PKB/AKT and Integrin function

PKB/Akt has previously been reported to be involved in the activation of both αvβ3 and α5β1, in addition to other integrins (Byzova et al., 2000) and in the cancer cell line HT1080, PKB/Akt is found at the leading edge of migrating cells and is involved in cell migration in a PI(3)K dependent manner (Kim et al., 2001). Furthermore, PI(3)K is reported to be involved in regulating inside out signalling of integrins (Kovacsovics et al., 1995 and Zhang et al., 1996), although the precise mechanisms by which this occurs is unknown. PI(3)K activity may activate integrins via the recruitment of PH domain
containing proteins to the membrane, which then influence integrin cytoplasmic tails and cause subsequent heterodimer activation. PKB/Akt may do this and a recent study has revealed that it can directly phosphorylate threonine (Thr-753) of the β3 integrin cytodomain (Kirk et al., 2000).

In conclusion, PI(3)K and PKB/Akt are necessary for integrin recycling events and they are involved in both the PDGF stimulated Rab4 dependent recycling of αvβ3 from the early endosome and the recycling of both αvβ3 and α5β1 from the perinuclear recycling compartment. These integrin recycling pathways are crucial for spreading on to vitronectin and fibronectin indicating that PI(3)K and PKB/Akt modulate integrin function.

An investigation of the PKB/Akt substrates located within endosomes and on endosomally-located integrin complexes will be needed to provide candidates for the mechanism of integrin regulation by PKB/Akt. Furthermore, it would be interesting to study the role of PKB/Akt in Rab activation and to ascertain if PKB/Akt indeed acts upstream of Rab4 in integrin recycling events.
Chapter Seven: General Discussion
Chapter 7: General Discussion

7.1 SUMMARY

The Rab family of GTPases regulate key targeting events in the endo/exocytic pathway and here I reveal that they are involved in integrin trafficking. In serum starved cells, internalised αvβ3 and α5β1 integrins were transported through Rab4-positive, early endosomes and arrived at the perinuclear recycling compartment approximately 30 minutes after endocytosis. From the recycling compartment, integrin is recycled in a Rab11 dependent manner in what I have termed a ‘long loop’ recycling event. Following treatment with PDGF, αvβ3 integrin, but not α5β1, was rapidly recycled directly back to the plasma membrane via a Rab4 dependent step. This rapid ‘short loop’ recycling pathway directed αvβ3 to numerous puncta across the dorsal surface of the cell and the integrin only became incorporated into focal complexes at later times of PDGF stimulation. Inhibition of short loop recycling of αvβ3 using dominant negative Rab4 mutants impaired cell spreading onto VN (a ligand for αvβ3) but not on FN (a ligand for both α5β1 and αvβ3). This indicates that Rab4 dependent recycling events are necessary for the function of αvβ3 integrin.

Signalling kinases regulating the ability of integrins to engage the extracellular matrix may be recruited to integrin cytoplasmic tails. Following addition of PDGF to serum-starved fibroblasts, active ERK1 coimmunoprecipitated with αvβ3 integrin and was seen to co-localise with αvβ3 in numerous small puncta distributed evenly over the cell surface. Only later did these punctate complexes redistribute to focal complexes in the peripheral lamellae. The association of ERK1 with αvβ3 was ablated by the MEK inhibitor PD98059, however this compound had no effect on the Rab4-dependent flux of integrin from early endosomes to the plasma membrane indicating that integrin recycling and the recruitment of active ERK1 were not inter-dependent. PD98059 also reduced cell spreading on vitronectin indicating that αvβ3 must both recycle to the plasma membrane via the Rab4 pathway and recruit ERK1 in order to function efficiently.

Regulated recycling of αvβ3 was not restricted to fibroblasts, but also occurred in HUVECs. Activation of either KDR or Flt1 with VEGFs stimulated αvβ3 recycling to the plasma membrane, whereas the recycling of α5β1 appeared not to be regulated. Furthermore, αvβ5 did not participate in an endo/exocytic cycle but resided at the plasma
membrane. Albeit that activation of KDR and Flt1 produced similar results with respect to integrin recycling, the activation of these two receptors elicited different cytoskeletal responses. Activation of Flt1 with PIGF resulted in the formation of stress fibres, whereas, treatment with VEGFD to activate KDR, produced lamellar ruffles.

Work contained within this thesis has also revealed an involvement of the PI3 kinase/ PKB/Akt signalling axis in integrin recycling events. Wortmannin treatment and blockade of PKB/Akt using dominant negative proteins inhibited both PDGF-stimulated recycling of αvβ3 from the early endosome and long loop recycling of αvβ3 and α5β1 and correspondingly blocked cell spreading on both VN and FN. Additionally, constitutively active PKB/Akt stimulated the flux of αvβ3 from the early endosome to the plasma membrane indicating that this kinase is necessary and sufficient for integrin recycling.

7.2 HOW COULD THE ENDOCYTIC PATHWAY CONTRIBUTE TO MAINTAINENCE OF αVβ3 ACTIVITY?

The inside-out activation of integrins is clearly essential for many aspects of cell biology, but the interesting questions are firstly, how does the endocytic pathway contribute towards maintaining integrin function? And secondly, how are integrins actually sorted in the endocytic pathway?

7.2.1 Endosome acidification and ligand-receptor dissociation

Experiments using fluorescent reporter dyes have shown that the luminal pH of early endosomes is somewhat lower than the extracellular media and that of clathrin coated vesicles. The low pH of endosomes is maintained by a class of vacuolar protein translocating ATPases (V-ATPases) (Nelson et al., 2000). Although the role of endosome acidification is not completely understood, it is important for several aspects of intracellular trafficking. A well-established function of low endosome pH is to dissociate lysosomally-destined ligands from receptors that are returned to the plasma membrane. This occurs during the assimilation of LDL by the α2-macroglobulin receptor (Yamashiro et al., 1989) and is necessary for the release of Fe³⁺ from transferrin (Bakkeren et al., 1987). There are other examples of this process that would be expected to impinge on cell:matrix interactions. For instance, endocytosis and degradation of vitronectin has been shown to be mediated by αvβ5 (Memmo et al., 1998). Following addition of
exogenous vitronectin to fibroblasts, the ligand is internalised into intracellular vesicles. Initially αvβ5 and vitronectin co-localise, but 30 min following internalisation the receptor and ligand part company and the vitronectin proceeds to lysosomes (Memmo et al., 1998). It is possible that the integrin is then recycled to the plasma membrane, but the workers did not measure this directly. If αvβ3 were internalised with bound VN it is likely that the integrin and ligand would separate in the early endosome shortly following internalisation. αvβ3 could then be returned to the plasma membrane with its ligand pocket empty of VN and therefore competent to engage the matrix, whilst the ligand could be trafficked to the lysosomes for degradation. However if such a mechanism were to operate, the particular requirement for Rab4 over Rab11 in maintenance of αvβ3 function suggests the possibility that Rab4 may initiate the sorting of αvβ3 into a particularly acidic early endosomal subdomains prior to recycling, whereas the long loop recycling pathway would not. Indeed it is known that the average pH of recycling compartment is less acidic (pH 6.5) (Yamashiro & Maxfield, 1987) than that of the sorting endosome (pH 6.0) (Gagescu et al., 2000).

In addition to clearing receptors of bound ligands, endosome acidification has been shown to be necessary to mediate recycling of internalised receptors back to the plasma membrane. Indeed pharmacological manipulation of endosomal pH using Bafilomycin, a selective inhibitor of the V-ATPase, induces rapid translocation of GLUT4 to the plasma membrane, indicating that endosome acidification may be considered to be part of the signal transduction pathway controlling regulated receptor recycling (Chinni & Shisheva, 1999). A number of signalling pathways have been shown to result in increased endosome acidification (Zen et al., 1992) and recently a regulatory subunit of the V-ATPase E has been shown to bind to the DH homology (DH) domain of Sos1 (Miura et al., 2001). This association may form part of the mechanism whereby endosome acidification could be driven by receptor tyrosine kinases. Miura et al. (2001) have reported that overexpression of V-ATPase in COS cells enhanced the exchange of GTP onto Rac1 (Miura et al., 2001). Given that I have found that recycling of αvβ3 modulates its function, and that engagement of active integrins is known to activate Rac1 (Price et al., 1998) it would be interesting to determine whether αvβ3 is involved in mediating the influence of endosomal pH over Rac1 function.

In order to test hypotheses involving the influence of endosomal pH over αvβ3 function it will be necessary to monitor the pH of endosomal compartments &
subdomains through which the integrin travels. This could be achieved by using pH-sensitive variants of GFP (pHluorins) (Robey et al., 1998) fused to the extracellular domain of β3 integrin, and/or αvβ3 pHluorin-tagged ligands such as osteopontin (17-314) or fibronectin repeat 1.

It is now becoming clear that the coordinated endo/exocytosis may regulate cell migration via impinging on the function of another vitronectin receptor, the uPA receptor (uPAR). When bound to uPA, the uPAR remains at the plasma membrane (Vassalli et al., 1985, Stopelli et al., 1985 and Cubellis et al., 1986). However when associated with the plasminogen activator inhibitor (PAI-1), the uPAR/uPA/PAI-1 complex is internalised (Cubellis et al., 1990, Estreicher et al., 1990 and Jensen et al., 1990). As uPAR is a GPI-anchored protein, internalisation is achieved by ‘piggy backing’ onto the α2-macroglobulin receptor, and it then follows the clathrin-mediated internalisation pathway appropriate for this transmembrane receptor (Nykjaer et al., 1992, Conese et al., 1995 and Heegaard et al., 1995). Following internalisation the uPAR/PAI-1 parts company with the uPAR; the former being trafficked to lysosomes and degraded whilst the receptor is recycled to the plasma membrane cleared of its ligand and competent to bind more active uPA (Nykjaer et al., 1997). It has been postulated that the ability of uPAR to recycle back to the plasma membrane thus re-activated may perform an important function in the attachment/detachment cycle during cycle migration (Nykjaer et al., 1997). It is possible therefore that PDGF regulated, Rab4-dependent recycling of αvβ3 also forms part of such a coordinated attachment/detachment regime that operates during cell migration.

7.2.2 Retrieval of damaged integrins from the cell surface

During its lifetime, an integrin heterodimer it will be utilised by the cell for multiple rounds of matrix engagement and disengagement. At some point during its lifetime it is inevitable that the heterodimer will become damaged by oxidation, or cross-linked by transamidation of glutamines/asparagines (possibly by tissue transglutaminases), and this would lead to integrin misfolding and loss of proper heterodimer function (Barsigian et al., 1991 and Akimov et al., 2000). It would be expedient, therefore, for the cell to have a mechanism for scanning the cell surface for damaged integrins and sending them to the lysosomes for degradation. The endo/exocytic cycling of integrin is particularly rapid and the internalisation rate measured both by others and myself (i.e. 50% of PM α5β1/αvβ3 are internalised within 10 mins in the
presence of PMQ), could in principle result in the complete passage of the plasma membrane integrin through the endosomes within 30 min. It is possible that this could facilitate the detection and removal of damaged integrin from the cell surface.

Molecular chaperone proteins, such as calnexin, largely reside in the ER and these ensure the correct folding of polypeptide chains prior to their transport to the Golgi and the cell surface. One way in which these molecular chaperones operate is by binding to unfolded polypeptides tracts and this can assist folding (Fink, 1999), but also they act as molecular 'policeman' to retain misfolded proteins in the ER (Ellgaard & Helenius, 2001). A number of integrins, for instance $\alpha 6\beta 4$, $\alpha 5\beta 1$ & $\alpha 4\beta 1$, are known to associate with calnexin (Rigot et al., 1999 and Lenter & Vestweber, 1994). In addition to facilitating their folding in the ER, this has been proposed to modulate $\beta 1$ surface levels during keratinocyte terminal differentiation by retaining $\beta 1$ integrins in an intracellular compartment following which they are transported to lysosomes and degraded (Hotchin et al., 1995). There is evidence that chaperones and chaperone like proteins are abundant in endocytic compartments, and more recently it has become apparent that a fraction of calnexin is not retained in the ER but trafficked to the cell surface whereupon it is dynamically turned over by cycles of endo/exocytosis (Okazaki et al., 2000). It is possible that calnexin, and calreticulin, (a luminal homologue of calnexin which is known to bind to $\alpha$ chain cytoplasmic tails (Rojianni et al., 1991 and Leung-Hagestenijn et al., 1994) and has been implicated in integrin inside-out activation (Hagestenijn et al., 1994 and Coppolino et al., 1997)) and other molecular chaperones may act as ‘policemen’ to retain misfolded or damaged integrin within the early endosome, thus preventing their return to the plasma membrane and directing them to the lysosomes for degradation. A particular advantage would be conferred by doing this at the level of the endosome as it would prevent the accumulation of cross-linked or damaged integrin at the plasma membrane where it may initiate outside-in signalling leading to a phagocytosis response and possible disruption of the cell migratory machinery.

The high affinity receptor for IgG, FcyRI, engages in a rapid constitutive endo/exocytic cycle and this enables the receptor to scan the cell surface for the presence of cross-linked immune complexes (Harrison et al., 1994, Davis et al., 1995). Doing this at the level of the endosome allows the removal of small immune complexes from the cell surface without allowing them to engage at the plasma membrane and illicit phagocytosis and the undesirable recruitment of signalling molecules associated with this process.
**7.2.3 The role of endo/exocytosis in generating polarised distribution of surface integrin**

In migrating cells mechanisms must exist to move integrins from the back of the cell towards the advancing lamellipodium. Two mechanisms have been proposed that would achieve this. These represent opposite ends of a mechanistic spectrum, but are not mutually exclusive. The first model shown in Fig. 7.1 proposes that integrins disengage at the cell rear and move laterally in the plasma membrane to be re-engaged at the leading edge without entering the cell (Regen *et al.*, 1992 and Palecek *et al.*, 1996). However, the considerable activity of integrin endo/exocytosis cycle that has been measured by myself and others (Roberts *et al.*, 2001, Bretscher, 1992 and Lawson & Maxfield 1995, Gao *et al.*, 2000, Fabbri *et al.*, 1999, and Ng *et al.*, 1999) would make it unlikely that an unengaged integrin would reside at the cell surface for sufficient time to complete this cycle without being internalised and recycled several times.

Several workers have shown that integrin-containing vesicles move from the rear of the cell to the perinuclear recycling compartment and this has led to the proposal that exocytic vesicles move integrin directly form the rear of the cell to the lamellipodia, possibly via a perinuclear endosomal compartment as shown in Fig. 7.2 (Laukaitis *et al.*, 2001, Regen *et al.*, 1992, Lawson & Maxfield 1995 and Bretscher & Aguado-Velasco 1998). A brief discussion of the possible relationship between cell migration and phagocytosis is needed to fully describe this model. Phagocytic cells generally move towards the object of their phagocytosis and there is a mechanistic relationship between this migration and the ingestion of the particle. For instance both processes involve the extension of cellular processes towards an extracellular stimulus and they utilise Rho GTPase-based cell signalling and actin polymerisation mechanisms. Prior to the initiation of phagocytosis, the V-SNARE, VAMP-3 resides in the perinuclear recycling compartment where it co-localises with the TFN-R (Bajno *et al.*, 2000). Shortly following the engagement of an opsonised particle, however, focal exocytosis of VAMP-3-containing vesicles occurs in the vicinity of the newly forming phagocytic cup (Bajno *et al.*, 2000) suggesting that the vectoral transport of endocytic vesicles may contribute to the formation of polarised membrane extensions such as a lamellipodium. Indeed it has been known for some time that the TFN-R is trafficked to the tips of the lamellopodia and exocytosed at the leading edge of fibroblasts (Hopkins *et al.*, 1994). Additionally the membranes of the EGF-induced lamella are enriched in both TFN-R and α2 macro-
globulin receptors following activation of Rac (Bretscher & Aguado-Velasco, 1998). However it is notable that none of these studies report the focal exocytosis of integrins at the leading edge of migrating cells. Infact the finding of Laukaitis et al. (2001) suggests that integrin containing vesicles actually seem to move back from the leading edge back towards the perinuclear recycling compartment (Laukaitis et al., 2001). This combination of these reports and the data contained within this thesis lead me to propose a third model, which combines aspects of Figs 7.1 and 7.2. This model proposes that the endo/exocytic cycle contributing to the forward movement of integrin in a cell migrating toward a chemotactic stimulus and is illustrated in Fig.7.3. Early endosomes are distributed relatively evenly throughout the cell, whereas, the perinuclear recycling compartment is orientated towards the direction of migration (Pierini et al., 2000). In the presence of high PDGF, αvβ3 is not transported to the perinuclear recycling compartment, but is returned directly from early endosomes to the plasma membrane. Therefore at the front of the cell where the concentration of chemoattractant is greatest one would expect integrin to be retained at the front of the cell and not transported back to the perinuclear recycling compartment. By contrast, at the rear of the cell, αvβ3 would be expected to be transported forwards to the perinuclear recycling compartment. Thus the engagement of a short loop cycle at the front of the cell and a long loop cycle for integrin internalised at the cell rear would conspire to move integrin forward within the cell with respect to the concentration of chemoattractant.

7.2.4 Lipid rafts

The endo/exocytic recycling of integrins may serve as a mechanism to localise integrins to specialized areas of the plasma membrane such as those rich in spingolipids, cholesterol and caveolin1. These lipid rafts are often thought of as sites for initiation of signal transduction (Simons & Toomre, 2000), although it has been suggested that they serve as sites of endocytosis (Ikonen, 2001) and may also down-regulate signalling (Aman et al., 2001). The recruitment of integrin to membrane rafts, and the remodeling of lipid rafts by growth factors, may serve to bring integrins into contact with associating proteins that mediate integrin function. αvβ3 integrin has been shown to form a complex with IAP and heterotrimeric G proteins in lipid rafts and it has been shown that cholesterol is critical for the formation of this complex and subsequent signalling (Green
Fig. 7.1 Lateral diffusion of integrins. This model proposes that integrins disengage at the cell rear and move laterally in the plasma membrane to be re-engaged at the leading edge without entering the cell.
Fig. 7.2 The formation of focal complexes by vectoral vesicular transport of integrins. It is thought that the new material at the leading edge is, in part, membrane recycled from the back of the cell and that focal adhesions can be formed by integrins undergoing endocytosis at the cell rear followed by directional recycling to the leading edge.
Fig. 7.3. Migrating fibroblasts in a chemotactic gradient. Early endosomes are distributed relatively evenly throughout the cell, whereas, the perinuclear recycling compartment is orientated towards the direction of migration. In the presence of high PDGF, αvβ3 is not transported to the perinuclear recycling compartment, but is returned directly from early endosomes to the plasma membrane. Therefore at the front of the cell where the concentration of chemoattractant is greatest one would expect integrin to be retained at the front of the cell and not transported back to the perinuclear recycling compartment. By contrast, at the rear of the cell αvβ3 would be expected to be transported forwards to the perinuclear recycling compartment which is orientated toward the direction of cell movement. Thus the engagement of a short loop cycle, at the front of the cell and a long loop cycle for integrin internalised at the cell rear would conspire to move integrin forward within the cell with respect to the concentration of chemoattractant.
et al., 1999). A recent study has shown that upon addition of insulin-like growth factor, IAP moves from triton-insoluble fraction to the triton-soluble fraction, and this is accompanied by an increase in association with αvβ3 and which serves to activate the integrin (Maile et al., 2002). Another integrin-associated protein implicated in integrin activation is the uPAR receptor, and this receptor has been localised to lipid rafts where it forms a complex with β1 integrin and stabilises signalling complexes (Wei et al., 1999).

Endocytosis also provides a mechanism to bring surface receptors into the vicinity of their endosomally located effectors. For example the EGFR is active on the endosome, and adaptor molecules, such as Shc, have been found associated with EGFR at the cell surface and within endosomes, whereas others, such as Eps8, are found only with intracellular receptors (Burke et al., 2001). The assembly of integrin containing macromolecular complexes on endosomes may be important for integrin downstream signalling and it is possible that integrins associate with a different subset of protein on different intracellular compartments and this results in the activation of specific signalling cascades dependent on the location.

7.3 HOW IS SELECTIVITY ACHIEVED IN THE ENDOSONAL PATHWAY?

The mechanisms by which integrins are sorted in the endocytic pathway remains to be elucidated, however, several possibilities are discussed below.

7.3.1 Sorting Nexins

The sorting nexin family of phox domain containing proteins, interact with many types of membrane receptors and are involved in intracellular trafficking. For instance sorting nexin 1 (SNX1) was identified by yeast two-hybrid as a partner for cytoplasmic domain of the EGF receptor (Kurten et al., 1996). The SNX1 yeast homologue, Vps5p, has been identified as a component of the retromer (a complex which facilitates retrograde transport from the endosome to the TGN). This complex also contains Vps35p, Vps29p, Vps17p and Vps26p, and has been shown to be involved in the retrieval of a Vps10p (a carboxypeptidase Y receptor) from endosomes to the Golgi (Seaman et al., 1997, Seaman et al., 1998). Based on this, SNX1 has been proposed to have a role in directing EGF receptors to lysosomes for degradation and indeed overexpression of SNX1 results in accelerated EGF receptor degradation (Kurten et al., 1996). The transferrin receptor has also been shown to interact with SNX1 and the splice variant
SNX1A (Haft et al., 1998), and it is possible that this interaction may be important in determining the fate of the receptor; i.e. degraded verses recycled (Trowbridge et al., 1993). Other nexins have been identified and these include SNX15, which has been shown to associate with the PDGF receptor (Phillips et al., 2001) and SNX6, which associates with the TGF-β family of serine/threonine kinases (Parks et al., 2001), and it is possible that sorting nexins could interact with integrin receptors. The precise mechanisms by which the cargo is selected for a transport vesicle is still unknown, although given that sortin nexins display a specificity for transmembrane receptors, it is possible that these proteins select the proteins to be conveyed within transport vesicles.

### 7.3.2 Endosomal lipid domains

In addition to protein sorting in the endocytic pathway there is a large body of evidence to suggest that lipids themselves undergo sorting to create internal membrane domains, which then participate in protein sorting events (Mukherjee & Maxfield, 2000). For example, the charged lipid lysobisphosphatidic acid (LBPA) has been shown to be concentrated in the internal membranes of the late endosome, and these LBPA enriched membranes were critical for the correct sorting of transmembrane proteins such as the mannose 6- phosphate receptor from the late endosome to the TGN (Kobayashi et al., 1998). Lipid sorting has also been shown to occur in the endocytic recycling compartments, which have been shown to contain large pools of cholesterol (Mukherjee et al., 1998). In a study measuring the endocytic trafficking of GPI anchored proteins, it was found that these lipid anchored proteins are retained about three times longer in the recycling endosomes compared to transmembrane proteins such as the transferrin receptor (Mayor et al., 1998). Furthermore, this retention was lost when cells were grown in media that depletes intracellular cholesterol (Mayor et al., 1998). It is possible that the association of integrins with other proteins i.e. such as uPAR, which preferentially associate with specific enriched lipid domains forms part of the mechanism by which integrins are sorted. It would be interesting to investigate integrin recycling under cholesterol depletion to ascertain the effect on integrin activation.

### 7.3.3 Rab microdomains

Rab5, Rab4, and Rab11 have been localised to distinct microdomains on endosomes (Sonnichsen et al., 2000) and it thought that these domains are organised by
the Rabs themselves and other regulatory proteins through local recruitment of specific effectors. One may hypothesise that a Rab4 restricted environment on the membrane contributes to direct plasma membrane recycling, and indeed areas of Rab4 microdomains have been observed forming vesicular structures in A431 cells (Sonnichsen et al., 2001). The transferrin receptor was found to traffic sequentially through Rab5, Rab4 and Rab11 microdomains, and treatment of cells with wortmannin caused a prolonged association of transferrin with Rab5 domains, indicating that the advancement from Rab5 to Rab4 domains is regulated by PI(3)Ks (Sonnichsen et al., 2001). Conversely, positive mechanisms may exist to prolong the association of receptors with Rab4 domains, perhaps via interaction with Rab effectors or other vesicle associated proteins, and this could result in the incorporation of receptors into recycling vesicles. In order to determine whether αvβ3 is recruited to Rab4 microdomains on early endosomes, it would be necessary to study the behaviour of GFP tagged integrins and Rab proteins in live cells following growth factor addition.

7.3.4 Other microdomains

Clathrin-coated endosomal buds have been previously described as structures that are distinguished from plasma membrane derived clathrin-coated pits by size (60 nm and 100 nm, respectively), by continuity with endosomes, and a lack alpha-adaptin (Stoorvogel et al., 1996). Clathrin coated endosomal buds have also been shown to contain transferrin receptors, to carry endogenous dynamin and furthermore, expression of an exogenous temperature sensitive dynamin accumulated on the clathrin coated buds on recycling endosomes and interfered with transferrin recycling (van Dam & Stoorvogel, 2002). Although adaptor complexes that exclusively associate with endosomes have not yet been identified, the presence of γ-adaptin (Stoorvogel et al., 1996) and β3 adaptin (Dell Angelica et al., 1998) has been demonstrated on endosomal clathrin-coated buds. During clathrin-mediated endocytosis at the plasma membrane, the μ chains of the AP2 adaptin complex have been shown to bind to cytoplasmic tails of selected receptors and recruitment them to clathrin-coated pits (see Heilker et al., 1999 for a review). Other μ isoforms or novel unidentified adaptor complexes may exist that act on endosomes, and it is possible that these adaptor complexes could contact integrin cytoplasmic tails and recruit integrins to endosomal clathrin coated buds. The AP1 complex has been shown to contain different μ isoforms, μ1A and μ1B, which function at the TGN for transport to
endosomes and to the basolateral membrane respectively (Folsch et al., 2001), and it is possible that different μ subunit isoforms acting at the endosome could result in the sorting of integrins for recycling back to the plasma membrane or for progression to the perinuclear recycling compartment.

### 7.3.5 Integrin sorting motifs

The cytoplasmic domains of many cell surface receptors contain di-leucine or tyrosine based signals that are required for their endo/exocytic cycling, however, is unclear which cytoplasmic motifs are necessary for integrin recycling. Fabbri et al. (2000) identified a YXX0 motif that was important in β2 integrin recycling, however this motif is restricted to β2 integrins. Integrin tails contain NPXY motifs, which have been shown to be important in the endocytosis of some receptors, however this motif is not necessary for integrin endocytosis (Vignoud et al., 1994), and it remains to be determined if this it is involved in integrin recycling. As NPXY motifs are highly conserved across the integrin family and present in β1, β3 and β5 integrins, (which I have shown to have different trafficking properties), it is unlikely that this motif is involved in integrin recycling. Further to this it is now known that the NPXY motif of β3 is involved in association with the talin head and therefore most probably has a role in attaching αvβ3 to the actin cytoskeleton in focal complexes. Given this it is likely that other cytoplasmic sequences are necessary for integrin recycling, and these may determine whether the integrin is to be recycled from early endosomes or from the perinuclear recycling compartment. To investigate this possibility chimeric cytoplasmic domains, in which residues from the β3 sequence are systematically replaced with those from β1, could be generated, placed into mammalian expression vectors and assayed for their ability to take part in short or long loop recycling. Furthermore, these chimeric cytoplasmic domains could be made as GST fusion proteins and incubated with PDGF-stimulated cell lysate. This would allow the complement of proteins recruited to integrin cytodomains under growth factor stimulation to be determined. By this approach one could identify important residues involved in ERK1 recruitment to αvβ3, or the association of any other unidentified proteins, which may be important in integrin recycling or activation.
7.3.6 Recruitment of kinases

Several kinases have been implicated in GLUT4 endo/exocytic trafficking. PKB/Akt and the atypical PKCs, PKCζ and PKCλ, have been shown to be recruited to the GLUT4 compartment (Calera et al., 1998, Standaert et al., 1999). Many workers have reported the involvement of PKB/Akt and PKCζ in GLUT4 recycling (Wang et al., 1999, Hill et al., 1999, Foran et al., 1999, Ueki et al., 1998, Hajduch et al., 1998, Calera et al., 1998, Hill et al., 1999, Braiman et al., 2001 and Standaert et al., 1999), for instance, overexpression of PKCζ has been shown to stimulate GLUT4 translocation and conversely, dominant negative PKCζ blocks transporter delivery in both adipocytes and myotubes (Braiman et al., 2001). It is possible that kinases, such as PKB and atypical PKCs, are recruited to integrins and these physical associations direct αvβ3 recycling. To investigate this, proteins co-immunoprecipitating with αvβ3 in PDGF stimulated fibroblasts could be screened to identify kinases and their involvement in integrin recycling could be assessed using dominant negative approaches.

7.3.7 SNARE complexes

Membrane fusion events are mediated by SNAREs and SNARE associated proteins that bring into close proximity transport vesicles and their target membranes and ultimately leads to their fusion. An interaction of the t-SNARES, SNAP-23 and syntaxin 4, on the plasma membrane with vesicle-associated synaptobrevin-2 (Sbr-2) and/or cellubrevin (Cbr), has been proposed to provide the targeting and/or fusion apparatus for insulin-stimulated translocation of GLUT4 to the plasma membrane (Foran et al., 1999). Constitutively active PKB has been shown to drive GLUT4 translocation in a manner that is dependent on Sbr-2/Cbr and SNAP-23 (Foran et al., 1999). It has been shown that phosphorylation of certain docking and fusion proteins may be important in their regulation (Linial, 1997). PKB/Akt has an established role in regulating GLUT4 traffic and it is possible that PKB/Akt is required for this. Indeed, PKB β/Akt-2 is known to be recruited to GLUT4 vesicles in an insulin dependent manner, where it phosphorylates components of GLUT4 containing vesicles, which may represent SNARE/VAMP proteins (Kupriyanova & Kandror, 1999). In addition to this PKCζ has been shown to be recruited to GLUT4 vesicles where it phosphorylates the v-SNARE, VAMP2 (Braiman et al., 2001). It would be interesting to determine whether PKB/Akt or PKCζ is recruited to αvβ3 containing vesicles upon PDGF treatment, and whether this results in the
phosphorylation of vesicular components required for fusion of αvβ3 recycling vesicles with the plasma membrane.

7.3.8 Motor proteins

There is evidence to suggest that the interaction of motor proteins with Rab complexes facilitates the movement of vesicles or organelles to the plasma membrane (discussed in chapter 1.7.1.4). Insulin stimulated GLUT4 transport is dependent upon microtubule and actin-based cytoskeletal structures (Fletcher et al., 2000, Olson et al., 2001, Tsakiridis et al., 1994 and Omata et al., 2000) and a kinesin motor has been found to direct the translocation of GLUT4 to the plasma membrane (Emoto et al., 2001). Rab4 has been shown to interact with the dynein light intermediate chain-1 in a yeast two-hybrid screen (Bielli et al., 2001) and this suggests that this motor is involved in αvβ3 recycling. Furthermore PKCζ, a kinase implicated in GLUT4 trafficking, has been shown to generate cell polarity during astrocyte migration and that it is most likely achieve this by regulating the function of dynein (Etienne-Manneville & Hall, 2001). It is therefore possible that atypical PKCs could influence Rab-dependent events by phosphorylating the motors or adaptors that recruit recycling vesicles onto the microtubular network.
Appendix
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Appendix
SPECIAL NOTE

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THE BEST AVAILABLE IMAGE HAS BEEN ACHIEVED.
PDGF-regulated rab4-dependent recycling of αvβ3 integrin from early endosomes is necessary for cell adhesion and spreading

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Background: It has been postulated that the regulation of integrin vesicular traffic facilitates cell migration by internalizing integrins at the rear of the cell and transporting them forward within vesicles for exocytosis at the leading edge to form new contacts with the extracellular matrix. The rab family of GTPases control key targeting events in the endo/exocytic pathway; therefore, these GTPases may be involved in the regulation of cell-matrix contact assembly.

Results: The endo/exocytic cycle of αvβ3 and α5β1 integrins was studied using mouse 3T3 fibroblast cell lines. In serum-starved cells, internalized integrins were transported through rab4-positive, early endosomes and arrived at the rab11-positive, perinuclear recycling compartment approximately 30 min after endocytosis. From the recycling compartment, integrins were recycled to the plasma membrane in a rab11-dependent fashion. Following treatment with PDGF, αvβ3 integrin, but not α5β1, was rapidly recycled directly back to the plasma membrane from the early endosomes via a rab4-dependent mechanism without the involvement of rab11. This rapid recycling pathway directed αvβ3 to numerous small puncta distributed evenly across the dorsal surface of the cell, and the integrin only became localized into focal complexes at later times following PDGF addition. Interestingly, inhibition of PDGF-stimulated αvβ3 recycling using dominant-negative rab4 mutants compromised cell adhesion and spreading on vitronectin (a ligand for αvβ3), but adhesion to fibronectin (a ligand for α5β1 and αvβ3) was unchanged.

Conclusions: We propose that growth factor-regulated, rab4-dependent recycling of αvβ3 integrin from early endosomes to the plasma membrane is a critical upstream event in the assembly of cell-matrix contacts.

Background
In order for cells to spread on or migrate across flat surfaces, integrin-containing focal complexes must form rapidly at the peripheral or leading lamellae. Formation of these focal complexes requires integrin engagement but is also driven by growth factors such as PDGF [1]. Many surface receptors, including integrins, participate in an endo/exocytic cycle [2]. They are internalized, delivered to endosomes, and then recycled to the plasma membrane for reutilization. It has been suggested that this cycle may facilitate focal complex assembly by internalizing integrins at the rear of the cell and transporting them forward within vesicles for exocytosis at the leading lamellae [3].

Previous studies have shown that the integrin endo/exocytic cycle is functionally important. Fabbri et al. [4] have shown that a YXXΦ motif in the cytoplasmic tail of β2 integrin is essential for recycling to the plasma membrane following internalization and that disruption of this motif inhibits β2-dependent cell migration. Furthermore, β1 integrins are internalized via a dynamin-dependent step, and inhibition of β1 integrin internalization by expression of dominant-negative dynamin also reduces cell motility [5]. It is unclear, however, whether cell-signaling pathways coordinate cell motility by regulating the recycling of integrins.

Transferrin receptor (TFN-R) recycling operates in two distinct time domains; a short circuit recycling pathway directly from early endosomes to the plasma membrane [6] and an indirect route involving transit through the perinuclear recycling compartment [7]. The rab family of small GTPases control key targeting events in these recycling pathways. Rab 11 localizes to the perinuclear recycling compartment and has been shown to control recycling from this compartment as well as transport to the trans-Golgi network [8-10]. Rab4, on the other hand, is localized predominantly to early endosomes [6] and, to a lesser extent, to recycling endosomes where it colocalizes.


with rab11 [11, 12], and it is thought to be involved in recycling from both of these compartments. ARF6, a GTPase known to regulate cell spreading and motility, has also been shown to regulate endosomal recycling via a pathway distinct from those regulated by rab GTPases [13, 14]. Although some studies have shown that β1 integrins colocalize with rab11 and the transferrin receptor [5], it is unclear, however, which rab- or ARF-dependent steps are involved in the endo/exocytic cycle of integrins.

Here, we show that PDGF regulates the recycling rate for αvβ3, but not for α5β1. This process involves rab4 and does not require rab11 or ARF6; thus, it defines a mechanism whereby growth factors can regulate integrins during cell adhesion and spreading.

**Results**

**Internalization of integrins**

Preliminary experiments indicated that treatment with PDGF rapidly increased the levels of αvβ3 integrin at the plasma membrane (see the Supplementary material available with this article online). We developed assays for integrin endocytosis and recycling to determine whether PDGF regulation of these processes could account for this change in αvβ3 surface levels. Integrin internalization was determined by surface labeling Swiss 3T3 fibroblasts with NHS-SS-biotin at 4°C, followed by incubation at 37°C for various times. Biotin was removed from proteins remaining on the cell surface by exposure to Sodium 2-mercaptoethanesulphonate (MesNa) at 4°C, and internalized integrin was assessed by immunoprecipitation, followed by Western blotting with streptavidin. Both αvβ3 and α5β1 heterodimers were internalized with similar kinetics, their internal pools reaching a steady level by 10 min (Figure 1a,b). Using capture-ELISA to quantify biotinylated integrins, we found that the addition of the

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**Internalization of integrins.** (a,b) Serum-starved Swiss 3T3 fibroblasts were surface labeled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4°C and were warmed to 37°C for the times indicated. Biotin was released from proteins remaining at the cell surface by MesNa treatment at 4°C, the cells were lysed, and integrins were immunoprecipitated with (a) anti-β3 or (b) anti-α5 monoclonal antibodies. Immobilized material was then analyzed by 6% SDS-PAGE, followed by Western blotting with peroxidase-conjugated streptavidin. The positions of α5, αv, β1, and β3 integrin chains are indicated. (c,d) Surface-labeled cells were warmed to 37°C in the absence (open triangle) or presence (solid triangle) of 0.6 μM PMA for the times indicated. Biotin was released from proteins remaining at the cell surface, and biotinylated integrin was determined by capture-ELISA using microtiter wells coated with (c) anti-β3 or (d) anti-α5 integrin monoclonal antibodies. (e,f) Surface-labeled cells were warmed to 37°C in the presence of 0.6 μM PMA and in the absence (open circle) and presence (solid square) of 10 ng/ml PDGF-BB for the times indicated. Internalized integrin was determined as for (c) and (d) using microtiter wells coated with (e) anti-β3 or (f) anti-α5 integrin monoclonal antibodies. (Mean ± SEM from three separate experiments).
receptor recycling inhibitor, primaquine (PMQ) [15], increased the measured internalization rate of αβ3 and α5β1 integrin (Figure 1c,d), indicating that both integrins recycle back to the plasma membrane very shortly after internalization. Therefore, to measure the effect of PDGF on the endocytic rate of integrins, we performed internalization assays in the presence of PMQ. This analysis revealed that PDGF did not affect the endocytic rate for either αβ3 or α5β1 integrin (Figure 1e,f).

Integrin endosomal compartments

Recycling receptors do not normally accumulate in early endosomes but pass rapidly through them and proceed to the perinuclear recycling compartment where they accumulate before making the last step in recycling to the plasma membrane. Incubation times of 30–60 min at 37°C have been routinely used to load the perinuclear recycling compartment with internalized tracer [16]. Additionally, internalized receptor may be concentrated in the early endosomes by reducing the temperature to slow transport through the endosomal system [17, 18].

To monitor the trafficking of integrin through endosomal compartments, cell-surface αvβ3 was tagged using anti-β3 monoclonal antibody at 4°C. Internalization was initiated by raising the temperature to either 22°C for 15 min or to 37°C for 30 min. Following this, antibody remaining at the cell surface was removed by a low pH wash at 4°C, and αvβ3 integrin and rab proteins were visualized by immunofluorescence. Following the shorter internalization period, αvβ3 integrin became closely colocalized with rab4 in endocytic vesicles distributed relatively evenly about the cytoplasm (Figure 2a–c). During this time, αvβ3 did not reach the perinuclear recycling compartment and showed little colocalization with rab11 (Figure 2d–f). Longer internalization times resulted in the integrin being transported out of the rab4-positive compartment such that, following 30 min at 37°C, αvβ3 was observed to focus in the perinuclear region and colocalize with rab11 (Figure 2g–i). Similar results were obtained for α5β1 integrin (data not shown).

Recycling of integrins

We proceeded to monitor the recycling of integrins from early endosomes and the perinuclear recycling compartment using a pulse-chase approach. Cells were surface-labeled, and internalization was allowed to proceed for 15 min at 22°C to allow integrin to accumulate in rab4-positive early endosomes. Biotin was removed from proteins remaining on the cell surface by exposure to MesNa at 4°C, and internalized integrin was chased from the cells at 37°C for various times in the presence or absence of PDGF. At each time, the cells were reexposed to MesNa to remove biotin from integrins that had recycled back to the cell surface, and the level of biotinylated integrin remaining within the cell was assayed by capture-ELISA. PDGF stimulated the rate of αvβ3 recycling from the early endosomes by approximately 2-fold, such that, in the presence of the growth factor, all internalized integrin had returned to the plasma membrane within 10 min (Figure 3a). A limited amount of α5β1 recycled from this compartment, but this was unaffected by PDGF (Figure 3b).

To monitor recycling of tracer that accumulated in the perinuclear recycling compartment, cells were surface labeled, and internalization was allowed to proceed for 30 min at 37°C. Integrin recycling was then determined as for Figure 3a,b. The recycling rates were similar for both α5β1 and αvβ3 and were unaffected by PDGF (Figure 3c,d), indicating that αvβ3 is subject to PDGF-regulated recycling only when present in the rab4-positive early endosomes and that recycling from endocytic compartments distal to this is refractory to this kind of regulation.

Rab4 dependence of αvβ3 recycling

Rab4 is known to regulate the recycling of receptors from early endosomes to the plasma membrane [19]. To investigate the potential involvement of rab proteins in integrin recycling, we employed NIH 3T3 fibroblasts, which are similar to Swiss 3T3s but can be transfected to high efficiency. To measure recycling from transfected cells, we transiently expressed human integrins and detected them using human integrin-specific antibodies. The antibodies used were highly selective for havβ3 and havβ1 and did not crossreact with mouse integrins (Figure 4a). Additionally, when cells were cotransfected with rab4 and human integrins, both receptor and GTPase were expressed in the same cells (Figure 4b,c).

Treatment with PDGF increased recycling of havβ3 from early endosomes, and the expression of wild-type rab4 increased both the basal and PDGF-stimulated rates of havβ3 recycling (Figure 4d). havβ1, however, did not recycle directly from these endosomes (Figure 4e).

We employed two dominant-negative mutants to assess the rab4 dependence of αvβ3 recycling; S22Nrab4, which binds GDP poorly but is unable to bind GTP [20], and N121Irab4, which is unable to bind guanine nucleotide [21]. S22Nrab4 significantly reduced, and N121Irab4 completely abolished, PDGF-stimulated recycling of αvβ3 (Figure 4d). These data indicate an absolute requirement for rab4 in this process.

The data shown in Figure 3 suggest that αvβ3 must be present in rab4-positive early endosomes to be amenable to PDGF-regulated recycling. Transport through the recycling compartment is inhibited by dominant-negative mutants of rab11 [8, 9]. Dominant-negative N124Irab11 has no effect on PDGF-induced recycling of αvβ3 (Figure 4d), indicating that the growth factor regulates recycling...
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Figure 2

Visualization of integrin endosomal compartments. NIH 3T3 fibroblasts were transfected with hαv3 and hβ3 integrin in combination with (a–c) wild-type rab4 or (d–i) wild-type rab11. Surface αvβ3 was tagged by incubation with the mouse anti-hβ3 monoclonal antibody for 30 min at 4°C. Surface-bound antibody was allowed to internalize for (a–f) 15 min at 22°C or for (g–i) 30 min at 37°C, and the cells were rapidly cooled to 4°C. Antibody remaining at the cell surface was removed by a low-pH wash, and the cells were fixed and detergent permeabilized. Internalized antibody was visualized using (e,d,g; shown in green) FITC-conjugated anti-mouse, and the cells were counterstained with (b) rabbit anti-rab4 and (e,h) rabbit anti-rab11, followed by detection with Texas Red-conjugated anti-rabbit antibody (shown in red). Yellow indicates colocalization of the two fluorophores. The scale bar represents 16 μm.

Experiments employing a 30 min internalization period indicated that both hαvβ3 and hαβ1 integrin recycled at similar rates from the perinuclear recycling compartment, such that, during 30 min of chase, ≈60% of integrin had returned to the plasma membrane (Figure 4f,g), a value similar to that observed in Swiss 3T3s (shown in Figure 3c,d). In complete contrast to PDGF-regulated recycling of hαvβ3 from the early-sorting endosomes, recycling of hαvβ3 and hαβ1 from the perinuclear compartment was unaffected by N121Irab4 but strongly inhibited by dominant-negative N124Irab11 (Figure 4f,g).

Endosomal recycling can be regulated by the ARF subfamily of GTPases, notably ARF6. We therefore determined the effect of dominant-negative T27NARF6 on PDGF-dependent αvβ3 recycling. Consistent with previous observations that ARF6 regulates a membrane recycling pathway distinct from that controlled by rab4 [13], T27NARF6 did not inhibit αvβ3 recycling (Figure 4d).

Visualization of PDGF-dependent αvβ3 recycling

Treatment with PDGF for 10 min increased surface staining for αvβ3 and resulted in its localization to numerous small puncta distributed over the cell surface but sometimes enriched in lamellar ruffles (Figure 5c,d). Examination of optical slices from the confocal image indicate that these puncta were present primarily on the dorsal surface of the cell (data not shown). Following longer treatments with PDGF, αvβ3 was seen to be incorporated into larger complexes in a peripheral distribution that is characteristic of integrin-containing focal complexes (Figure 5e,f) [1].

The time course of the appearance of αvβ3-rich puncta parallels that measured for PDGF-dependent integrin recycling from early endosomes, suggesting that recycling integrin may be targeted to these puncta. Integrin recycling was visualized by tagging αvβ3 with an NHS-SS-biotin-labeled antibody that was allowed to internalize and recycle as shown in Figure 4d. PDGF stimulated the recycling of αvβ3-bound antibody (Figure 5i), and this was targeted to numerous small punctate complexes distributed over the cell surface (Figure 5k).
Figure 3

(a) $\alpha\beta_3$  

(b) $\alpha5\beta_1$

30 min internalization:

(c) $\alpha\beta_3$  

(d) $\alpha5\beta_1$

Recycling of $\alpha5\beta_1$ and $\alpha\beta_3$ integrins. Cells were surface labeled, and internalization was allowed to proceed for (a,b) 15 min at 22°C or for (c,d) 30 min at 37°C, and biotin was removed from receptors remaining at the cell surface by treatment with MesNa at 4°C. Cells were then rewarmed to 37°C for the times indicated in the absence (open circle) or presence (solid square) of 10 ng/ml PDGF-BB to allow recycling to the plasma membrane, followed by a second reduction with MesNa. Cells were lysed, and integrin-biotinylation was determined by capture-ELISA using microtiter wells coated with (a,c) anti-3 or (b,d) anti-5 integrin monoclonal antibodies. The proportion of integrin recycled to the plasma membrane is expressed as the percentage of the pool of integrin labeled during the internalization period (values are mean ± SEM from five separate experiments).

Given that PDGF-regulated recycling is dependent on rab4 and that early endosomal recycling of $\alpha\beta_3$ is directed to cell surface puncta, we determined whether rab4 inhibition would affect the appearance of these puncta. S22Nrab4 blocked the assembly of $\alpha\beta_3$-containing puncta (Figure 5g), but membrane ruffling was uncompromised (Figure 5h).

Taken together, these data indicate that recycling of $\alpha\beta_3$ to the plasma membrane via a rab4-dependent pathway directs this integrin to punctate plasma membrane complexes. These puncta subsequently organize into the more familiar integrin-containing focal complexes.

Involvement of rab4 in cell adhesion and spreading

To investigate the possibility that rab4-dependent $\alpha\beta_3$ recycling was necessary for cell adhesion or spreading, cells transfected with wild-type or dominant-negative rab4s were adhered to either vitronectin (VN), a good ligand for $\alpha\beta_3$, or fibronectin (FN), a ligand for both $\alpha5\beta_1$ and $\alpha\beta_3$, in the presence of PDGF. N121Irab4, which abrogated early endosomal recycling of $\alpha\beta_3$, inhibited the adhesion of transfected cells to VN (Figure 6a). Adhesion to FN was unaffected by N121Irab4, indicating that the inhibition was specific for $\alpha\beta_3$-mediated events. S22Nrab4, however, did not affect adhesion to VN (Figure 6a), implying that the partial blockade of $\alpha\beta_3$ recycling with this construct was insufficient to block adhesion.

However, partial inhibition of $\alpha\beta_3$ recycling by S22Nrab4 compromised spreading of cells following attachment to VN. Within 1 hr of plating, cells transfected with wild-type rab4 spread better than their untransfected neighbors (Figure 7a,b). In contrast, cells transfected with S22Nrab4 did not spread efficiently on VN and seemed unable to properly organize their actin cytoskeleton (Figure 7c,d). Spreading on FN was similar irrespective of transfection with wild-type rab4 (Figure 7e; indicated by arrow) or S22Nrab4 (Figure 7f; indicated by arrow), consistent with the observation that $\alpha5\beta_1$ recycling is unaffected by dominant-negative rab4. Quantification of cell area indicated that S22Nrab4 reduced spreading on VN by approximately 50% (Figure 6b), a value consistent with the reduction of $\alpha\beta_3$ recycling by this construct.

In contrast to dominant-negative rab4s, N124Irab11 did not inhibit the adhesion of cells to either matrix protein (Figure 6a) and even increased spreading onto VN (Figure 6b). Therefore, direct recycling of integrin from early endosomes to the plasma membrane, and not that from the perinuclear recycling compartment, is critical for $\alpha\beta_3$ function.

Discussion

We have characterized regulation of the endo/exocytic cycle of $\alpha\beta_3$ integrin by PDGF and found that it plays a role in the functioning of the integrin during cell adhesion and spreading. In the absence of serum, $\alpha\beta_3$ integrin is internalized and passes through the early endosomes to arrive at the perinuclear recycling compartment approximately 30 min after internalization. The integrin is then recycled to the plasma membrane in a rab11-dependent fashion. However, following treatment with PDGF, $\alpha\beta_3$ integrin is recycled directly back to the plasma membrane from early endosomes in a rab4-dependent fashion with-
Rab4 dependence of αvβ3 recycling. (a) NIH 3T3 fibroblasts were transfected with human α and β3 (αvβ3) or human α5 and β1 (α5β1) integrins or empty vector controls (mock). Cells were surface labeled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4°C and were lysed. Lysates were immunoprecipitated (I.P.) with anti-human β3 (hβ3) or anti-human α5 (hα5) monoclonal antibodies. Immobilized material was then analyzed by 6% SDS-PAGE, followed by Western blotting with peroxidase-conjugated streptavidin. The migration positions of human αv, β3, α5, and β1 integrin chains are indicated. (b,c) Cells were transfected with human αv and β3 integrins in combination with wild-type rab4. Following transfection, the cells were fixed, permeabilized, and costained for (b) human β3 integrin and (c) rab4 as for Figure 2. The scale bar represents 150 μm. (d-g) NIH 3T3 fibroblasts were transfected with (d,f) human αvβ3 or (e,g) α5β1 integrins either alone (Control) or in combination with wild-type rab4 (wt-rab4), S22N-rab4, N121I-rab4, N124I-rab11, and T27N-ARF6 as indicated. Cells were surface labeled with 0.2 mg/ml NHS-SS-biotin for 30 min at 4°C, and internalization was allowed to proceed for (d,e) 15 min at 22°C or for (f,g) 30 min at 37°C. Cells were exposed to MesNa at 4°C, and internalized integrin was chased back to the cell surface at (d,e) 37°C for 10 min in the absence (open bars; Basal) and presence (solid bars) of PDGF. Cells were then reexposed to MesNa, and biotinylated integrin was determined by capture-ELISA using microtiter wells coated with (d,f) anti-human β3 or (e,g) anti-human α5 monoclonal antibodies. Values are mean ± SEM from at least three separate experiments.

out the involvement of rab11 or ARF6. This direct recycling pathway delivers αvβ3 to numerous small puncta distributed evenly across the dorsal surface of the cell, and the integrin only later becomes localized into focal complexes. α5β1 integrin is not regulated in this way, but undergoes rab11-dependent endo/exocytic cycling even in the presence of PDGF. Furthermore, dominant-negative rab4s, but not rab11s, reduce adhesion and spreading on VN, demonstrating that rapid recycling from early endosomes is required for αvβ3 integrin-dependent processes to proceed efficiently.

Regulation of recycling from early endosomes
Glut4 and E-cadherin are examples of proteins recycled to the plasma membrane in response to stimulation. E-cadherin recycles from early endosomes in response to cell-cell contact [22]. Insulin stimulation of Glut4 recycling has been shown to be dependent on rab4 [23], suggesting that this GTPase is a target for regulation of receptor recycling by growth factors. Rab4 is proposed to be necessary for the formation of vesicles involved in recycling proteins from the early endosome to the cell surface. Overexpression of wild-type rab4 reduces the ability of...
Visualization of PDGF-dependent recycling of αvβ3 integrin. (a–h) NIH 3T3 fibroblasts were transfected with hav03 integrin either (a–f) alone or (g,h) in combination with S22Nrab4. Following transfection, the cells were serum starved for 30 min and then challenged with 10 ng/ml PDGF-BB for (c,d,g,h) 10 min or for (e,f) 30 min, or they were (a,b) allowed to remain quiescent. Cells were fixed in 2% paraformaldehyde, surface αvβ3 was visualized by indirect immunofluorescence, and F-actin was counterstained with Texas Red-conjugated phalloidin. Surface-only integrin staining was obtained by the addition of the primary antibody prior to the detergent-permeabilization step. The scale bar represents 20 μm. (i) Anti-β3 antibody was biotinylated with NHS-SS-biotin and bound to the surface of the cells at 4°C. Biotinylated antibody was allowed to internalize for 15 min at 22°C, and the cells were exposed to MesNa at 4°C to remove biotin from antibody remaining on the plasma membrane. Internalized antibody was chased back to the cell surface at 37°C for 10 min in the absence and presence of 10 ng/ml PDGF-BB. Cells were then reexposed to MesNa, and biotinylated antibody was determined by capture-ELISA using microtiter wells coated with anti-hamster monoclonal antibody. (j,k) Biotinylated antibody was bound, internalized, and recycled in the (j: Basal) absence and (k) presence of PDGF as for (i). Following this, the cells were fixed, and recycled antibody was visualized using FITC-conjugated streptavidin (surface-only labeling being ensured by the omission of a detergent-permeabilization step). The scale bar represents 16 μm.

the TFN-R to reach acidic endosomes, presumably by rapidly mistargeting internalized receptors directly to the plasma membrane [19]. Consistent with this, we found that overexpression of wild-type rab4 enhanced PDGF-dependent rapid recycling of αvβ3, while dominant-negative rab4 mutants opposed this. The pulse-chase experiments presented in Figures 3 and 4 indicate that, in order to be amenable to PDGF-regulated recycling, αvβ3 must be present in early endosomes at the time of growth factor addition. Following the addition of PDGF, αvβ3 may recycle directly from the early endosomes or, alternatively, may proceed to the plasma membrane via an indirect route through the perinuclear recycling compartment. Previous studies showed that transport through, and exit from, the perinuclear recycling compartment requires the function of rab11 [8, 9]. N124Irab11 has no effect on the ability of PDGF to trigger αvβ3 recycling, indicating that the direct route to the plasma membrane is regulated by the growth factor.

PDGF regulation of rab4 may occur via activation of PI 3-kinases that can regulate guanine nucleotide exchange on rab4 [24]. We have found that inhibition of PI 3-kinase with wortmannin, albeit unable to affect αvβ3 endocytosis, potently inhibited its recycling (data not shown). Additionally, PKB/Akt, a kinase activated downstream of PI 3-kinase, regulates rab function in macrophages [25]. We are currently investigating the involvement of PI 3-kinases and PKB/Akt in growth factor-regulated αvβ3 recycling.

αvβ3 recycling and the regulation of cell adhesion and motility

Tagging αvβ3 with antibodies revealed that PDGF-induced recycling of αvβ3 occurs over the dorsal cell surface and is not targeted directly to focal contacts. The early endosomal recycling pathway may, therefore, play a general role in regulating integrin-mediated events. Indeed, inhibition of PDGF-regulated αvβ3 recycling using dominant-negative rab4 mutants impaired cell adhesion and nonpolarized spreading. This suggests that this aspect of regulated vesicular transport may facilitate integrin activa-
The effect of dominant-negative rab4s on cell adhesion and spreading. NIH 3T3 cells were transfected with wild-type rab4 (wtrab4), S22Nrab4, N121rab4, or N124rab11 in combination with a β-galactosidase transfection marker. The cells were then briefly trypsinized and allowed to adhere to either vitronectin (VN) or fibronectin (FN) in the presence of 10 ng/ml PDGF-BB for 1 hr. Following this, the unattached cells were washed off with ice-cold PBS, and attached cells were fixed and stained for β-galactosidase expression. (a) The number of β-galactosidase-expressing cells adherent to the VN or FN matrices was expressed as a proportion of those adherent to poly-L-lysine in the same experiment. (b) Adherent cells were then photographed with a digital camera, and the area of transfected cells was determined by delineation of the cell envelope using "NIH image" software. The data are expressed as a percentage of the cell area of wild-type rab4-expressing cells following spreading on VN. (Values are mean ± SEM).

Conclusions
We have shown that PDGF stimulates rab4-dependent recycling of αvβ3 integrin from early endosomes to the cell surface. Inhibition of this process using dominant-negative rab4 mutants impaired cell adhesion and spreading on ligands for αvβ3. These data describe for the first time a mechanism through which growth factors can regulate rather than mediate vectorial delivery to the leading edge of migrating cells.

It has been suggested that factors such as PDGF promote cell spreading by modulating the affinity of the integrins for their ligands, a mechanism that has been termed inside-out signaling [26]. The precise and rapid regulation of the affinity of integrins for their ligands is a feature of many members of this family. A classic example of this is the activation of the platelet receptor αIIbβ3 following thrombin stimulation. Upon stimulation, αIIbβ3 undergoes a conformational change rendering it competent to bind ligand, and the total levels of active receptor exposed at the cell surface are increased. The affinity of both α4β1 [27] and the β2 integrins αLβ2 and αMβ2 [28] for their ligands is also regulated by exposure of cells to various stimuli. Although certain signaling pathways, including PI 3-kinase activation, have been implicated in mediating inside-out activation, the mechanism through which this is achieved remains unclear. Here, we have found that the rapid recycling of αvβ3 to the cell surface is dependent on rab4 and that this can be regulated by PDGF, a growth factor that promotes cell spreading and motility. This rapid recycling pathway must be necessary for integrin function, as dominant-negative rab4 mutants block αvβ3-mediated cell adhesion and spreading on vitronectin. Therefore, modulating the flux of integrin to and from the cell surface is a mechanism through which the cell can achieve the inside-out activation of integrins. It will be interesting to determine whether the same mechanism can regulate other integrins.
late integrin recycling and, furthermore, that this has functional consequences during cell adhesion and spreading.

Materials and methods

Cell culture and transfection

Swiss and NIH 3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) with 10% fetal calf serum (Globepharm) and 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Life Technologies) at 37°C with 5% CO₂. For transient transfection experiments, NIH 3T3 fibroblasts were grown to 50% confluence, fed with fresh DMEM containing 10% fetal calf serum, and transfected with integrin, rab, and ARF6 constructs (all cDNAs were ligated into pcDNA3; see the Supplementary material for details of constructs) using Fugene 6 (Roche) according to the manufacturer's instructions. The ratio of Fugene 6 to DNA was maintained at 3 μl Fugene 6:1 μg DNA. Integrin recycling and cell adhesion/spreading assays were carried out 24 hr posttransfection.

Internalization and recycling

Internalization and recycling of integrins was measured using a modification of the method described in [29].

Internalization

Cells were serum starved for 30 min, transferred to ice, washed twice in cold PBS, and surface labeled at 4°C with 0.2 mg/ml NHS-SS-biotin (Pierce) in PBS for 30 min. Labeled cells were washed in cold PBS and transferred to DMEM at 37°C with or without 10 ng/ml PDGF-BB (PeproTech) in the presence and absence of 0.6 μM primapine to allow internalization. At the indicated times, the medium was aspirated, and the dishes were rapidly transferred to ice and washed twice with ice-cold PBS. Biotin was removed from proteins remaining at the cell surface by incubation with a solution containing 20 mM MesNa in 50 mM Tris (pH 8.6) and 100 mM NaCl for 15 min at 4°C [30]. MesNa was quenched by the addition of 20 mM iodoacetamide (IAA) for 10 min, and the cells were lysed in 200 mM NaCl, 75 mM Tris, 15 mM NaF, 1.5 mM Na₃VO₄, 7.5 mM EDTA, 7.5 mM EGTA, 1.5% Triton X-100, 0.75% igepal CA-630, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 1 mM 4-(2-Aminothyl)benzenesulphonyl fluoride (AEBSF). Lysates were passed three times through a 27G needle and were clarified by centrifugation at 10,000 × g for 10 min. Supernatants were corrected to equivalent protein concentrations and levels of biotinylated integrin were determined by capture-ELISA, or integrins were isolated by immunoprecipitation and analyzed by SDS-PAGE.

Recycling

Following surface labeling, cells were transferred to serum-free DMEM at 22°C for 15 min or 30 min at 37°C to allow internalization of tracer into early endosomes and the perinuclear recycling compartment, respectively. Cells were returned to ice and washed twice with cold PBS, and biotin was removed from proteins remaining at the cell surface by reduction with MesNa. The internalized fraction was then chased from the cells by returning them to 37°C in serum-free DMEM in the absence or presence of 10 ng/ml PDGF-BB. At the indicated times, cells were returned to ice and biotin was removed from recycled proteins by a second reduction with MesNa. Biotinylated integrins were then determined by capture-ELISA.

Capture-ELISA

Maxisorb 96-well plates (Life Technologies) were coated overnight with 5 μg/ml appropriate anti-integrin antibodies (see the Supplementary material for full antibody information) in 0.05 M Na₂CO₃ (pH 9.6) at 4°C and were blocked in PBS containing 0.05% Tween-20 (PBS-T) with 5% BSA for 1 hr at room temperature. Integrins were captured by overnight incubation of 50 μl cell lysate at 4°C. Unbound material was removed by extensive washing with PBS-T, and wells were incubated with streptavidin-conjugated horseradish peroxidase (Amersham) in PBS-T containing 1% BSA for 1 hr at 4°C. Following further washing, biotinylated integrins were detected by a chromogenic reaction with ortho-phenylenediamine.

Immunoprecipitations

Magnetic beads (Dynal) conjugated to anti-integrin antibodies were incubated with lysates overnight at 4°C with constant rotation. Beads were washed six times with lysis buffer, and immunosolated material was eluted by boiling for 10 min in nonreducing Laemmlin sample buffer. Integrin chains were resolved by 6% nonreducing SDS-PAGE and visualized by Western blotting with peroxidase-conjugated streptavidin.

Immunofluorescence microscopy

Cells were plated onto glass coverslips and grown to 50%-70% confluency over 3 days and were transfected 24 hr prior to experimentation where appropriate. Cells were serum starved for 30 min and treated with 10 ng/ml PDGF-BB for an additional 10 or 30 min prior to fixation in 2% paraformaldehyde in PBS for 20 min at room temperature. Following fixation, non specific binding was blocked for 1 hr in PBS containing 10% FCS (PBS-FCS). The cells were then incubated with anti-β3 monoclonal

Figure 7

The effect of S22Nrab4 on cell spreading. NIH 3T3 fibroblasts were transfected with human αv and β3 integrins in combination with (a,b,e) wild-type rab4 (WTrab4) or (c,d,f) S22Nrab4. The cells were then briefly trypsinized and were allowed to adhere to either (a-d) vitronectin or (e,f) fibronectin in the presence of 10 ng/ml PDGF-BB for 1 hr. Cells were fixed in 2% paraformaldehyde and double stained for F-actin and cell-surface human β3 integrin. The arrows in (c) indicate transfected cells. The scale bar represents 40 μm.
antibodies at 5 μg/ml for 1 hr at room temperature. Following this, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and then reblocked in PBS-FC5. Detection was by FITC-conjugated secondary antibodies. The actin cytoskeleton was counterstained with Texas Red-conjugated phaloxidin in PBS for 10 min at room temperature.

For tracking the internalization of αvβ3 integrin, NIH 3T3 fibroblasts were transfected with hox and hβ3 integrin in combination with wild-type rab4 or wild-type rab11. Following serum starvation, surface αvβ3 was tagged by incubation with mouse anti-hβ3 monoclonal antibody for 30 min at 4°C in PBS containing 1% BSA. Surface-bound antibody was allowed to internalize for 15 min at 22°C or for 30 min at 37°C, and the cells were rapidly cooled to 4°C. Antibody remaining at the cell surface was removed by incubation in acid-PBS (corrected to pH 4.0 by the addition of HCl) at 4°C for 6 min. The cells were then fixed in 2% paraformaldehyde and were detergent permeabilized. Internalized antibody was visualized using FITC-conjugated anti-mouse, and the cells were counterstained with rabbit anti-rab4 and rabbit anti-rab11, followed by detection with Texas Red-conjugated anti-rabbit antibody.

Cell adhesion and spreading assays
Tissue culture plates (Nunc, 24-well) were coated with fibronectin. Cell adhesion and spreading assays were performed on fibronectin-coated dishes for 1 hr at 37°C. Following serum starvation, surface αvβ3 was tagged by incubation with mouse anti-hβ3 monoclonal antibody for 30 min at 4°C in PBS containing 1% BSA. Surface-bound antibody was allowed to internalize for 15 min at 22°C or for 30 min at 37°C, and the cells were rapidly cooled to 4°C. Antibody remaining at the cell surface was removed by incubation in acid-PBS (corrected to pH 4.0 by the addition of HCl) at 4°C for 6 min. The cells were then fixed in 2% paraformaldehyde and were detergent permeabilized. Internalized antibody was visualized using FITC-conjugated anti-mouse, and the cells were counterstained with rabbit anti-rab4 and rabbit anti-rab11, followed by detection with Texas Red-conjugated anti-rabbit antibody.

Supplementary material
Supplementary material including important data documenting the effect of various growth factors on the surface expression of αvβ3 and α5β1 integrins on serum-starved NIH 3T3 fibroblasts is available at http://images.cellpress.com/supmat/supmat.htm. Additional methodological information on the sources and generation of antibodies and cDNAs is available there.

References


