The Effect of a Squamous Cell Carcinoma Associated β1 Integrin Mutation on Cell Behavior

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Abstract

Integrins are the main receptors for extracellular matrix proteins and are responsible for mediating attachment of most cell types to their surrounding matrix. In addition integrins regulate growth and differentiation of many cell types, including epidermal keratinocytes, through a variety of signalling pathways. Integrin loss or overexpression contributes to the pathogenesis of benign epidermal disorders and influences the incidence and prognosis of squamous cell carcinomas (SCC) and other tumours. However, no integrin mutations have yet been reported in tumours of any origin. In my thesis I have studied the SCC4 cell line isolated from a human oral SCC. This cell line has normal integrin expression and is poorly differentiated. When these cells are infected with a retrovirus encoding the wild-type chick β1 integrin they regain the ability to differentiate. I found that these cells are heterozygous for a β1 mutation, T188I, mapping to the A domain of the subunit. T188I results in increased ligand binding, irrespective of the partner α subunit, both in solid phase assays with recombinant protein and in living cells. The mutation promotes cell attachment and spreading but not invasion or motility. It also alters integrin signalling as shown by increased levels of MAPK phosphorylation. When introduced into the SCC4 cell line the mutant β1 integrin fails to stimulate differentiation. Activation of β1 integrins in normal keratinocytes also suppresses differentiation.

To discover how frequently similar mutations occur in SCC I screened 124 skin tumours for mutations in exons surrounding T188I. A single alanine to valine substitution was found in a single case. My results establish mutation as a mechanism by which integrins can contribute to tumour development and provide new insights into how integrins regulate keratinocyte differentiation.
Acknowledgments

I would like to thank Fiona, my supervisor, for all her help over the last four years. I would also like to thank Simon and Liz for all the times they have fed cells and helped out far beyond the call of duty. Also the various members of bay three over the last few years, John, Liz, Robin and Kristen for making it such as fun (and often surreal) place to work. The other integrin people in the lab (Ingo, Douglas, Sam and Dave) have been of great help, as have all the other members of the lab.

The support staff at LIF have been of immense help, especially Cathy, Ayad, Gary and Derrick from the FACS lab, Colin, Debbie and Peter from microscopy and all the crew of the equipment park and cell production.

I would also like to acknowledge the help provided by Viv Perkins, Alistair Henry, Paul Stephens and Martyn Robinson of CellTech PLC, Judith Jones of UMDS and Claire Taylor of Cancer Research UK Leeds.

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<tr>
<td>AM12</td>
<td>Gag, pol +env AM12 packaging cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cβ1</td>
<td>Chick β1 integrin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation antigen</td>
</tr>
<tr>
<td>Col</td>
<td>Collagen</td>
</tr>
<tr>
<td>DCS</td>
<td>Donor calf serum</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modification of Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FAD</td>
<td>F12 +adenine +DMEM</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>Fn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>hβ1</td>
<td>Human β1 integrin</td>
</tr>
<tr>
<td>HICE</td>
<td>Hydroxycortisone, insulin, cholera enterotoxin and EGF</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IVS</td>
<td>Intron Variable Sequence</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>Ln</td>
<td>Laminin</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MT</td>
<td>Mutant</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>Vn</td>
<td>Vitronectin</td>
</tr>
<tr>
<td>VWF</td>
<td>Von Willebrand Factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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Overview

The importance of the β1 family of integrin extracellular matrix receptors in regulating the terminal differentiation of human keratinocytes has been known for many years. In my thesis I describe the effects of an integrin mutation on the behaviour of a cell line derived from a human squamous cell carcinoma. This introduction will begin with an overview of integrins and then proceed to discuss their role in the skin and how they regulate keratinocyte behaviour.

1.1 Integrins

Integrins are a family of cation dependent extracellular matrix (ECM) receptors. An integrin consists of a heterodimer of a single α and β subunit (Hynes, 1992). Each subunit is a large (>100kDa) integral membrane glycoprotein (Tamkun et al., 1986). Integrins have been implicated in a diverse range of processes including platelet aggregation, phagocyte adhesiveness, leucocyte extravasation and angiogenesis (Hynes, 1987; Hynes, 1992). In all of these cases the essential property of the integrin is its ability to bind extracellular proteins and mediate the adhesion of the cell. The term integrin was coined “to denote its role as an integral membrane complex involved in the transmembrane association between the extracellular matrix and the cytoskeleton” (Tamkun et al., 1986).
Integrins are amongst the most widespread family of proteins known with every nucleated human cell type expressing at least one family member. Integrin like proteins are found in many organisms. *Drosophila* has 4 α (αPS1, αPS2, αPS3 and αv) and 2 β (βPS and βv) subunits (Gotwals *et al.*, 1994). These show changing patterns of expression during development. Their functional importance is shown by the effects of mutations of βPS (*lethal myospheroid*), αPS1 (*multiple edematous wings*) and αPS2 (*inflated*) which result in embryonic lethality due to defects in muscle junctions (Gotwals *et al.*, 1994). Two integrins homologous to α5 and αv have been found in *C. elegans* (Sulston *et al.*, 1992). Even primitive organisms such as the sponge have genes that have high degrees of similarity to mammalian integrins (Wimmer *et al.*, 1999). The importance of integrins, particularly the β1 family is further illustrated by the β1 knockout phenotype which is lethal prior to implantation of the embryo (Fassler and Meyer, 1995; Stephens *et al.*, 1995).

Integrins function by binding to defined motifs in their ligands (such as the RGD or REDV motifs in fibronectin, (Pierschbacher and Ruoslahti, 1984; Humphries *et al.*, 1986a; Sonnenberg, 1993)) and clustering into complexes such as focal adhesions, focal contacts or hemidesmosomes where they associated with either the actin or keratin cytoskeleton. Signalling molecules can be recruited to their cytoplasmic tails and lead to cytoskeletal rearrangement and signalling (outside in signalling) (Giancotti and Ruoslahti, 1999; Schwartz, 2001; Schwartz and Ginsberg, 2002). Integrins can also modulate their affinity for ligands in response to signals from the cell (inside out signalling) by cytoplasmic proteins binding the integrin tails and propagating conformational changes up the structure to the ligand binding domain.
and by clustering (Yauch et al., 1997; van Kooyk and Figdor, 2000; Hogg and Leitinger, 2001).

1.1.1 Integrin families

Integrins can be loosely divided into families depending on their subunit composition (Hynes, 1992) (Table 1.1). While this is a convenient division it does not show the complexity of the tissue distribution and ligand specificity of the different dimers, nor does it necessarily equate with their function.

<table>
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<tr>
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<th>Cell types (examples)</th>
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<td>α1 CD49a/CD29 VLA-1</td>
<td>Collagen</td>
<td>Fibroblasts, Activated T&amp;B lymphocytes</td>
</tr>
<tr>
<td>α2 CD49b/CD29 VLA-2</td>
<td>Collagen</td>
<td>Fibroblasts, Platelets, Endothelial and Epithelial cells, Monocytes</td>
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<tr>
<td>α3 CD49c/CD29 VLA-3</td>
<td>Laminin</td>
<td>Fibroblasts, Epithelial cells,</td>
</tr>
<tr>
<td>α4 CD49d/CD29 VLA-4</td>
<td>Fibronectin, VCAM-1</td>
<td>Melanocytes, Lymphocytes, monocytes</td>
</tr>
<tr>
<td>α5 CD49e/CD29 VLA-5</td>
<td>Fibronectin</td>
<td>Fibroblasts, Endothelial and epithelial cells, monocytes</td>
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<tr>
<td>α6 CD49f/CD29 VLA-6</td>
<td>Laminin</td>
<td>Monocytes, T lymphocytes, platelets</td>
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<tr>
<td>α7</td>
<td>Laminin</td>
<td>Muscle</td>
</tr>
<tr>
<td>α8</td>
<td>Fibronectin, Tenascin</td>
<td>Neurons, Epithelial cells</td>
</tr>
<tr>
<td>α9</td>
<td>Tenascin</td>
<td>Muscle, hepatocytes</td>
</tr>
<tr>
<td>αv CD51/CD29</td>
<td>Fibronectin, vitronectin</td>
<td>Fibroblasts</td>
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The β1 integrin family, ligands and typical cells on which they are expressed
### Alternate Nomenclature

<table>
<thead>
<tr>
<th>αv</th>
<th>Ligands</th>
<th>Cell types (examples)</th>
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<tr>
<td>β1</td>
<td>CD51/CD29 Fibronectin, Vitronectin</td>
<td>Fibroblasts</td>
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<tr>
<td>β3</td>
<td>CD51/CD61 Fibrinogen, vWFA, Thrombospondin, Vitronectin</td>
<td>Fibroblasts, Platelets, Endothelial and Epithelial cells, Monocytes</td>
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<td>β5</td>
<td>Vitronectin</td>
<td>Epithelial cells, Fibroblasts</td>
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<tr>
<td>β6</td>
<td>Fibronectin</td>
<td>Epithelial cells</td>
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<tr>
<td>β8</td>
<td>Fibronectin, Laminin</td>
<td>Sensory neurones</td>
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The αv integrin family, ligands and typical cells on which they are expressed.

<table>
<thead>
<tr>
<th>β2</th>
<th>Ligands</th>
<th>Cell types (examples)</th>
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<tr>
<td>αL</td>
<td>CD11a/CD18, LFA-1</td>
<td>All leucocytes</td>
</tr>
<tr>
<td>αM</td>
<td>CD11b/CD18, Mac-1, CR3, Mo1</td>
<td>Monocytes, Granulocytes, CD8+ve T cells, NK cells</td>
</tr>
<tr>
<td>αX</td>
<td>CD11c/CD18, CR4, LeuM5</td>
<td>Monocytes, Granulocytes, activated B lymphocytes, NK cells</td>
</tr>
<tr>
<td>αD</td>
<td>ICAM-3</td>
<td>Monocytes, Granulocytes, most lymphocytes</td>
</tr>
</tbody>
</table>

The β2 integrin family

<table>
<thead>
<tr>
<th>α4</th>
<th>Ligands</th>
<th>Cell types (examples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β7</td>
<td>LPAM-1</td>
<td>T &amp; B lymphocyte subpopulations</td>
</tr>
<tr>
<td>αIIβ</td>
<td>GpIIb/IIIa Fibrinectin, vWFA</td>
<td>Platelets</td>
</tr>
<tr>
<td>α6</td>
<td>β4</td>
<td>Laminin</td>
</tr>
<tr>
<td>αE</td>
<td>β7 HML-1</td>
<td>E-Cadherin</td>
</tr>
</tbody>
</table>

The "orphan" integrins

**Table 1.1:** Human integrin dimers divided by subunit composition showing typical ligands and cells on which they are expressed.
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1.2 Integrin Structure and Function

At its simplest an integrin dimer resembles a globular head and pair of stalks leading
to the plasma membrane. This structure was first seen by electron microscopy
(Nermut et al., 1988). The recent solution of the αvβ3 extracellular domain crystal
structure has given a clearer picture of the structure of an integrin (Xiong et al.,
2001). The receptor is a large multi-domain structure capable of undergoing radical
conformational changes which allows it to react both to signals from the cell and
from the surrounding matrix (Xiong et al., 2002).

1.2.1 α subunit

The α integrin subunit consists of multiple domains (Fig 1.1). At the extracellular
terminus there is a large β propeller structure made up of seven blades of four β
sheets (Springer, 1997). Two of these blades (2 and 3) are involved in forming part
of the ligand binding site in the majority of integrins (Mould et al., 2000). Four of
these blades (4-7) also contain calcium-binding motifs. These were once thought to
be involved directly in ligand binding however it has now been shown that these lie
on the underside of the propeller, well away from the regions interacting with either
the β chain or the ligand (Xiong et al., 2002).

In a subset of integrins, mostly collagen receptors ( α1, α2) or leucocyte integrins
(αL, αM, αX, αE) the α subunit has an extra 200 amino acid domain inserted above
the β propeller, between loops 2 and 3 (Zang et al., 2000). This I domain (for
“inserted”) is a von Wilebrand Factor A domain (Lee et al., 1995b) and appears to be
the sole site of ligand binding in these integrins. The structure of this domain has
been extensively studied, indeed it was the first part of the structure to be resolved at
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The stalk region of the α subunit comprises a series of 3 β sandwich domains known as the thigh and the calf 1 and 2 domains (Xiong et al., 2001) (Fig 1.1). A single transmembrane region connects the extracellular domains to the cytoplasmic tail of the subunit. In a subset of α subunits the extracellular region is cleaved at a point in the calf 2 domain just before the transmembrane region and the subunit is then held intact by a disulphide bond (α 3,5,6,7,8). α4 also exists in a cleaved form (α4 80/70) however this cleavage is further out at the base of the propeller domain (Rubio et al., 1992; Teixido et al., 1992). A truncated form of α6, α6p, has also been reported which is synthesised from a normal, full length, α6 mRNA (Davis et al., 2001). This smaller form of α6 lacks the first 13 exons including the ligand binding domains. The authors suggest that it is formed either by post-transcriptional modification of the full length α6 or that it results from translation of the mRNA starting from a second internal translation start site. The role of this form is not well understood but it appears to be upregulated in differentiating keratinocytes and it has been suggested to be a dominant negative form of the subunit involved in down regulating cell adhesion (Davis et al., 2001).

The cytoplasmic domains of the α subunits are not highly conserved although all start with a KXGFFKR motif. The tails range from the 67 amino acid α7 to the 15 amino acid α1. The variation in α subunit tail sequence is thought to be indicative of the presence of α subunit specific binding proteins that allow the different effects seen upon ligation of different dimers that contain the same β subunit. No direct
interactions between α subunit tails and cytoplasmic components are known except for the high affinity interaction of α4 with paxillin (Liu et al., 1999) and an association with the chaperone calnexin prior to heterodimer assembly (Lenter and Vestweber, 1994). Sequences in the α subunit tail are thought to negatively regulate integrin activation as experiments have shown that truncation of the α subunit tail can cause activation of the ligand binding activity of the dimer (Hibbs et al., 1991; Chan et al., 1992; Bauer et al., 1993; Rabb et al., 1993).
Fig 1.1: Structure of the αv subunit. α-helices in red, β-sheets in yellow, random coil in grey. Based on 1JV2 (Xiong et al., 2001).
1.2.2 β subunit

All β subunits have a highly conserved complex structure (Fig 1.2). The subunit consists of several domains, a ligand binding domain, an Ig fold, several cysteine rich domains that comprise the “stalk”, a short transmembrane domain and a cytoplasmic domain (Xiong et al., 2001).

The ligand binding domain is at the amino terminus and is in the form of a von Willebrand factor A fold (Tuckwell and Humphries, 1997) and will be referred to as the βA domain. It is also known as the I-like domain due to its similarity to the I domain found in some α subunits although it contains two insertions that are not found in the α subunit. The βA domain has been shown to be the domain responsible for recognising the RGD sequence (Puzon-McLaughlin and Takada, 1996; Tozer et al., 1996). The domain contains three cation-binding sites: the MIDAS site, which is the primary site of ligand interaction (Tozer et al., 1996), the ADMIDAS (ADjacent to MIDAS) site recently shown in the crystal structure (Xiong et al., 2001) and a third cation, LIMBS, that is acquired upon ligand binding (Xiong et al., 2002). The MIDAS site is thought to be normally occupied by Mg2+ but can also accept Mn2+ and Ca2+ but these cause changes in conformation leading to changes in ligand binding consistent with changes in activation of the receptor. Mutagenesis of the side-chains that co-ordinate the cation cause loss of function of the integrin dimer (Tozer et al., 1996). It is thought that the ADMIDAS site plays a role in stabilising the upper surface of the domain upon ligand binding. The third cation site known as LIMBS (Ligand-associated Metal Binding Site) is not present in the unliganded integrin but is generated by the conformational changes caused by ligand binding. It
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is then thought to stabilise the ligand bound state (Xiong et al., 2002). Another important area of the ligand binding domain is the specificity loop, a small cysteine bridged loop not found in other vWFA domains which has been shown to control the specificity of both the ligand binding and signalling performed by the receptor. This loop also cross-links to integrin peptide ligands indicating it forms part of the ligand binding surface (Bitan et al., 2000; Yahalom et al., 2002). Substitution of this loop in β1 for the corresponding sequence from β3 has been shown to alter ligand specificity (Takagi et al., 1997), signalling (Miao et al., 2002) and to influence folding and dimerisation (Takagi et al., 2002). Another region, the 3_10 helix, is also not found in α subunit I domains. An isoleucine sidechain from this loop fits into the cavity at the centre of the β-propeller of the α subunit and is crucial for subunit dimerisation (Xiong et al., 2001).

The region that lies below the βA domain is a hybrid Ig fold consisting partly of the N terminus of the protein and partly from the region that lies C terminal to the βA domain (Xiong et al., 2001). This domain makes extensive contacts across the lower surface of the βA domain. It is thought that there is little movement between these two structures. The extreme N terminus of the protein forms a PSI (Plexin, Semaphorin and Integrin) domain and this connects the N terminus back onto itself to the EGF-I domain in the stalk below the Hybrid domain. To date the atomic level structure of this domain has not been determined.

The stalk of the β subunit comprises four EGF like units which together are often referred to as the cysteine rich region as each unit contains multiple internal disulphide bonds (Shih, 1993; Calvete et al., 1991). The four units appear to
divide into 2 tandem repeats (1 and 2, 3 and 4). The junction between the two units inside the tandem repeat appears inflexible and includes a disulphide bridge as well as H-bonding whereas the interface between units 2 and 3 appears more flexible (Xiong et al., 2001). In addition to the EGF-like repeats there is a final extracellular domain known as βTD (Xiong et al., 2001). This comprises a four stranded β sheet and a N terminal helix and has little homology to any other known structure. The stalk region contains the epitopes for many of the affinity reporter antibodies (Bazzoni et al., 1995; Luque et al., 1996). These antibodies function by binding to an epitope that only becomes exposed once the integrin undergoes structural rearrangement following ligand binding.

The cytoplasmic domain of β subunits is usually short, around 30-50 amino acids. Apart from β4 and β8 the cytoplasmic domains of the remaining six known β subunits are highly conserved, probably reflecting the importance of their role as ECM receptors. The β4 subunit has a large cytoplasmic domain consisting of an additional thousand amino acids which reflects its role in hemidesmososome formation and connection to the intermediate filament network (Spinardi et al., 1993). Other β subunit cytoplasmic domains have 3 groups of conserved residues, normally designated cyto 1, 2 and 3 (Reszka et al., 1992). The region immediately adjacent to the membrane, before the cyto-1 region, has been implicated as a negative regulator of integrin activation (Hughes et al., 1992). Cyto 1 is a region of 10 amino acids near the membrane. This has been shown to be required for binding of the focal adhesion components FAK, filamin and α-actinin (Otey et al., 1993; Schaller and Parsons, 1994; Lewis and Schwartz, 1995; Sharma et al., 1995). Cyto 2 and 3 are NPXY motifs. These have been shown to be sites of phosphorylation of the tail and
disruption of these sites can modulated integrin function, preventing recruitment to focal adhesions (Bodeau et al., 2001; Mulrooney et al., 2001). All 3 domains are vital for proper function of the integrin, deletion/disruption of any causes the integrin to lose the ability to mediate cell attachment although certain mutations can result in an increased affinity for ligand (Hughes et al., 1992).
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Fig 1.2: Structure of the β3 subunit extracellular domain. α-helixes in red, β-sheets in yellow, random coil in grey. Based on 1JV2 (Xiong et al., 2001). The PSI domain is not shown as the atomic structure has not been resolved. Inset is an alternative view of the A domain in the ligand bound form (based on 1L5G (Xiong et al., 2002)) showing the specificity loop, the $3_{10}$ helix and the LIMBS, MIDAS and ADMIDAS cation sites (blue spheres, left to right)
1.2.3 Ligand binding and activation

The heterodimeric structure of integrins is more complex than many other types of cell adhesion molecule and this increased complexity allows modulation of integrin function in a more subtle manner than simple up or down regulation at the protein level. One of the requirements of integrin function in many situations is the necessity to perform their adhesive functions in a very rapid and specific manner, often in limited time frames and locations. Integrins achieve this regulation of their activity through two main methods, receptor clustering and changes in receptor conformation (Hynes, 1992; van Kooyk and Figdor, 2000; Hogg and Leitinger, 2001).

Integrin clustering is often associated with integrin ligation and this clustering of receptors leads to an increased avidity of the cell for its ligand. As an example showing the importance of clustering, deletion of the α4 cytoplasmic domain prevents the clustering of α4β1 into focal contacts and hence prevents cell attachment despite not preventing the binding of the truncated integrin to VCAM-1 (Yauch et al., 1997). In this case the clustering of the receptor appears to be the important step in regulating the interaction between the cell and the ECM. In other cases structural rearrangements in the receptor itself lead to changes in affinity for ligand. The platelet integrin αIIbβ3 exists in a low affinity form on unactivated platelets. In this state it is capable of binding to immobilised fibrinogen but not to any of its soluble ligands. This means the platelet can only bind to pre-existing sites of clotting. Exposure of the platelet to activating stimuli result in the αIIbβ3 receptor switching into a high affinity state capable of binding to soluble ligands (Kieffer and Phillips, 1990; Phillips et al., 1991). Similar activation events have been observed in
the β2 integrins (Arnaout, 1990; Larson and Springer, 1990). The multiple conformational states can be measured by the use of reporter antibodies that specifically recognise one of the affinity states (Luque et al., 1996) and other antibodies have been produced that are capable of inducing or stabilising a high affinity conformation (Kovach et al., 1992; Petruzzelli et al., 1995). Different activating antibodies have also been reported to promote different active states recognising different ligands (Ortlepp et al., 1995)

There is no current consensus of whether the conformational shift or receptor clustering is the initial event in integrin activation. It is possible that ligand binding to activated, high affinity integrins causes recruitment of further receptors resulting in clustering. It is also conceivable that the initial interactions are between low affinity integrins and ligand and that clustering of these receptors results in changes in conformation leading to strengthening of the interaction due to increases in both avidity and affinity. The two mechanisms of integrin adhesion regulation are likely to have a synergistic effect with conformational changes increasing individual integrin-ligand interactions and integrin clustering increasing the avidity of the interaction and supporting cell attachment. It is also likely that whether integrin clustering or changes in conformation is the dominant factor in activation varies depending on the integrin dimer in question.

The crystal structures of individual integrin domains have helped to reveal the changes in conformation that occur on activation and ligand binding. Comparisons of the crystals of α subunit I domains in the presence of Mg2+ and Mn2+ (Lee et al., 1995a; Qu and Leahy, 1996) and collagen ligands (Emsley et al., 2000) as well as
mutants that lock the domain in either the low or high affinity conformation (Shimaoka et al., 2000; Shimaoka et al., 2001) have provided a mechanism for regulation of I domains. The crystals of the extracellular portion of the \( \alpha \nu \beta 3 \) in the presence of an RGD peptide have shed light on how changes are transmitted throughout the structure (Xiong et al., 2002).

Information on the mechanisms of regulation of integrin activation has also been obtained through studies using antibody epitopes that either cross the boundary between the \( \alpha \) and \( \beta \) chains (Zang et al., 2000), block function (Greve and Gottlieb, 1982; Mould et al., 1997; Shih et al., 1997; Mould et al., 2000), report affinity (Bazzoni et al., 1995; Lu et al., 2001a; Mould et al., 2002) or activate the integrin (Faull et al., 1996). Other experiments involved the use of ligand mimetic peptides to map the critical residues in the ligand binding domain (Humphries et al., 2000; Mould et al., 2000). These results led to a number of models of different regions of the integrin structure being proposed (Huang and Springer, 1997; Tuckwell and Humphries, 1997; Huang et al., 2000a; You et al., 2002).

The quaternary structure of the dimer has recently been resolved in both the ligand bound and an unbound form for the \( \alpha \nu \beta 3 \) dimer (Xiong et al., 2001; Xiong et al., 2002). The interaction of the \( \beta \) subunit A domain and the \( \beta \) propeller domain closely resembles the structure of the G-protein \( \alpha / \beta \) subunit interface with the 3\textsubscript{10} helix of the \( \beta A \) domain located in the gap in the axis of the \( \beta \)-propeller domain (Xiong et al., 2001). The two \( \alpha \nu \beta 3 \) crystal structures only included the extracellular domains of \( \alpha \nu \beta 3 \). Because of this the effect that the ligand induced conformational change has on the cytoplasmic domains is still not well understood. The role of the cytoplasmic
domains in conducting signals both into the cell and out to the ligand binding domains is crucial in understanding integrin function (Takagi et al., 2001).

In integrins where an α subunit I domain is the principal site of ligand binding the I domain undergoes some very specific changes upon activation (Fig 1.3). In the α2 I domain a single turn of helix is transferred from helix C to helix α6 which allows the opening of the ligand binding domain (Emsley et al., 2000). The MIDAS cation moves, its co-ordination sphere changes and it interacts directly with the ligand. Most notably small changes in other regions of the structure result in a large movement of the C terminal α7 helix which drops downwards by 10 Å in the liganded structure (Emsley et al., 2000). Mutated domains where this helix has been restrained in either the up or down position show that this movement is capable of activating or inactivating the entire I-domain (Lu et al., 2001b; Shimaoka et al., 2001). The movement of the α7 helix is thought to allow the conformational transition to be passed down the structure. Recent work has shown that a sequence bottom of this domain acts as a ligand for the β subunit A domain and that the interplay between these two domains is vital for function of the integrin (Alonso et al., 2002).
Fig 1.3: Conformational changes in the 1 domain of the α2 subunit (after (Emsley et al., 2000)). On ligand binding the cation moves towards helix α5, the αC helix unwinds and transfers a loop to α6 and the α7 helix is displaced downwards and away from α1. α-helix in red, β sheets in yellow and random coil in grey. Cations shown as blue spheres. Prepared using SWISSPdB viewer from structures 1AOX (left) and 1DZI (right).
In subunits lacking an α subunit I domain the principal site of ligand binding is the β subunit A domain. This domain is similar in structure to the α I domain, however activation appears to occur in a different manner. In the β A domain the major events are rearrangement of the cation binding sites with the MIDAS site becoming occupied and co-ordinating with the ligand (Xiong et al., 2002). The LIMBS cation binding site forms and becomes occupied to stabilise the conformation and the region containing the specificity loop moves towards the MIDAS site (Xiong et al., 2002). It has also been suggested that the α1 helix may become displaced in a similar fashion to the α7 movement seen in α subunit I domains (Mould et al., 2002).

Sequences in the second and the third repeats of the α subunit β-propeller domain play a role in defining ligand specificity by interacting with “synergy sequences” in integrin ligands that surround the RGD motif (Mould et al., 1997; Humphries et al., 2000; Mould et al., 2000).

In addition to the local changes around the ligand binding sites the integrin undergoes large scale structural rearrangement. The inactive form of the integrin seen in the αvβ3 crystal is bent back on itself at two hinge regions, one in each subunit (Xiong et al., 2001). On activation the integrin is thought to straighten into the classic “globular head with stalks” conformation seen in electron-micrographs (Nermut et al., 1988; Beglova et al., 2002). This switch exposes a large region of the stalk, which is hidden in the closed conformation, and many activation reporter antibodies bind to epitopes in this region (Luque et al., 1996). It is likely that this conformational transition is passed through the membranes and leads to relative changes in the position of the cytoplasmic domains and thus influences integrin signalling.
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1.3 Integrin Interacting Proteins, Signalling and Focal Adhesion Assembly

Integrins are capable of assembling large complexes of proteins through interactions mediated by their cytoplasmic, transmembrane and extracellular domains. These complexes function not only as structural junctions between the cell and ECM but also control complex signalling systems allowing the cell to respond to changes in its environment.

1.3.1 Transmembrane and extracellular Integrin interacting proteins

Integrins mediate several processes through cis-interactions with other membrane proteins. Caveolin has been shown to interact with a subset of α subunits (α1, α5, αv) via the integrin transmembrane domains. Its ability to bind Src family kinases, such as Fyn, allows a signalling pathway to ERK1/2 to be activated (Wary et al., 1998). This pathway is associated with caveolin’s ability to bind to cholesterol and glycosphingolipids and organise membrane rafts that are rich in the Src family members which are both myristoylated and palmitoylated such as Fyn, Yes and Lck (Harder and Simons, 1997). Integrins which are in the active conformation have been shown to interact with these lipid rafts in certain cell types (Leitinger and Hogg, 2002).

IAP (CD47) is a large 5 transmembrane Ig family protein which is widely expressed. It has been shown to act as a receptor for thrombospondin. It interacts with α2β1 (Wang and Frazier, 1998) and αvβ3 (Lindberg et al., 1996) and appears to act to regulate cell migration by signalling through pathways involving FAK and heterotrimeric G proteins.
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Many \( \alpha \) integrin subunits form highly stable interactions with members of the tetraspanin or transmembrane-4 superfamily (TM4SF). These interactions are mediated through the one of the extracellular loops of the tetraspanin and the extracellular domain of the \( \alpha \) subunit although the exact point of interaction seems to differ in different tetraspanin/integrin complexes (Hemler \textit{et al.}, 1996; Yauch \textit{et al.}, 2000). The cytoplasmic domains of the tetraspanins are associated with signalling pathways involving PI-4-K and PKC and have been implicated in processes including cell spreading, migration, MMP induction and differentiation in different cell types (Hemler, 1998; Sugiura and Berditchevski, 1999; Stipp and Hemler, 2000; Berditchevski, 2001).

1.3.2 Integrin cytoplasmic domain binding proteins.

The interactions between integrin cytoplasmic domains and their associated adapter proteins has been extensively studied. However because of the complex nature of these interactions, coupled with the fact that many of them appear to be transient, rely on phosphorylation by other proteins, or require multiple proteins to be present to be stable, they are not fully understood. One of the best characterised is that of paxillin with the \( \alpha 4 \) cytoplasmic domain (Liu \textit{et al.}, 1999; Liu and Ginsberg, 2000). In this case the interaction is high affinity, allowing the two proteins to be co-immuno-precipitated. Paxillin is also thought to be capable of forming a weaker interaction with some \( \beta \) subunits and interacts with a large number of proteins present in the large complex which assembles around the \( \beta \) subunit tail (Turner, 2000). \( \alpha \)-actinin, filamin, tensin and talin are all structural components of the focal
adhesions which interact with the β subunit tail (reviewed in (Petit and Thiery, 2000)).

Other proteins that interact directly with the β cytoplasmic domain have been identified by yeast two hybrid screens; ICAP, RACK-1, caspase-8 and ILK. ICAP has been shown to interact with the region between the cyto-2 and 3 motifs of β1 (Chang et al., 1997; Zhang and Hemler, 1999). Overexpression of ICAP inhibits cell spreading but this is reversed by overexpression of constitutively active Cdc42. ICAP interacts with both cdc42 and Rac as an inhibitor of GDP dissociation (Degani et al., 2002).

RACK-1 interacts with an area just before the cyto-1 motif and has been shown to co-immuno-precipitate with β subunits under certain conditions (Liliental and Chang, 1998). RACK-1 is an adapter protein for the active form of PKC and recruits activated PKCα and ε (Liliental and Chang, 1998; Besson et al., 2002). PKC isoforms are involved in processes governing transport of β subunits to the membrane (Ng et al., 1999). PKCα may interact directly with some β subunits through the NPXY motifs in the β1 tail (Parsons et al., 2002).

Caspase-8 is reported to bind directly to the membrane proximal region of unligated β1 and β5 subunits (Stupack et al., 2001).

ILK (Integrin Linked Kinase) was initially identified as a kinase that binds directly to the β1 tail (Hannigan et al., 1996). ILK has been implicated in processes including anchorage dependent growth (Attwell et al., 2000), suppression of apoptosis (Persad...
et al., 2001) and tumorigenicity (Marotta et al., 2001). Over expression of ILK induces an epithelial/mesenchymal transition in mammary epithelial cells (Somasiri et al., 2001). There is also evidence that ILK can promote β-catenin signalling (Tan et al., 2001). The kinase activity of ILK does not appear to be essential for its function in integrin signalling (Zervas and Brown, 2002) and it is now thought to act primarily as an adapter molecule (Tu et al., 1999; Guo and Wu, 2002).

The kinase FAK (Focal Adhesion Kinase) has also been reported to bind to the β1 tail (Lewis and Schwartz, 1995; Hannigan et al., 1996) and has a role in focal adhesion assembly/disassembly and in integrin mediated signalling.
1.3.3 Focal Adhesions

Focal adhesions were first identified in the 1970s as regions of the cell that were in close contact with the substratum (Abercrombie et al., 1971; Couchman and Rees, 1979). These are large protein structures that assemble around integrin tails to mediate adhesion and motility. Focal adhesions can be divided into the two types: focal contacts and fibrillar adhesions (Zamir et al., 1999). Focal contacts are the “classical” focal adhesion, enriched in scaffolding proteins such as talin and paxillin and are highly tyrosine phosphorylated. Fibrillar adhesions are enriched for tensin, another scaffold protein, and are less highly phosphorylated.

Fig 1.4: Keratinocytes expressing endogenous WT human integrin (Red) and YPRF mutant chick integrin (Green) spread on collagen. WT integrin forms large focal adhesions. The YPRF disrupts the cyto-2 motif in the β1 tail and this prevents recruitment of the chick subunit to these focal adhesions.
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Fig 1.5 : Schematic structure and components of (A) focal contacts and (B) fibrillar adhesions.
Focal contacts

The structure of the focal contact (Fig 1.5A) is provided by a series of scaffolding proteins such as talin, vinculin, filamin and α-actinin upon which is built a series of signalling systems. Talin is present as a dimer and may associate directly with the β subunit tail. Cells expressing integrins that lack the binding sites for α-actinin and talin are deficient in migration and adhesion (Bodeau et al., 2001). In culture focal contacts appear to form at the periphery of the cell and can be promoted by plating the cells onto rigid matrixes such as glass bound vitronectin. These contacts apply significant amounts of force onto the matrix generated by myosin II driven cytoskeleton contraction (Zamir et al., 1999).

Fibrillar adhesions

Fibrillar adhesions are more elongated structures (Hynes and Destree, 1978) containing tensin, a protein with an actin filament capping function (Lo et al., 1994; Chuang et al., 1995) (Fig 1.5B). These adhesions also appear to be simpler, and not to involve as many other proteins as the talin containing focal contacts (Katz et al., 2000; Zamir et al., 2000). They form at sites of low matrix rigidity and tend to migrate towards the centre of the cell under the contractile force of the cytoskeleton. As they move the carry the matrix components bound to the integrins with them. It is thought that this is responsible for generation of the elongated fibrils of fibronectin seen under cells in culture (Katz et al., 2000).
1.3.4 Focal adhesion assembly/disassembly

As previously described focal adhesions are large complexes of proteins, the primary role of which is to anchor the cell to the basement membrane. However this strong adhesion has the effect of severely limiting the migration of the cell. In order for effective cell movement the stability of focal adhesions has to be tightly regulated (Rodriguez Fernandez et al., 1992; Rodriguez Fernandez et al., 1993), allowing the cell to attach to the matrix, apply sufficient force across the adhesion to move the cell body over it then detach from the matrix at the rear of the cell and recycle components to the leading edge.

The initial step of focal adhesion formation is clustering of integrin subunits. This process is highly dependent on the β cytoplasmic tail and is mediated by adapters such as talin binding to the cyto2/3 motifs (Horwitz et al., 1986). Subunits in which these motifs are disrupted are incapable of being recruited to integrin clusters and are present diffusely across the whole membrane surface (Solowska et al., 1989; Hayashi et al., 1990). FAK, talin, tensin, α-actinin and vinculin are recruited to early adhesion structures followed by their associated signalling molecules. Once the focal adhesion has begun to assemble around the clustered integrins the complex associates with actin fibres. This process is regulated by the Rho GTPase and its interactions with p140Dia, PI-5-Kinase and Rho Kinase (ROCK) ((Ridley and Hall, 1992), reviewed in (Petit and Thiery, 2000; Geiger et al., 2001)). ROCK has numerous targets including myosin phosphatase and myosin light chain. Phosphorylation of these proteins allows myosin II to interact with F-actin causing contraction of stress fibres. The generation of tension through the stress fibre matrix allows the cell to generate contractile force and propel itself over the matrix. This
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tension is also essential for the proper formation of the focal adhesion. Experiments have shown that the type of adhesion that forms, either fibrillar adhesion or focal contact, is directly dependent on the amount of contractile force that the matrix can support. If the matrix is non-rigid little tension is exerted on the focal adhesion as the cell deformations the matrix. If the matrix is rigid the force across the focal adhesion increases and this leads to recruitment of proteins such as talin and vinculin which stabilise the structure (Katz et al., 2000; Zamir et al., 2000; Riveline et al., 2001). The degree of phosphorylation seen in the structure also appears to be dependent on the degree of tension (Jockusch et al., 1995; Zamir et al., 1999).

At the rear of the cell many focal adhesions appear to physically break off from the cell and remain in contact with the matrix. However most focal adhesions are disassembled at the rear of the cell and their components recycled. Deactivation of Rho has been shown to lead to focal adhesion disassembly. This can be triggered by phosphorylation of Rho by PKA (Lang et al., 1996). Activation of Cdc42/Rac effectors such as N-WASP and PAK (p21 activated kinase) also induce stress fibre and focal adhesion disassembly by inactivating myosin light chain kinase. Activation of tyrosine kinases has also been linked to focal adhesion disassembly. V-Src transformed cells show increased phosphorylation of focal adhesion proteins but show a rounded morphology and disorganised focal contacts (Petit and Thiery, 2000). Phosphorylation of Src targets such as FAK and paxillin can also lead to their degradation suggesting that Src is important both in focal adhesion disassembly as well as signalling (Petit and Thiery, 2000).
1.3.5 Integrin Signalling

Unlike many other cell surface receptors integrins have no intrinsic kinase activity. For many years it was thought that, because of this, they had no signalling function beyond their role in cell adhesion and focal adhesion formation. It is now understood that, by recruiting a range of adapter proteins and kinases through interactions with both extracellular and cytoplasmic domains, integrins are involved in many different signalling pathways (reviewed in Giancotti and Ruoslahti, 1999; Schwartz, 2001; Schwartz and Ginsberg, 2002).

One of the major kinases involved in integrin mediated signalling is the Focal Adhesion Kinase (FAK). As its name suggests FAK plays an important role both in focal adhesion formation and disassembly but it is also involved in several signalling cascades leading from the integrin. One of the best characterised roles for FAK is in FAK mediated MAPK activation (Fig 1.6A). FAK is initially recruited to focal adhesions through its interactions with talin and vinculin (Chen et al., 1995) and possibly the β subunit tail (Lewis and Schwartz, 1995). Auto phosphorylation of Y397 activates FAK as a tyrosine kinase. This allows binding of the membrane bound kinase Src via an SH2 domain interaction with Y397 (Schaller et al., 1994; Schlaepfer et al., 1994). Further phosphorylation events lead to the recruitment of the adapter proteins such as CAS and CRK leading to activation of the JNK pathway. Recruitment of the Grb2/SOS complex leads to activation of the ERK1/2 MAP Kinase via Ras and Raf (reviewed in Giancotti and Ruoslahti, 1999).

Integrin mediated ERK1/2 activation can also occur independently of FAK activation (Wary et al., 1998) (Fig 1.6A). In this pathway Shc binds to the Fyn SH3 domain
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leading to phosphorylation of Shc and recruitment of the Grb2/SOS complex. This allows activation of Ras and triggers the MAPK cascade. The two pathways leading to Ras are thought to act in tandem, Shc initially triggering the high levels of MAPK activation seen shortly after adhesion and FAK causing the sustained activation following it (reviewed in Giancotti and Ruoslahti, 1999).

Integrin ligation can influence signalling to MAPK stimulated by ligation of the EGF receptor. Studies have shown that in the absence of integrin ligation EGF induced signalling is largely inhibited (Miyamoto et al., 1996), in contrast integrin derived signalling to MAPK can occur in the absence of EGF, albeit at lower levels than when growth factors are present. Other work has suggested an association between the EGF receptor and various integrins (Miyamoto et al., 1996). It has been shown that co-clustering of the EGF receptor and the α2β1 integrin at sites of cell-cell contact induce tyrosine phosphorylation and activation of the EGF receptor in the absence of its ligand (Moro et al., 1998; Yu et al., 2000). EGF receptor signalling uses many of the same components found in integrin mediated signalling pathways, such as Shc, Grb and SOS so a synergistic role for these two pathways is likely. In addition the PDGF (Schneller et al., 1997; Woodard et al., 1998) and VEGF (Soldi et al., 1999) receptors have also been reported to form complexes with integrins.

PI-3-Kinase plays a role in integrin signalling downstream of FAK (Fig 1.6B). This lipid kinase is an important regulator of adhesion and migration and activates the anti-apoptotic protein kinase Akt (Chen et al., 1996). PTEN, a tumour suppresser lipid/protein phosphatase acts as a negative effector on these pathways, acting to reduce phosphorylation in the absence of integrin activation (Parekh et al., 2000).
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Figure 1.6 (A) FAK dependent and independent activation of ERK by ligation of integrins. (after (Giancotti and Ruoslahti, 1999)) (B) Integrin signalling to Akt through FAK/PI3K. PTEN is thought to act as a repressor on this pathway.
1.4 Structure and Function of the Skin

Human skin consists of several layers (Fig 1.7). The outermost layer is the epidermis. Below the epidermis lies a basement membrane that separates the epidermis from the underlying dermis. The epidermis and the dermis interdigitate, forming the rete ridge and dermal papillae (Fig 1.7).

1.4.1 The Dermis

The most common cells in the dermis are fibroblasts although there are also numerous blood vessels and nerve endings. The dermis can be divided into two layers. The upper layer, the papillary dermis, is a loosely arranged matrix of type I and III collagen fibres (Goldsmith, 1991). Below this lies the reticular dermis, a layer of densely packed collagen I fibres along with filamentous type V and VI collagens. Elastin is also present in this layer and this provides the elastic strength of the skin (Goldsmith, 1991). The dermis also contains large amounts of mucopolysaccharides, primarily hyaluronates and dermatan sulphates. These occupy the space between the collagen fibres (Nasemann et al., 1983). Below the dermis lies a layer of subcutaneous fat that separates the skin from the underlying muscles.


**Fig 1.7:** Schematic representation of the structure of human skin.

### 1.4.2 The Epidermis

The epidermis is the main barrier between the body and the surrounding environment (Fig 1.8). It is predominantly made up of keratinocytes although smaller numbers of melanocytes (pigment cells) (Jimbow *et al.*, 1991), Merkel cells (sensory cells) and Langerhans cells (a type of antigen presenting cell) (Hauser *et al.*, 1991) are also present. The outer layers of the epidermis consist of tough squames that provide the barrier function of the skin. Below these are layers of living cells that arise from the population of dividing keratinocytes in the basal layer. Within the epidermis and dermis are also found hair follicles, apocrine and eccrine sweat glands and sebaceous glands (Odland, 1991). These are all derived from the epidermis.
1.4.2 The basal layer keratinocytes

The cells of the basal layer (Fig 1.8) are in contact with an underlying membrane comprised of a range of ECM proteins. The most abundant are collagens, especially type IV collagen (Timpl, 1989; Odland, 1991). In addition there are large amounts of laminins and other proteoglycans (Burgeson and Christiano, 1997). Fibronectin is not normally present except in wounded epidermis (Mosher, 1989; Fine, 1994). The basement membrane components are synthesised both by keratinocytes and dermal fibroblasts (Marinkovich et al., 1993). The basal keratinocytes adhere to the basal membrane through β4 mediated hemidesmosomes and β1 mediated focal adhesions (Carter et al., 1990a; Carter et al., 1990b; Stepp et al., 1990).
1.4.3 The upper epidermal layers

The various layers of the skin can be distinguished by the range of proteins they express.

The spinous layer comprises the first 4-8 cell layers above the basal layer and is characterised by an increase in cell size and an increase in desmosomal junctions between the keratinocytes (Holbrook, 1994). Most suprabasal layers express involucrin (Rice and Green, 1979), a protein synthesised early in the differentiation pathway, which is part of the cornified envelope of a mature squame.

The granular layer is identified by the synthesis of electron dense keratohyalin granules of loricrin and profilaggrin (Holbrook, 1994). Loricrin is a component of the cornified layer (Mehrel et al., 1990) and profilaggrin is the precursor to filaggrin, a protein involved in the aggregation of keratin filaments (Rothnagel et al., 1987; Rothnagel and Steinert, 1990).

The final layer of the skin, the cornified layer, consists of flattened squames. Cornified squames are extremely flat, being approximately 40 μm in diameter but only 0.5 μm in thickness. These cells have lost their organelles and their nucleus and function as a barrier to desiccation and mechanical damage (Nemes and Steinert, 1999). The contents of a squame are largely keratins surrounded by a 12 nm thick insoluble cornified envelope that is deposited beneath the remains of the plasma membrane (Holbrook, 1994). These squames are continually being shed from the surface of the skin.
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The cells of all layers adhere to each other in a calcium dependent manner through adherens junctions and desmosomes (Cowin, 1994). Adherens junctions, also known as zonula adherens are found as a belt around the cell and appear as electron dense regions where the membranes of adjacent cells run parallel about 20 nm apart (Geiger and Ginsberg, 1991). This type of cell-cell junction is found in many tissues including the epithelium (Geiger et al., 1987).

The intracellular section of adherens junctions consists of dimers of cadherin family proteins (Bussemakers et al., 1993; Kemler, 1993). These are transmembrane proteins that form calcium dependent head-to-tail dimers through their extracellular domain with corresponding proteins from adjacent cells. Keratinocytes of all epidermal layers express E-cadherin while P-cadherin is expressed only in the basal layer (Hirai et al., 1989). Adherens junctions link to the actin cytoskeleton through vinculin, α-actinin and catenin family proteins (Kemler, 1993).

Desmosomes link adjacent cells through desmoglein and desmocollin, members of the cadherin superfamily (Schwarz et al., 1990; Kowalczyk et al., 1999). In vivo desmosomes cover a large proportion of the membrane surface of keratinocytes (Cowin, 1994). Desmosomes are attached to the keratin cytoskeleton through a group of proteins termed plakins. This junction effectively interconnects the keratin cytoskeletons of surrounding keratinocytes into one mesh, increasing the ability of the skin to resist damage.
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Gap junctions are also present in the keratinocytes. These are small transmembrane channels that permit the transfer of small molecules between adjacent cells (Evans, 1988). These are found in all layers of the epidermis.

1.4.4 Squamous Cell Carcinoma

Squamous cell carcinomas (SCC) are common tumours of the skin and epithelia (Fig 1.9). The incidence of SCC is so high and the mortality rate so low that, along with basal cell carcinomas of the skin, they are excluded from the national statistical analysis of cancer cases. The combined incidence of these two types of non-melanoma skin cancer was over 40,000 in England and Wales in 1997 and around 20% of these were recorded as SCC. Less than 500 mortalities per year are recorded as being directly due non-melanoma skin cancer (Source: National Office of Statistics).

SCC most frequently arise from other skin lesions such as actinic keratosis or benign papilloma although they can also form due to exposure to chemical carcinogens or radiation (Nasemann et al., 1983). Most SCC do not show great metastatic potential with less than 3% of patients showing any secondary tumours. The exceptions to this are carcinoma of the oral cavity and especially the lower lip of which 11% are metastatic. SCC induced by chemical or radiation exposure also frequently metastasise (Lever and Elder, 1997).
Fig 1.9 Histology of (A) Normal human skin and (B) squamous cell carcinoma. (C) SCC showing keratin pearls (arrow heads).
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Treatment is normally by excision followed by chemo- or radiotherapy and survival rates are normally 90% over 5 years if treated early. If the condition is not treated promptly large scale tissue destruction, can occur, especially in oral tumours (Nasemann et al., 1983). SCC show disruption of the normal skin architecture with islands of keratinocytes present within the dermis and loss or disruption of the basement membrane (Wheater, 1991). The keratinocyte differentiation program is also perturbed. Well-differentiated SCC produce large amounts of keratin and frequently large masses of protein known as keratin pearls can be seen (Fig 1.9C). Poorly differentiated tumours show reduced keratin expression (Wheater, 1991). Spindle like cells may be present in more severe undifferentiated tumours (McGee et al., 1992)

1.5 Epidermal Integrins

Keratinocytes express a range of integrin subunits. In vivo α2, α3, α6, α9 and αv are expressed along with the β1, β4 and β5 subunits (Watt and Hertle, 1994; Watt, 2002). These make up the α2β1 collagen receptor, the α3β1 laminin receptor, α6β4 laminin receptor, α9β1 tenascin receptor and the αvβ5 vitronectin receptor. Upregulation of α5β1 and αvβ6 is seen in wounded or hyperproliferative epidermis as well as in culture (Adams and Watt, 1991; Watt and Hertle, 1994). αvβ8 is also reported to be present (Stepp, 1999). Despite expressing both the α6,αv and β1 subunits neither α6β1 or αvβ1 is expressed in keratinocytes (Adams and Watt, 1991).

In normal, undamaged epidermis integrin expression is restricted to the basal layer of keratinocytes in the epidermis and outer root sheath of the hair follicle. The
exception to this is αvβ8 which is expressed only in the suprabasal layers of the epidermis (Stepp, 1999). α6β4 is concentrated on the basal membrane of keratinocytes where it forms hemidesmosomes, anchoring the cell to the basement membrane (Watt and Hertle, 1994). Wholemount labelling of basal keratinocytes shows some focal adhesion like clusters of β1 integrins interspersed with the hemidesmosomes on the basal surface of the cell but most β1 integrins are present in a ring around the cell periphery (Jensen et al., 1999). Although β1 integrins appear to be concentrated at cell-cell borders this is likely to be a result of the heavily interdigitated membranes at these sites rather than due to any role in cell-cell adhesion (Braga et al., 1998). In wounded or hyperproliferative skin integrin expression may be seen in the suprabasal layers of the epidermis (Watt and Hertle, 1994).

Alternative splicing events occur in the integrin subunits expressed by keratinocytes. The most common β1 expressed is β1A although keratinocytes are also one of the few cell types to express β1B. This subunit has a unique 12 amino acid sequence that replaces the last 21 amino acids of β1A. β1B is expressed on the cell surface but does not mediate cell adhesion and acts in a dominant negative manner against β1A function in transfection experiments (Balzac et al., 1994). The importance of β1B is questionable as it is expressed at very low levels in human keratinocytes (Kee et al., 2000) and is not conserved in the mouse genome (de Melker and Sonnenberg, 1999).

α6A is the normal α6 form expressed in keratinocytes (Hogervorst et al., 1993). However the α6B subunit is found in some keratinocyte cell lines derived from mouse tumours (Tennenbaum et al., 1995). β4A is the most common variant
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expressed in keratinocytes although trace amounts of β4B (Hogervorst et al., 1993) and β4E are present. β4E has a shorter (232 amino acid) tail and lacks the structures required for hemidesmosome formation (van Leusden et al., 1997; de Melker and Sonnenberg, 1999).

1.5.1 Functions of Epidermal Integrins

The primary function of integrins in the epidermis is to secure the cells of the basal layer to the basement membrane through their interactions with the actin and keratin cytoskeletons. Integrins also play a vital role in mediating keratinocyte migration over ECM (Watt and Hertle, 1994; Watt, 2002). α3β1 is required at the leading edge of the cell for keratinocyte migration over laminin (DiPersio et al., 1997; Goldfinger et al., 1999). In migrating cells α6β4 is present at the leading of edge of the cell where it can be associated with membrane protrusions and show a reduced association with hemidesmosomes on the basal surface (Nguyen et al., 2000; Mercurio et al., 2001).

Mutation of specific integrin subunits has confirmed the role of the different subunits in mediating adhesion and migration in vivo (Table 1.2). Deletion of either the α6 or β4 subunits in mice result in a loss of hemidesmosomes, severe blistering of the skin and other epithelia and death die shortly after birth (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). This closely mimics the human condition junctional epidermolysis bullosa where mutations in the α6β4 integrin result in severe blistering of the skin.
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<table>
<thead>
<tr>
<th>Subunit</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3</td>
<td>Disorganised basement membrane, occasional blistering.</td>
<td>(DiPersio et al., 1997)</td>
</tr>
<tr>
<td>β6</td>
<td>Juvenile hair loss</td>
<td>(Huang et al., 1996)</td>
</tr>
<tr>
<td>α9</td>
<td>No defects</td>
<td>(Huang et al., 2000c)</td>
</tr>
<tr>
<td>β5</td>
<td>Reduced migration of keratinocytes</td>
<td>(Huang et al., 2000b)</td>
</tr>
<tr>
<td>β1</td>
<td>Floxed β1 x K5Cre</td>
<td>(Grose et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Abnormal hair follicles, hair loss, blistering, reduced proliferation and abnormal differentiation, disruption of basement membrane, impaired wound healing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Floxed β1 x K14Cre</td>
<td>(Brakebusch et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Blistering, basement membrane disruption, reduced hemidesmosomes, thin epidermis, reduced hair follicles, reduced α6β4</td>
<td></td>
</tr>
<tr>
<td>α6</td>
<td>Severe blistering</td>
<td>(Georges-Labouesse et al., 1996)</td>
</tr>
<tr>
<td>β4</td>
<td>Severe blistering</td>
<td>(Dowling et al., 1996; van der Neut et al., 1996)</td>
</tr>
<tr>
<td>α3+α6</td>
<td>Blistering</td>
<td>(DiPersio et al., 2000)</td>
</tr>
<tr>
<td>α2</td>
<td>No defects</td>
<td>(Holtkotter et al., 2002)</td>
</tr>
</tbody>
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Table 1.2: Epidermal Phenotypes of integrin transgenic mice (Watt, 2002)

Loss of α3 by targeted deletion in mice also causes a disruption of the basement membrane and occasional epidermal blistering on the feet and legs (DiPersio et al., 1997). The double knockout of α3β1 and α6β4 does not result in a more severe phenotype than either alone (DiPersio et al., 2000). α2, α9 and β5 knockouts do not result in skin phenotypes (Huang et al., 2000b; Huang et al., 2000c; Holtkotter et al., 2002) although loss of β5 does result in severely impaired migration of keratinocytes (Huang et al., 2000b).

The β1 knockout is early embryonic lethal (Fassler and Meyer, 1995; Stephens et al., 1995). Mice expressing floxed β1 alleles have been generated and crossed with mice...
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expressing Cre under the control of either the keratin 5 or 14 promoter. This results in Cre activity in the basal layer of the epidermis (Brakebusch et al., 2000; Raghavan et al., 2000). These mice suffer from epidermal blistering but not to the degree reported in the α6β4 null mice. Wound healing studies performed on these mice confirm that β1 is essential for keratinocyte migration in vivo (Grose et al., 2002). Mouse keratinocytes lacking β1 due to K5-Cre expression show an increase in the proportion of cells expressing differentiation markers, the number of cells expressing involucrin increased from 1% to 20-40% (Grose et al., 2002). Other than the increase in the number of differentiated cells the differentiation program itself appears to proceed normally. The mice also show a general reduction in proliferative cells in their hair follicles and by 7 weeks lack both hair follicles and sebaceous glands (Brakebusch et al., 2000). No change in the differentiation program was seen in K14-Cre mice, however the proportion of differentiated cells was not measured (Raghavan et al., 2000).

1.5.2 Epidermal Stem Cells.

Epidermal stem cells are a sub-population of keratinocytes that are responsible for renewing the epidermis. The cells of the interfollicular epidermis, the hair follicle and the sebaceous glands all arise from the same population of stem cells (Watt, 2001). Human epidermal stem cells can be identified in culture and in the epidermis by their high expression of the β1 integrin subunit (Jones and Watt, 1993; Jensen et al., 1999). High β1 expressing cells appear to be clustered into groups at the tip of the dermal papillae and are surrounded by areas of lower expressing cells (Jensen et al., 1999) (Fig 1.10A). In hair follicles a region of high β1 expression, called the
bulge region, is also thought to contain stem cells (Jones et al., 1995; Akiyama et al., 2000). In the mouse various locations for stem cells have been suggested including the bulge region of the hair follicle (Cotsarelis et al., 1990), the inter-follicular epidermis (Allen and Potten, 1974) and the outer root sheath (Lenoir et al., 1988).

The cells surrounding the areas of high β1 expression are the offspring of stem cells, known as transit amplifying cells. These cells have left the stem cell compartment and divide a limited number of times before withdrawing from the cell cycle and differentiating (Watt, 2001)(Fig 1.10B). These cells are known as transit amplifying cells. These cells have lower β1 integrin levels and are less adhesive to ECM than stem cells (Bickenbach and Chism, 1998).

The high level of β1 integrin in human epidermal stem cells has two significant roles in maintaining the stem cell compartment. Expression of a chimera of the CD8 extracellular domains and the β1 integrin cytoplasmic domain has a dominant negative effect on the endogenous β1 integrin. Keratinocytes expressing this construct show reduced levels of endogenous β1, reduced β1 mediated adhesion and reduced colony forming efficiency in culture (Zhu et al., 1999). This effect appears to be due to changes in integrin signalling to MAPK, making the cells behave as transit amplifying cells. Expression of a constitutively active MAPK or over expression of WT β1 integrin restores normal function in these cells (Zhu et al., 1999). The high level of β1 also makes stem cells less migratory than their offspring and helps ensure the segregation of the stem and transit cell compartments (Jensen et al., 1999).
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Fig 1.10 (A) β1 high expressing cells are found in clusters within epidermal whole mounts with the highest expression at the centre of the cluster (inset). Scale bar 100 μm. (Jensen et al., 1999) (B) The fate of epidermal stem cells. (Zhu et al., 1999)
1.5.3 Integrins and Squamous Cell Carcinoma

SCC, in human and mouse, often show considerable variation in integrin expression both between tumours and between regions of the same tumour. Normal expression, over expression and total or focal loss of the normal keratinocyte integrin subunits has been observed as well as de novo expression of integrins such as αvβ6. (Peltonen et al., 1989; Jones et al., 1993; Jones et al., 1997; Bagutti et al., 1998).

Changes in expression of α6β4 have been implicated in epithelial carcinogenesis. Over expression of this integrin in the layers of the tumour that are not adjacent to the stroma is associated with poor prognosis in human disease (Van Waes et al., 1991) and a high risk of malignant conversion in mouse carcinogenesis (Tennenbaum et al., 1995). Loss of α6β4 in the layers of tumour adjacent to the stroma is associated with a loss of the basement membrane (Downer et al., 1993). In many tumours α6β4 expression is maintained but becomes associated with the actin cytoskeleton to promote invasion and migration (Mercurio et al., 2001).

Changes in integrin expression, especially the induction of αvβ6, are often linked to direct changes in cell motility through induction of matrix metalloproteinases (Thomas et al., 2001).

The expression of integrins in suprabasal keratinocytes also appears to result in changes in tumorigenesis. By expressing integrin subunits under the control of the involucrin promoter mice can be generated that express various integrins in the suprabasal layers of the skin. Expression of integrins in suprabasal keratinocytes
does not lead to spontaneous tumours. However, after treatment with DMBA to initiate Ras mutations followed by TPA to promote clonal expansion effects of integrin expression on tumour development can be seen. In normal mice benign papilloma appear first, some of these progress to SCC but others spontaneously regress. In mice expressing suprabasal α3β1 the rate of conversion from papilloma to SCC is reduced. Expression of suprabasal α6β4 increases the formation of both types of tumour. Suprabasal α2β1 has no apparent effect on SCC formation (Owens and Watt, 2001). Although skin tumours are derived from the proliferative cells in the basal layer this shows that the cells surrounding the tumour are capable of influencing the tumours development.

Integrin expression is also linked to differentiation in SCC cell lines. Transfection of αv into a cell line lacking this subunit has been shown to restore the ability of the cell to undergo terminal differentiation and inhibits anchorage dependent growth (Jones et al., 1996).

1.5.4 Regulation of terminal differentiation

Cultured keratinocytes that are placed in suspension withdraw from the cell cycle and undergo terminal differentiation (Green, 1977; Adams and Watt, 1989). After 24 hours up to 80% of cells will express involucrin, compared to 5-20% in adherent cultures. This process can be partially inhibited by ligation of the α5β1 integrin, either by fibronectin or anti-β1 antibodies (Adams and Watt, 1989; Watt et al., 1993) (Fig 1.11). Monovalent antibody Fab fragments and RGD peptides also cause suppression of suspension induced differentiation showing that receptor ligation
rather than clustering is the essential factor (Adams and Watt, 1989). The suppression of differentiation does not involve polymerisation of the actin cytoskeleton as cytochalasin D treatment does not prevent the effect (Adams and Watt, 1989; Watt and Jones, 1993). Addition of integrin ligands can only prevent differentiation if added within a few hours of the start of suspension culture. After 5 hours the cells have committed to differentiation and cannot then be rescued (Adams and Watt, 1989).

![Diagram]

**Fig 1.11** Suppression of suspension induced differentiation by $\beta_1$ integrin ligation.

Function of the $\beta_1$ integrin is regulated at two levels during keratinocyte differentiation. In the initial stages of suspension induced differentiation keratinocytes become less adhesive due to inactivation of their $\beta_1$ integrin (Hotchin *et al.*, 1993). This inactivation can be reversed by the use of activating antibodies to the $\beta_1$ subunit. However, the down regulation of integrin function occurs after the
cell has committed to differentiation and the use of activating antibodies to reverse this does not reverse commitment (Hotchin et al., 1993). After 24 hours in suspension the β1 mRNA is down regulated leading to loss of the subunit (Hotchin and Watt, 1992; Hotchin et al., 1995).

While there are clearly parallels between suspension induced differentiation and anoikis the two are distinct processes and primary human keratinocytes do not undergo apoptosis in suspension (Gandarillas et al., 1999). Deletion of β1 does not induce apoptosis in the skin (Brakebusch et al., 2000). While there are reports that loss of β4 causes apoptosis (Dowling et al., 1996) α6 null epidermis does not show an increase in apoptotic cells (DiPersio et al., 2000). Although there is evidence that unligated integrins can recruit caspase-8 to the plasma membrane and thereby stimulate apoptosis in adherent cells (Stupack et al., 2001) this is not seen in mice where a variety of integrins are expressed suprabasally in the absence of ligand (Carroll et al., 1995; Romero et al., 1999; Owens and Watt, 2001).

The role of integrins in the regulation of keratinocyte differentiation has been further investigated by the use of chick β1 integrin (cβ1) constructs retrovirally expressed in human keratinocytes. The cβ1 subunit is highly homologous to the human β1 subunit, dimerises with the endogenous α subunits and functions identically to the endogenous subunit when retrovirally expressed in keratinocytes (Levy et al., 1998). Experiments with mutant constructs of the cβ1 have helped to reveal the specific regions of the β1 subunit that are involved in regulation of differentiation (Fig 1.12) (Levy et al., 2000).
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Fig 1.12 The 8 cytoplasmic domain and D154A extracellular domain cβ1 constructs used to examine the role of the β1 subunit in regulation of differentiation (Levy et al., 2000).

These experiments show that disruption of the cyto-2 motif in the integrin cytoplasmic domain removes the ability of the integrin to be recruited to focal adhesions and to mediate cell attachment to ECM. However this does not remove the ability of the integrin to suppress suspension induced differentiation. Deletion of either amino acids 759-771 or 771-790 makes the integrin non-functional in any of the assays. Disruption of the MIDAS motif in the βA domain by the D154A substitution prevents the integrin from binding ligand. This prevents the integrin supporting cell attachment but because the focal adhesion targeting motif in cyto-2 is intact it is still recruited to existing focal adhesions. This mutant subunit has lost the ability to suppress suspension induced differentiation. These results reiterate that the important event in regulation of terminal differentiation is ligand binding and that sequences involved in receptor clustering are not required (Levy et al., 2000).
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SCC4, a keratinocyte cell line derived from a human oral squamous cell carcinoma, normally differentiates at a low rate in culture (<1%) but does not differentiate further in suspension culture (Rheinwald and Beckett, 1981; Levy et al., 2000). When this cell line is infected with WT cβ1 integrin 10-15% of the cells begin to differentiate. In this case the restoration of differentiation in not due to replacement of a lost integrin subunit as the SCC4 line has normal β1 expression (Sugiyama et al., 1993). When these cells are infected with the same panel of mutant cβ1 subunits all the constructs that are capable of suppressing suspension induced differentiation in normal keratinocytes are capable of inducing differentiation in SCC4 in adherent culture (Fig 1.12). All constructs that were unable to affect differentiation in keratinocytes failed to induce differentiation in SCC4 (Levy et al., 2000).

1.6 Aims

The β1 subunit is an important regulator of keratinocyte differentiation (Adams and Watt, 1989; Adams and Watt, 1993) and the SCC4 cell line is a useful tool to study this process (Levy et al., 2000). The aim of this project was to investigate the SCC4 differentiation phenotype. Initial observations suggested either a defect in the signalling pathway leading from the endogenous β1 integrin or a defect in the integrin itself. To investigate these ideas I decided to investigate the signalling hypothesis by plating SCC4 expressing the cβ1 WT integrin on surfaces coated with anti-cβ1 or anti-human β1 to investigate the signalling events triggered by the two subunits. To look for a defect in the endogenous β1 I generated a cDNA of the β1 gene expressed in the SCC4, sequenced it and looked for mutations that could affect function.
Chapter 2

Materials and Methods

2.1 Cell Biology

2.1.1 General solutions

The central cell services of Cancer Research UK fund provided sterile distilled, de-ionised water and solutions that are indicated by “CR-UK”. All reagents used were tissue culture grade and sterile.

*Phosphate buffered saline (PBS, CR-UK)*

8 g NaCl, 0.35 g KCl, 1.43 g Na$_2$HPO$_4$ and 0.25 g KH$_2$PO$_4$ were dissolved in 1 l dH$_2$O, adjusted to pH 7.2 and autoclaved. PBS$_{ABC}$ was PBS supplemented with 1 mM CaCl$_2$ and 1 mM MgCl$_2$

*Tris Buffered Saline (TBS)*

10x Stock solution was prepared by dissolving 24.2g Trizma base and 80g NaCl in 1L dH$_2$O. The pH was adjusted to 7.6 and the solution was sterile filtered.

*EDTA solution (versene, CR-UK)*

8g NaCl, 0.2g KCl, 1.15g Na$_2$HPO$_4$, 0.2g KH$_2$PO$_4$ and 0.2g ethyldiaminotetraacetic acid disodium salt (EDTA) and 1.5ml 1% (w/v) phenol red solution were dissolved in 1L dH$_2$O. The pH was adjusted to 7.2 and the solution was autoclaved.
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Trypsin solution (CR-UK)

8 g NaCl, 0.1 g Na2HPO4, 1 g D-glucose, 3 g Trizma base, 2 ml 19% (w/v) KCl solution and 1.5 ml of 1% phenol red solution were dissolved in 200 ml dH2O. The pH was adjusted to 7.7 and 0.06 g penicillin and 0.1 g streptomycin (Gibco BRL) were added. 2.5 ml pig trypsin (Difco 1:250) was dissolved in 250 ml dH2O. Air was bubbled through the solution until the trypsin dissolved. The trypsin solution was then added to the tris buffered saline, made up to 1 l with dH2O. The solution was sterilised through filtration through a 0.22 μm filter and stored at -20°C.

Mitomycin C stock solution

Mitomycin C is an inhibitor of DNA synthesis and nuclear division. It is used to metabolically inactivate J2-3T3 cells to form a feeder layer for the culture of primary keratinocytes. A 100x stock solution was prepared by dissolving 4 mg of mitomycin C powder (Sigma) in 10 ml PBS. The final concentration used was 4 μg/ml.

Puromycin Stock solution

100 mg puromycin powder was dissolved in 100 ml PBS, sterilised using a 0.22 μm filter and stored at -20°C.

Polybrene Stock solution

1000x stock was prepared at 5 mg/ml in sterile conditions and stored in frozen aliquots.
2.1.2 J2-3T3 and J2-3T3 Puro Cell Culture

J2-3T3 feeder cells (J2F) are a 3T3 derived clone selected for its ability to support keratinocyte growth (Rheinwald and Green, 1975). 75 cm² flasks of J2 cells were washed with versene, incubated with versene/trypsin to detach cells then passaged 1:10 and grown to confluence. J2-3T3-puro (J2P) cells are J2F cells infected with the pBabe-puro retrovirus and were used to culture keratinocytes in medium containing puromycin. Cultures of feeders could be maintained for 2-3 months before transformed cells appeared which made them unsuitable for keratincyte culture.

J2-3T3 and J2-3T3 Puro culture medium (E4+DCS)

J2-3T3 cells were cultured in Dulbecco’s modification of Eagle’s medium (DMEM) (E4, CR-UK) suplimented with 10% (v/v) donor calf serum (DCS, Gibco BRL). For J2-3T3 Puro cells (J2-3T3 transfected with the puromycin resistance gene) the medium was supplemented with 2.5 µg/ml puromycin. Medium was stored at 4°C until use.

Freezing and thawing of J2-3T3 cells

Cells were harvested as above then resuspended in DCS containing 10% (v/v) sterile dimethyl sulphoxide (Gibco BRL). 1ml aliquouts were then frozen in each cryovial overnight at −70°C in insulated containers. One 75 cm² flask was aliquoted into 3-5 cryovials. The vials were then transferred to liquid nitrogen storage. Thawing of the cells was performed by transferring the tubes to a 37°C water bath. As soon as the cell suspension was thawed it was added to 10ml of medium and centrifuged at 1,000 rpm for 5 minutes. The cells were then resuspended in medium and plated onto 25 cm² flasks.
2.1.3 Culture of human epidermal keratinocytes

Isolation of primary human keratinocytes

Neonatal foreskins were kindly provided by Dr Cohen of the Fitzroy Clinic, London. Isolation of primary keratinocytes was carried out as soon as possible after circumcision (Levy et al., 1998). Under sterile conditions, using a pair of forceps and curved scissors, a piece of foreskin was trimmed of dermal and fatty tissues. The foreskin was cut into pieces of about 5 mm$^2$ and transferred into a Wheaton Cellstir (Jencons) containing 5 ml trypsin and 5 ml versene and stirred over a magnetic stirrer at 37°C. Dissociated cells were collected every 30 minutes and added to 5 ml keratinocyte culture medium. The number of cells obtained was estimated using a haemocytometer. Dissociation of cells from the tissue was continued with addition of fresh versene and trypsin solution. This procedure was repeated 2 to 3 times before the number of cells obtained started to decrease. The yield from a neonatal foreskin was usually between 1-5x10$^7$ cells. Feeder cells had been plated onto 25 cm$^2$ flasks in readiness. Isolated cells were pooled, pelleted and plated at a density of 10$^5$ cells per 25 cm$^2$ flask. Cells were cultured until just confluent. One flask of cells was tested for mycoplasma infection by the CR-UK Cell Production Unit, while the remaining cells were harvested and frozen at 10$^6$ cells/ml as for J2-3T3 cells. Keratinocytes from 3 isolations were used; km, kp and kc.
Keratinocyte culture medium \((FAD+FCS+HICE)\)

FAD powder (Imperial Labs.) consisting of 1 part Ham's F12 and 3 parts DMEM+180 μM adenine (final conc.) was supplemented with 3.07 g/l NaHCO₃, 100 IU/L penicillin and 100 μg/l streptomycin. FAD medium (CR-UK) was bubbled with CO₂ until acidic in pH before sterilising by filtration through a 0.22 μm filter. Medium was stored at 4°C until use.

Stock solutions of additives were prepared. \(10^{-5}\) M cholera enterotoxin (ICN) was stored at -20°C. 100 μg/ml recombinant human epidermal growth factor (Sigma) was prepared by first dissolving in 1/10 volume 0.1M acetic acid (BDH) before adding to FAD medium containing 10% (v/v) batch tested fetal calf serum (FCS, Imperial Labs) and stored at -20°C. The additives were combined into a 1000x cocktail (HCE): 1ml hydrocortisone, 100 μl cholera enterotoxin and 1 ml epidermal growth factor stock solutions were added to 7.9 ml FAD medium with 10% FCS and stored at -20°C. The final concentrations in the medium were \(10^{-10}\) M cholera enterotoxin, 0.5 g/ml hydrocortisone and 10 ng/ml epidermal growth factor. 1000x insulin stock solution (5 mg/ml in 5 mM HCl, Sigma) was stored at -20°C. The final concentration in the medium was 5 μg/ml insulin.

Complete keratinocyte medium \((FAD+FCS+HICE)\) was prepared by adding 10% (v/v) FCS, cocktail and insulin solutions to the FAD medium prior to use. For keratinocytes infected with retrovirus puromycin was added to the medium at 1 μg/ml. Complete medium was stored at 4°C for up to 14 days.
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Preparation of J2-3T3 cells as feeder cells

The culture of human keratinocytes requires co-cultivation with mitotically inactive J2-3T3 cells which are referred to as feeder cells (Rheinwald and Green, 1975). Feeder cells were incubated with 4 μg/ml mitomycin C for 2-3 hrs at 37°C. Cells were then harvested and plated onto flasks as described earlier. Cells from a confluent 75 cm² flask were plated onto between 3x 75 cm² flasks or 9x 25 cm² flasks. J2-puro cells were used as feeder cells when keratinocytes were infected with retroviruses.

Serial culture of human keratinocytes

Frozen keratinocytes (passage 1-4) were thawed as described for J2-3T3 cells. The strains used were named km, kq, and kn. Each strain corresponds to keratinocytes isolated from a single individual. The usual number of cells seeded in 1x 75 cm² was 2x10^5 actively growing cells; 5x10^5 cells from a frozen cryotube were plated in a 75 cm² flask to allow for loss of viability resulting from freezing and thawing. Fresh medium was applied every 2 days. A day prior to any experimental manipulation, keratinocytes were fed with fresh medium.

Keratinocytes were passaged just before they reached confluence. The cultures were rinsed once with versene and then incubated with versene for 5-10 minutes at 37°C. This treatment caused any remaining feeder cells to detach. Keratinocytes would round up but would not detach from the flask. The versene solution was discarded and the remaining keratinocytes were incubated in 5ml trypsin/versene solution (1 part trypsin and 4 parts versene) at 37°C for about 10 minutes, until all keratinocytes had detached from the flask. 5ml medium was added to the suspension and the
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number of cells was counted using a haemocytometer. The cells were pelleted and resuspended in medium as described and plated onto flasks with feeder cells; $10^5$ cells were added to a $25 \text{ cm}^2$ flask or $2 \times 10^5$ cells were added to a $75 \text{ cm}^2$ flask.

2.1.4 Squamous cell carcinoma cells

SCC Cell Culture

The SCC cell lines used in this work were originally derived from patients with advanced oral squamous cell carcinomas (Rheinwald and Beckett, 1981). These lines were cultured in the same manner as normal primary keratinocytes.

2.1.5 GD25 β1 null mouse fibroblasts

GD25 culture medium (E4+DCS)

GD25 cells were cultured in Dulbecco’s modification of Eagle’s medium (DMEM) (E4, CR-UK) supplemented with 10% (v/v) donor calf serum (DCS, Gibco BRL). For GD25 cells infected with puromycin resistant constructs the medium was supplemented with 2.5 μg/ml puromycin.

GD25 cell culture.

GD25 cells (Wennerberg et al., 1996) are a fibroblast line derived from β1 integrin null ES cells. When GD25 cells approached confluence they were harvested by washing in versene then incubating in 1:4 trypsin/versene at 37°C from 5 minutes. The cells were pelleted at 1,000 rpm for 5 minutes then resuspended in medium. Cells were subcultured at dilutions of 1:5 or 1:10.
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2.1.6 Retroviral Producer Cells

Retroviral producer cell culture

Helper free ecotropic packaging cells GP + envAM12 (abbreviated as AM12), were obtained from Dr P. Patel of the Institute of Cancer Research, London. These cells were designed in conjunction with the pBabe puro vector to reduce the risk of generation of wild type Mo MuLVirus via homologous recombination (Markowitz et al., 1988a; Markowitz et al., 1988b). The packaging cells were cultured in E4 medium supplemented with 10% (v/v) FCS (Sigma). For infected retroviral producer cells the culture medium was supplemented with 2.5 μg/ml puromycin. Phoenix ecotropic packaging cells were obtained from the American tissue culture collection. Phoenix is a second-generation retrovirus producer lines derived from the human embryonic kidney 293 cell line for the generation of helper free ecotropic and amphotropic retroviruses. The lines had been created by placing into 293T cells constructs capable of producing gag-pol, and envelope protein for ecotropic and amphotropic viruses. For both the gag-pol and envelope constructs non-moloney promoters were used to minimize recombination potential. Different promoters for gag-pol and envelope were used to reduce their inter-recombination potential. Gag-pol was introduced with hygromycin as the co-selectable marker and the envelope proteins were introduced with diptheria resistance as the co-selectable marker.

Transfection of phoenix cells.

Ecotropic virus producer cells lines were generated by transfecting the phoenix packaging cells with retroviral vector, and then using the virus containing supernatant to infect GD25 or AM12. This two step protocol gives higher viral titres than from normally transfected producers (Sally Lowell, personal communication)
and allows production of amphotrophic virus for infection of human cells. The transfection process used was a calcium phosphate precipitation infection protocol (Nolan protocol). 8x10^6 phoenix cells were seeded onto a 100 mm dish the day before transfection. 5 minutes prior to transfection, chloroquine was added to each plate at 25 μM. Chloroquine increases the transfection efficiency by neutralizing vesicle pH and thereby inhibiting lysosomal DNAse. 10 μg DNA, 438 μl dH2O, 61 μl 2 M CaCl2 were mixed and brought to a 500 μl total volume with dH2O. 0.5mL 2xHBS was added, mixed by bubbling air through the mixture and the HBS/DNA was added dropwise into 1 ml medium on the cells.

24 hours post-transfection the medium was changed. Retroviral supernatants were collected at 65- to 90 hours after infection, filtered though a 0.45 μm filter to remove cells and added to GD25 or AM12 cells.

Infection of AM12 and GD25 cells

Producer lines that are generated by retroviral infection have higher viral titres than those generated by transfection (Morgenstern and Land, 1990b; Morgenstern and Land, 1990a), hence virus released into the culture medium by confluent phoenix cells was used to produce stable high titre AM12 producer cells. AM12 or GD25 cells were seeded on 100 mm dishes at 1-2x10^5 density the day before infection. 2.5 ml infection medium (virus-containing medium collected from the phoenix cells, supplemented with 5 μg/ml polybrene; Sigma) was added to the cells. After 12h infection at 37°C the infection medium was replaced with fresh culture medium (E4 + FCS). The selection medium containing 2.5 μg/ml puromycin was applied 48h later and changed every 2 days until cells reached confluence.
Where required the lines produced by this process where FACsorted to give populations with high surface expression of the transferred construct.

2.1.7 MOLT-4

Molt-4 is a lymphocyte cell line derived from a patient with acute lymphoblastic leukaemia. These cells were provided by ICRF cell production. These cells were used as a control in HUTS21 epitope expression experiments.

2.2 Assays of proliferation and differentiation

2.2.1 Proliferation Assay

Cultures of SCC4 cells expressing integrin constructs were set up with $1 \times 10^5$ cells in each well of a 24 well tissue culture dish. At intervals over the next 17 days triplicate wells were harvested in small volumes of liquid and the number of cells determined by counting on a haemocytometer.

2.2.2 SCC4 Differentiation Assay.

$1 \times 10^5$ SCC4 cells expressing integrin constructs were plated into a 25 cm$^2$ flask with J2 feeders and cultured until just preconfluent. The culture wash then harvested as normal by Cytsin/versene, washed in PBS several times and resuspended at $1 \times 10^6$ cells/ml in PBS. Small amounts of each culture were then air dried onto duplicate wells of an 8 well slide. Multiple slides were prepared. The slides were then fixed in 4% formaldehyde for 10 minutes and stored in PBS. Just prior to staining the cells were permeabilised in cold methanol for 10 minutes. The slides were then stained using DH-1 or SY5 (human involucrin) or SQ13C (human cornifin). Multiple fields for each condition were counted directly. Fields totalling three hundred cells were counted in each case.
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2.2.3 Suspension differentiation assay

*PolyHEMA coating of dishes*

To minimise attachment of cells to dishes bacteriological grade plastic petri dishes were coated with polyHEMA (type NCC, cell culture grade, Hydro Medical). A 10% polyHEMA (w/v) solution in 95% ethanol was prepared by mixing end over end overnight at room temperature. To coat dishes stock solution was diluted to 0.4% in ethanol/aceton (1:1). 5 ml of solution was added to a 100 mm dish and swirled repeatedly to coat the base of the dish and then removed immediately. This was repeated once. Coated dishes were left under sterile conditions to dry before use.

*Methyl cellulose medium*

3.5 g methyl cellulose (Sigma) was autoclaved in a 400 ml centrifuge tube. 180 mM FAD was heated to 60 °C and added to the tube. The contents were stirred for 30 minutes at 30 °C and then overnight at 4°C (Green, 1977). After addition of 20 ml FCS the medium was centrifuged at 9,500 rpm at 4 °C. The medium was then aliquotted and stored at -20°C until use. Immediately before use the medium was supplemented with HICE as for keratinocyte medium.

*Suspension culture*

Newly confluent cultures of keratinocytes were harvested and resuspended at $10^6$ cells/ml in complete FAD medium. The cells were then added slowly to the methyl cellulose medium simultaneously swirling the medium to ensure homogenous distribution of the cells. The final concentration of cells was $10^5$ cells/ml. 50 ml of cell suspension was poured into polyHEMA coated dishes and cultured overnight at 37 °C. Cells were recovered by diluting the methyl cellulose with 10 volumes of versene.
and centrifuged in a 250 ml conical bottom Beckman bottle at 2,000 rpm for 10 minutes at 4 °C. The recovered cells were then dried onto coverslips and stained for differentiation markers in the same manner as SCC4 cells.

2.3 Adhesion Assays

2.3.1 ECM proteins

Bovine plasma fibronectin was purchased from Sigma-Aldrich. Lamin was also sourced from Sigma-Aldrich. Collagen was supplied by Vitrogen Corp. Vitronectin was purified from human plasma by the method of Cheresh et al. Briefly 100ml plasma was clotted by addition of 2 ml of 1 M CaCl₂. The clot was removed and the serum filtered. 0.5 ml of 0.2 M PMSF and 2.5 ml of 0.2 M EDTA was added and the plasma run over a sepharose 4B column. The flow through was then run on a heparin-sepharose column (equilibrated in 10 mM Na₂PO₄, 0.13 M NaCl, 5 mM EDTA pH 7.7) to remove fibronectin and other heparin binding proteins. The plasma was then treated with 8M urea incubated at room temperature for 2 hr and recirculated over a second heparin column, equilibrated in 10 mM Na₂PO₄, 8 M urea, 5 mM EDTA pH 7.7 for 12 hours to isolate the vitronectin. The column was then washed in 100ml of the equilibration buffer, then 100ml 10mM Na₂PO₄, 8 M urea, 5 mM EDTA, 0.13 M NaCl pH 7.7. 1 column volume (5ml) of 10 mM Na₂PO₄, 8 M urea, 5 mM EDTA, 0.13 M NaCl, 10 mM β-mercaptoethanol was run onto the column and the column incubated overnight at room temperature. The column was eluted with 10 mM Na₂PO₄, 8 M urea, 5 mM EDTA, 0.13 M NaCl. Fractions of eluted protein were pooled and analysed by SDS-PAGE to determine purity and A280 to measure concentration. Over 10 mg of vitronectin was isolated from 100 ml plasma.
Elution curve for Vitronectin

Fig 2.1 Elution of Vn from heparin sepharose column. Fraction 6 is not plotted as A280 was > 3.

2.3.2 Cell Attachment Assay

96 well plates were coated overnight at 4°C with ECM proteins in PBS at appropriate concentrations. 100 µl of the ECM solution was applied to each well. The plate was then blocked by incubation with 5% heat denatured BSA in PBS for 1 hr at 37°C. The plate was then washed three times in PBS and used immediately.

Preconfluent flasks of cells were treated with 5 µM of CellTracker dye (Molecular Probes) in serum free medium for 20 minutes at 37°C. The cells were then washed twice and placed in full medium for a further 20 minutes. The cells were then harvested using trypsin/versene, washed in complete medium and then in TBS twice.
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The cells were finally resuspended at $5 \times 10^5$ cell/ml in TBS. Mg2+, Mn2+, Ca2+ or EDTA (1 mM final concentration) was added as appropriate and the 100 μl of the cell suspension was added to each well. The plate was incubated at 37°C for an appropriate length of time and then washed twice with TBS. The fluorescence from each well was measured on a fluorescent plate reader and then quantitated by comparison with a standard curve prepared from the same cell suspension.

2.3.3 Recombinant Integrin Adhesion Assay

These methods were used by Drs A Henry and V Perkins at CellTech, Slough to screen for affinity changes in mutant integrins.

Integrin cDNAs were cloned into the vector pEE12.2h which contains the human γ1 Fc domain as a SalI/EcoR1 genomic fragment. Separate vectors encoding the extracellular regions of the human α5, αv, wild type β1 and T188I β1 integrin subunits were constructed, essentially as described previously (Stephens et al., 2000). The appropriate α and β integrin vectors were transiently co-expressed in CHOL761h cells. Solid phase binding to fibronectin coated plates was performed. Briefly, equal concentrations of WT and MT integrin supernatent were incubated in fibronectin coated 96 well plates, for 2 hr at room temperature. The plates were then washed and the bound integrin quantitated using a fluorescently labelled anti-Fc antibody.
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2.4 Cell motility and spreading

2.4.1 Time lapse microscsopy

In order to quantitate cell motility subconfluent cultures were harvested and plated onto dishes coated in fibronectin, at a density that allowed tracking of individual cells. Frames were taken every 2 minutes for 24 hours using microscopes fitted with a digital cameras. Motility was measured using Kinetic image analysis software and speed was calculated using a program written in Mathematica (Wolfram Research) by Dr. D. Zicha (CR-UK).

2.4.2 Determination of rate of spreading

Cells were plated onto fibronectin, allowed to attach and filmed as above while they spread over 3-4 hours. Every third frame was analysed and the number of small spherical unspread cells and the total number of cells was counted. From this the percentage of cells that had begun to spread was determined and plotted against time. Any cells that failed to spread at all during the assay were discounted as non-viable.

2.4.3 Cell motility towards serum

GD25 cells expressing the WT or MT integrin or empty vector were placed into the upper section of duplicate 6 well plate cell culture insert with 8 μm pores in serum free medium. The lower part of the well contained medium supplemented with 10 % serum. The cells were left to migrate for 6 hr after which the cell culture insert membranes were fixed in methanol and then either the upper or lower side of the membrane was wiped clean of cells. The membranes were then stained in crystal violet solution, washed twice in PBS and air dried. Three random fields were photographed using a digital camera and the number of pixels of each field stained
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by the crystal violet measured using the NIH-Image analysis program. The percentage of cells that had migrated through the membrane was determined by dividing the number of coloured pixels on the bottom side of the membrane by the total number of positive pixels on the top and bottom (For an example see Fig 5.3).

2.5 Invasion Assay

Invasion assays were performed basically as described (Thomas et al., 2001). GD25 cells expressing WT or T188I MT integrin or empty vector in serum free medium were mixed with matrigel to give 1:2 ratio of matrigel to medium. 100 µl of matrigel mixture containing 30,000 cells were then placed in the top part of a 24 well cell culture insert with 8 µm pores. 150 µl of serum free medium was placed on top and 750 µl serum containing medium in the lower half of the well. The cells were allowed to migrate for 2 days then the inserts were removed, the matrigel cleaned from the top part and the membranes fixed in methanol. The cells were then stained with crystal violet and the centre of each membrane photographed at 2.5x with a digital camera. The number of pixels occupied by the crystal violet stained cells was then quantitated using NIH-Image. This value was converted into cells/mm² (the average size of a single cell and the scale of the photograph having previously been determined).

2.6 Immunological Methods

2.6.1 General Solutions

*Gelvatol mounting solution*

The Gelvatol mounting solution was prepared as described by Harlow and Lane (Harlow and Lane, 1988). 2.4 g Gelvatol (Monsanto Chemicals) was mixed with 6 g
glycerol (Sigma) and vortexed; 6ml dH_2O was added, mixed and left to stand for 90 minutes at room temperature. 12.5 ml of 200 mM Tris-HCl, pH 8.5 was added and the solution was vortexed, heated to 50 °C and vortexed again. Heating and vortexing were repeated three times and the solution placed on an end over end mixer overnight at room temperature. The solution was then centrifuged at 2000 rpm for 10 minutes at room temperature and stored in aliquots at 4 °C.
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#### 2.6.2 Antibodies

<table>
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<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Species</th>
<th>Reference/Source</th>
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<tr>
<td>JG22</td>
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<td>Mouse</td>
<td>(Greve and Gottlieb, 1982)</td>
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<tr>
<td>V2E9</td>
<td>Anti β1 integrin</td>
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<td>(Hayashi et al., 1990)</td>
</tr>
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<td>(Dittel et al., 1993)</td>
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<tr>
<td>HUTS21</td>
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<td>(Luque et al., 1996)</td>
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<td>(van de Wiel-van Kemenade et al., 1992)</td>
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<td>Rat</td>
<td>(Bohsnack et al., 1990)</td>
</tr>
<tr>
<td>9EG7</td>
<td>Activating/Activating anti-β1</td>
<td>Mouse</td>
<td>(Lenter et al., 1993)</td>
</tr>
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<td>Phospho-ERK1/2</td>
<td>Rabbit</td>
<td>NEB</td>
</tr>
<tr>
<td></td>
<td>ERK 1/2</td>
<td>Mouse</td>
<td>Santa Cruz</td>
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<tr>
<td>SY-5</td>
<td>Human involucrin</td>
<td>Mouse</td>
<td>(Hudson et al., 1992)</td>
</tr>
<tr>
<td>DH-1</td>
<td>Human involucrin</td>
<td>Rabbit</td>
<td>(Dover and Watt, 1987)</td>
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<td>Human cornifin</td>
<td>Rabbit</td>
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<td>Goat</td>
<td>Molecular Probes</td>
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</tr>
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<td>Anti Rabbit HRP</td>
<td>Donkey</td>
<td>Amersham</td>
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*Table 2.1: Antibodies used in this thesis*
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2.6.3 Preparation of cells for immunofluorescence staining

Cells were cultured on tissue culture plastic microscope slides (Nunc) or on glass
coverslips (Chance Propper Ltd.). Coverslips were first boiled in 7x detergent (ICN)
for 30 minutes to remove silicone coating. They were rinsed thoroughly first in tap
water and then in dH$_2$O. Washed coverslips were rinsed briefly in absolute ethanol
and spread out on filter paper to dry completely before autoclaving. Alternatively the
washed coverslips were coated with ECM proteins overnight at 4°C. Cells were then
plated onto the coverslips and allowed to spread before fixation.

Fixation of cells

Culture medium was discarded from cells and they were rinsed in PBS before fixing
in either 4% paraformaldehyde solution or 4% formaldehyde solution for 15 minutes
at room temperature. The specimens were rinsed three times in PBS, and then
incubated in PBS abc containing 2% FCS to reduce binding to non-specific proteins,
for at least 30 minutes at room temperature. To stain cells for focal adhesion
components, cells were fixed for 10 minutes with 4% formaledehdye in PBS
containing 0.1% Triton X-100

2.6.4 Staining protocols

Tissue sections or cultured cells were incubated in blocking solution for 30 minutes
at room temperature. Primary antibodies were diluted in the blocking solution and
applied to cells for 45 minutes at room temperature. The cells were rinsed three
times in blocking solution and then incubated with the secondary antibody diluted in
blocking for 45 minutes at room temperature. The cells were then rinsed three times
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in blocking solution, once in PBS, and once in dH2O, before mounting in Gelvatol solution.

In co-localisation experiments using a double immunofluorescence method, cells were stained with two primary antibodies. Both primary antibodies were of distinct species and these were subsequently probed with species-specific secondary antibodies conjugated to different fluorophore. The antibodies were applied in the following sequence: first primary; first secondary; second primary; second secondary, with thorough washes in blocking solution between each antibody application.

2.7 Flow cytometry

2.7.1 Staining protocol

Single cell suspensions of cells isolated directly from cultures harvested with trypsin/EDTA solution were incubated in primary antibodies diluted in 2% FCS PBS at 4°C for 20 min, washed, and incubated on ice for 15 minutes with an AlexaFluor488 conjugated secondary antibodies. Cells were washed 4 times and then resuspended in PBSabc. Samples were analysed on a Becton-Dickinson FACScan.

2.7.2 Sorting on basis of extracellular epitope expression

Cells were prepared for sorting by the above staining procedure but in sterile conditions. Cells were sorted using a pre-sterilised Becton-Dickinson FACSVantage cell sorter and collected in chilled complete medium. The cells were then replated in fresh pre-warmed medium.
2.8 Biochemistry

2.8.1 Protein Extraction for MAPK assay

Adherent cells were washed twice in ice cold PBS then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 1% NP-40, 0.1% SDS, 0.5% Deoxycholate, 5 mM EDTA, 1% Triton X-100) supplemented with a protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail (Sigma). Lysates were then sonicated briefly and assayed for protein content using the BCA protein assay (Pierce).

2.8.2 Protein assay

To determine the amount of protein present in cell extract the BCA assay kit (Pierce) was used as per the manufacturers instructions.

2.9 Polyacrylamide Gel Electrophoresis (SDS Page)

2.9.1 General Solutions

Laemmli Sample Buffer

2x laemmli sample buffer (non reducing) comprised 125mM Tris-HCl, pH6.8, 2% SDS, 20% glycerol and 0.02% bromophenol blue. 2x reducing sample buffer had an additional 10% (v/v) β-mercaptoethanol

SDS-PAGE running buffer

50mM Trizma, 384mM Glycine and 0.1% SDS.
2.9.2 SDS-PAGE

Vertical gel electrophoresis apparatus (Atto Corp. or Hoefer scientific instruments) were used. 1.5 mm gels were prepared between glass plates using the method of Laemmli (Laemmli, 1970). The recipe for the gel is presented in table 2.2. After pouring the resolving gel a layer of 1ml dH₂O was applied to ensure a flat surface. After the gel had polymerised the layer of water was poured away and the stacking gel was applied. A 1.5 mm comb was used to form the sample wells and the gel allowed to set. The comb was then removed and the wells flushed with SDS-Page running buffer. Samples were applied to the wells. 10 μl rainbow markers (Amersham) were added to one well. The gel was then electrophoresed at 100-150V until the dye front had just left the bottom of the resolving gel. The gel was then removed and prepared for Western blotting.

### Stock solutions

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<th>Formula/Concentration</th>
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<td>30% acrylamide / 0.8% bisacrylamide (Millipore)</td>
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<td>B</td>
<td>3M Tris-HCl, pH8.8 (BDH)</td>
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<tr>
<td>C</td>
<td>10% SDS</td>
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<tr>
<td>D</td>
<td>2M Tris-HCl, pH6.8 (BDH)</td>
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<tr>
<td>AP</td>
<td>10% ammonium persulphate (Bio-Rad) in dH₂O</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine (Bio-Rad)</td>
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<th>Stock solutions</th>
<th>A (ml)</th>
<th>B (ml)</th>
<th>C (ml)</th>
<th>dH₂O (ml)</th>
<th>TEMED (μl)</th>
<th>AP (μl)</th>
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<td>Stacking</td>
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<td>7.775 ml</td>
<td>10 μl</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

Table 2.2: SDS-PAGE gel recipe

2.9.3 Western Blotting

*Semi-dry transfer buffer*

10x stock solution comprised 0.2 M Tris-HCl, pH 7.5 and 1.5 M Glycine. The solution was filtered through a 0.22 μm filter and stored at room temperature. The semi-dry transfer solution was made by diluting the stock 10 fold in 0.1 % SDS and 20% (v/v) methanol.

*Protocol*

After SDS-PAGE electrophorese proteins were transferred on a Millipore Immobilon PVDF (polyvinylfluoride) membrane (pre-wetted in methanol) using a semi-dry transfer unit (Trans-Blot-SD, Bio-Rad) for 1 hr at 200 mA. After transfer the membrane was briefly washed in TBS containing 0.05% Tween (TBST) then blocked in TBST containing 5% milk powder (Premier Brands UK Ltd) for 2 hr at room temperature to block non-specific binding of antibodies.

*Blotting with antibodies and ECL*

Membranes were incubated with primary antibodies diluted in 5% milk/TBST overnight at 4°C with agitation. The membrane was then washed with 5 changes of TBST. The membrane was then incubated with a horse-radish peroxidase (HRP) conjugated secondary antibody diluted in TBST for 1 hr. The membrane was then washed 5 times. Detection was performed using the ECL chemiluminescence reagent.
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(Amersham) as per the manufacturer’s instructions. If a membrane was to be reprobed they were incubated in 200 mM glycine pH2.5, 0.2% SDS in dH₂O for 20 minutes at room temperature then washed in TBST and reblocked in TBST/5% milk.

2.10 Molecular Biology

2.10.1 Bacterial Culture General Solutions

L-broth (ICRF)

L-broth comprised 1% Bacto-Tryptone (Difco), 0.5% yeast extract (Difco) and 170mM NaCl and was sterilised by autoclaving.

L-agar (ICRF)

L-agar comprised 1.5% bacto-agar (Difco, w/v) in L-broth. The agar was dissolved by heating in a microwave oven and allowed to cool to 50°C before adding the selection antibiotic. The solution was then poured into 100 mm bacteriological petri dishes and left to set on a level platform. Agar dishes were stored at 4°C, agar side up.

Ampicillin stock solution

Ampicillin (Sigma, stock 100 mg/ml in dH₂O) was used as a selection antibiotic and was added to LB or L-agar to a final concentration of 100μg/ml.

Kanamycin stock solution

Kanamycin (Sigma, 1000x at 25 mg/ml) was used as a selection antibiotic and was added to LB or L-agar to a final concentration of 100μg/ml.
2.10.2 Transformation of bacteria

*Heat shock transformation of TOP10 cells*

50 μl of competent bacteria were thawed at room temperature and then incubated on ice for 10 minutes. DNA (0.1 μg for ligations or 1 ng for plasmids) was added, mixed and left on ice for 30 minutes. The bacteria were then heat-shocked at 42°C for 45 seconds and transferred onto ice for 2 minutes. 4 volumes of SOC medium (CR-UK) were added and the tube was incubated at 37°C for 1 hour. 100 μl culture was spread out onto plates containing L-agar and either ampicillin or kanamycin as appropriate and incubated overnight at 37°C. Single colonies were picked with a sterile loop to inoculate LB media.

2.11 DNA techniques

2.11.1 General solutions

All reagents used were of molecular biology grade. When possible, solutions were autoclaved after preparation to destroy DNases.

*Tris/EDTA buffer (TE)*

TE was used as a general storage buffer for DNA and comprised 10 mM Tris-HCl and 1 mM EDTA, pH 8.0.

*Tris-acetate-EDTA buffer (TAE)*

A 50x stock solution was prepared by dissolving 242g Trizma base and 57.1 ml glacial acetic acid (BDH) in dH₂O. 100 ml 0.5M EDTA, pH 8.0 were added and the final volume was made up to 1 l.
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Agarose/TAE gel

This was used in the electrophoresis of DNA. 0.8-2% (w/v) ultra pure agarose (Gibco BRL) was melted in a microwave oven in 1x TAE buffer. Ethidium bromide was added at 0.05 µg/ml to agarose solution before casting in gel mould. Typically, DNA was electrophoresed at constant voltage of 80-100 V in 1x TAE buffer.

DNA loading buffer

6x DNA gel loading buffer comprised 0.25% bromophenol blue (Sigma), 0.25% xylene cyan (Sigma) and 15% Ficcol (Sigma) in dH2O.

2.11.2 Isolation of DNA from agarose gels

DNA bands were isolated from agarose gels using the Qiaquick gel extraction kit (Qiagen) as per the manufacturer’s instructions.

2.11.3 Maxi/mini preps

Plasmid preparation at the Maxi (500 mg) and Mini (5 mg) scale were performed using the appropriate Qiagen plasmid isolation kit as per the manufacturer’s instructions.

2.11.4 Genomic DNA preparation

Three 10 µm sections of frozen tissue were placed in a 1.5 ml eppendorf tube on dry ice. 200 µl of DNA extraction buffer (50 mM Tris pH8, 100 mM EDTA, 0.5% SDS) containing 5 mg/ml Proteinase K (Sigma) was added and the tube centrifuged breifly to pellet the tissue in the buffer. The Tube was then incubated at 55 °C for 4 hours. 200 µl of phenol/chloroform/iso-propyl alcohol (25:24:1 by volume, Amresco) was added and the tube vortexed. The tube was then centrifuged and the upper, aqueous
phase transferred to a fresh tube without disturbing the interface layer. Sodium acetate (pH 5.2) was then added to the aqueous layer to 0.3 M. 1 ml of ethanol was added and the tube placed at -20 °C to precipitate DNA. The tube was then centrifuged at 14,000 rpm, the pellet washed with 70 % ethanol, air dried and resuspended in 30 μl TE.

2.11.5 Restriction enzyme digestion

All restriction enzymes were purchased from NEB and used as per manufacturers instructions.

2.12 PCR techniques

2.12.1 PCR

PWO (Roche), an error checking polymerase was used as per the manufacturers instructions for producing blunt ended fragments. 2x PCR Master Mix (Promega) was used for producing small PCR fragments for sequencing.

2.12.2 Site directed mutagenesis

Site directed mutagenesis was performed using the QuickChange protocol (Sigma-Aldrich). Briefly, complementary primers containing the mutated sequence were used to generate single strands covering the entire template plasmid by PCR. These strands anneal to each other to form complete, nicked plasmids which were used to transform competent cells. Colonies were then picked, DNA prepared and analysed for the presence of the mutation by restriction analysis. Clones containing the
mutation were then sequenced. For production of the cβ1 T188I plasmid pBabe-puro, cβ1 WT was used as template, the T188I- and T188I+ primers for amplification and an EcoN1 restriction site was removed by mutagenesis.

### 2.12.3 RT-PCR

RT-PCR was performed using the SuperScriptII polymerase (Promega). Random hexamer primers were used to generate a first strand cDNA. Specific sections of DNA were then generated using gene specific primers.

### 2.12.4 Sequencing

Sequencing reactions were performed using BigDye Terminator mix and analysed by the staff of the equipment park at 44 Lincoln’s Inn Fields.

### 2.12.5 Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Specificity</th>
<th>Use</th>
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<tr>
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<td>Sequencing and PCR</td>
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<tr>
<td>Chapter 2</td>
<td>Materials and Methods</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------</td>
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<td></td>
</tr>
<tr>
<td><strong>B1.9</strong></td>
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<td><strong>B1.10</strong></td>
<td>TCCAGATATGCGCTGTT TTCCAACAAG</td>
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<td><strong>T188I+</strong></td>
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<tr>
<td><strong>T188I-</strong></td>
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<td>Sequencing, PCR and SSCP</td>
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<table>
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<th>Type of DNA</th>
<th>Taq</th>
<th>Method</th>
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<td>Sequencing, PCR and SSCP</td>
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<td>β1</td>
<td>Sequencing, PCR and SSCP</td>
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<tr>
<td>5R</td>
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<tr>
<td>7R</td>
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<td>Human genomic DNA</td>
<td>β1</td>
<td>Sequencing, PCR and SSCP</td>
</tr>
</tbody>
</table>

Table 2.3 Primers used in this thesis

2.12.6 SNP analysis

These methods were used to screen for single point mutations by Dr Claire Taylor, CR-UK Leeds

*PCR*

PCR amplification of genomic DNA was carried out using Amplitaq Gold polymerase (Applied Biosystems) in the buffer supplied by the manufacturer; 2.5-3.0 mM MgCl₂, 200 μM each dNTP and 0.5 pmol/μl each primer. For DNA sequencing PCR primers were unlabelled; for FSSCP both primers were 5’ labelled with FAM, HEX or NED. PCR was checked for yield and specificity by agarose gel electrophoresis as described in Sambrook et al., 1989. (Sambrook et al., 1989)
Fluorescent single strand conformation polymorphism (FSSCP) analysis.

Fluorescent PCR products were diluted with water by a factor of between 1 in 10 and 1 in 40, depending on the yield of the reaction. 1-2 μl of the diluted product was mixed with 0.5 μl of ROX-500 size standards (Applied Biosystems) and 10.5 μl of HiDi Formamide (Applied Biosystems). The samples were denatured at 95°C for two minutes and snap cooled on ice. FSSCP analysis was carried out on a 3100 Genetic Analyser (Applied Biosystems) at 18, and 30°C using 5% Genescan polymer containing 10% glycerol and 1xTBE. Data was analysed using Genescan 3.7.1 and Genotyper 2.5 software (Applied Biosystems). Mutation detection was by visual inspection of electropherogram traces which were examined independently by two observers.

DNA sequencing

Unincorporated primers and deoxynucleotides were removed from PCR products using shrimp alkaline phosphatase and exonuclease I (Amersham Pharmacia). PCR primers were used for all sequencing reactions. Sequencing reactions were carried out using ABI PRISM BigDye Terminator Cycle Sequencing Kit versions 2 and 3 (Applied Biosystems) with electrophoresis of the products on 310 and 3100 Genetic Analysers (Applied Biosystems). Data analysis was carried out using Sequence Analysis 3.0 (Applied Biosystems) and SeqMan (DNASTar) software and by visual inspection of electropherograms.
2.13 List of Suppliers and Distributors

Aldrich Chemical Company Ltd. Dorset, UK.
Amresco, Solon, Ohio, USA.
Amersham International, Amersham, Buckinghamshire, UK.
BDH Laboratory Supplies Inc., Hemel Hempstead, Hertfordshire, UK.
Beckman Instruments, Palo Alto, California, USA.
Becton-Dickinson, Lincoln Park, New Jersey, USA.
Bio 101 Inc. La Jolla, California, USA.
Bio-Rad Laboratories Inc. Hemel Hempstead, Hertfordshire, UK.
Boehringer Mannheim UK Ltd. Lewes, East Sussex, UK.
Calbiochem -Novabiochem (UK) Ltd. Nottingham, UK.
Carl Zeiss Ltd. Welwyn Garden City, Hertfordshire, UK.
Chance Propper Ltd., Swethwick, Warley, UK.
Clontech, Palo Alto, UK.
Coulter Electronics Ltd. Harpenden, Herts, UK.
DAKO A/S, Denmark.
Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa, USA.
Difco Laboratories, Manston, Wisconsin, USA.
Eastman Kodak Co. is distributed by Sigma Chemical Co.
EOS Electronic, South Glamorgan, Wales, UK.
European Collection of Animal Cell Cultures (ECACC), Salisbury, UK.
Flow Laboratories Ltd., Aryshire, Scotland, UK.
Genetics Research Instrumentation Ltd. Dunnmow, Essex, UK.
Gibco BRL/Life Technologies Ltd. Paisley, Renfrewshire, UK.
Hoefer Scientific Instruments is distributed by Biotech Instruments Ltd., Beds, UK.
Hydro Medical Sciences Division, Brunswick, New Jersey, USA.
ICN Pharmaceuticals Ltd. Thame, Oxon, UK.
Imperial Laboratories (Europe) Ltd. Andover, Hampshire, UK.
Integrated Separation Systems, Natick, Maryland, USA.
Jackson Immunoresearch Laboratories, Luton, Bedfordshire, UK.
Jencons, Leighton Buzzard, Beds, UK.
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Monsanto Chemicals. Springfield, Massachusetts, USA.
Millipore, Harrow, Middlesex, UK.
Molecular Probes, Leiden, Netherlands.
New England Biolabs (NEB). New York, USA.
Nunc A/S, Roskilde, Denmark.
Perkin-Elmer Co. Foster City, California, USA.
Pharmingen, San Diego, California, USA.
Pierce, Rockford, Illinois, USA.
Premier Brands UK Ltd. Knighton, Stafford, UK.
Promega UK Ltd. Southampton, UK.
Qiagen Ltd. Crawley, UK.
RND Systems, Abingdon, Oxford, UK.
Santa Cruz Biotech. Inc. Santa Cruz, California, USA.
Seikagaku Corp. Tokyo, Japan.
Serotec Ltd. Kidlington, Oxford, UK.
Scotlab Ltd. Coatbridge, Strathclyde, Scotland, UK.
Sigma Chemical Co. Poole, Dorset, UK.
Transduction Laboratories, Lexington, Kentucky, USA.
US Biochemical Corp. Cleveland, Ohio, USA.
Vector Laboratories, Burlingame, California, USA.
Whatman International Ltd. Maidstone, Kent, UK.
Zeneca Pharmaceuticals, Macclesfield, UK.
3.1 Introduction

In this chapter I confirm that the expression of a WT chick β1 subunit in the SCC4 cell line stimulates terminal differentiation. I also show that this cell line is heterozygous for a mutation in the A domain of the β1 integrin subunit.
3.2 Results

3.2.1 Infection of SCC4 cells with chick β1 subunits induces differentiation.

SCC4 keratinocytes were induced to express the wild-type chick β1 integrin subunit or an empty pBabe-puro retrovirus by co-culturing with AM12 retroviral producer cells for 48 hours followed by puromycin selection for 1 week. The expression levels of the subunit in the SCC4 were then checked by flow cytometry (Fig 3.1). The number of cells expressing the terminal differentiation markers involucrin and cornifin was then determined. SCC4 cells in adherent culture exhibit extremely low levels of differentiated cells as detected by involucrin staining (less than 1% are positive for involucrin). Upon infection with the chick WT integrin subunit there is an increase in involucrin positive cells to 8-15% (Fig 3.2A). Induction of cornifin expression was also observed (Fig 3.2B). The empty vector had no effect on either differentiation marker.

Fig 3.1: Expression of the cβ1 subunit in the SCC4 cells (grey trace negative control, black JG22-anti-cβ1)
Fig 3.2: (A) SCC4 cells infected with cβ1 WT upregulate the keratinocyte differentiation marker involucrin. Disaggregated colonies of SCC4, SCC4 infected with pBabe-puro or SCC4 infected with pBabe-puro cβ1 WT stained with SY-5, an antibody to human involucrin. Data representative of 3 experiments. (B) Similar results were obtained when stained for cornifin.
3.2.2 Expression of endogenous integrin is normal when compared to SCC12B2.

In previous studies restoration of differentiation has been linked to replacement of a missing integrin subunit (Jones et al., 1996). In order to confirm that the induction of involucrin by WT cβ1 in SCC4 was not due to replacement of a missing β1 integrin subunit SCC4 were disaggregated, stained with P5D2 (anti-human β1). The levels of surface β1 was measured by flow cytometry and compared to the levels found in SCC12B2 another SCC line that does not respond to infection with the cβ1 WT subunit. Both lines expressed similar levels of β1 subunit (Fig 3.3).

It has been previously shown that infection with the cβ1 does not cause changes in the normal α subunit profile of keratinocytes or SCC4 cells (Levy et al., 2000).
Chapter 3  Analysis of the SCC4 phenotype

Fig 3.3: The SCC4 cell line expresses equivalent levels of β1 integrin to the SCC12B2 line, a similar SCC derived culture that does not differentiate on infection with the WT subunit.

Fig 3.4: SCC4 is capable of forming focal adhesions on collagen coated surfaces. Stained with P5D2 (anti-human β1). Bar 20 μm.
3.2.3 SCC4 form focal adhesions when plated on ECM proteins.

To confirm that the endogenous integrin in the SCC4 cells forms focal adhesions, cells were plated on 20 μg/ml collagen and allowed to spread. Cells were then simultaneously fixed and permeabilised and stained for human β1 integrin. The SCC4 cells spread and formed large focal adhesions in a similar manner to normal keratinocytes. (Fig 3.4, Fig 1.4).

3.2.4 Binding of cβ1 on anti-cβ1 antibody coated surfaces causes clustering of the endogenous β1 subunits

One of the initial hypotheses of the project was that the SCC4 cells carried a defect in integrin signalling. One approach to investigate this is to plate SCC4 cells expressing the WT cβ1 or one of a range of mutant integrins onto surfaces coated anti cβ1 antibody (JG22) or anti hβ1 to specifically cluster either subunit. Cell lysates could then be prepared to analyse the signalling properties of the subunit. However rather than selectively clustering the cβ1 the endogenous β1 also formed clusters that resembled focal adhesions (Fig 3.5). This occurred even if YPRF cβ1 integrin subunits were used which are themselves incapable of being recruited to focal adhesions. If uninfected cells were used the cells did not adhere to the plate.
Fig 3.5 Clustering of endogenous β1 integrin occurs when SCC containing either WT or YPRF cβ1 constructs are plated on JG22 (anti-cβ1 antibody). Stained with A2B2 (anti-hβ1). Scale Bar 40 μm
3.2.5 Sequencing of β1 cDNA in SCC4.

To determine whether the induction of involucrin in the SCC4 line was due a defect in the endogenous β1 subunit the β1 cDNA was sequenced. mRNA was isolated from cultured SCC4 cells and a first strand cDNA synthesised using random primers. Three approximately 1 kb overlapping fragments covering the length of the 2.5 kb coding region of the β1 gene message were then synthesised using PWO, an error checking polymerase (Fig 3.6). The resulting fragments were cloned into the pCR-Blunt vector and used to transform competent bacteria. Clones from these were then sequenced using primers against the flanking regions of the vector. In addition the same fragments were generated from a culture of the keratinocytes.

Fig 3.6 Fragments of the β1 cDNA generated for sequencing showing primers used

It was found that the clones isolated from the SCC4 line differed from the published sequence at four points (Table 3.1).
### Chapter 3  
**Analysis of the SCC4 phenotype**

<table>
<thead>
<tr>
<th>Change in codon</th>
<th>Amino Acid Effect</th>
<th>% of SCC4 clones</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT-&gt;ACT</td>
<td>92 His-&gt;Thr</td>
<td>100% (6/6)</td>
<td>Error in published sequence/Common Polymorphism</td>
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<td>100% (6/6)</td>
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<td>100% (6/6)</td>
<td>Error in published sequence</td>
</tr>
</tbody>
</table>

**Table 3.1**: Sequence changes found in the SCC4 β1 cDNA fragments

Of the four changes found in the SCC4 cDNA the H92T substitution was deemed to be either a common polymorphism or an error in the original sequence (Argraves et al., 1987) as both the SCC4 and the normal keratinocytes were found to be homozygous for this change. The T133T substitution causes no change in the translated protein and as such was discarded as irrelevant in this context. The T195S substitution is most likely to be an error in the published sequence as every clone sequenced in all SCC, keratinocytes and normal DNAs that covered this point were found to have a serine in this position. In addition the β1 integrin gene for every other species sequenced contains a serine at this point.

Both WT and T188I containing clones were found in the SCC4 cDNA at approximately 50% proportions. I therefore concluded that the SCC4 was heterozygous for this mutation. Smaller fragments comprising of amino acids 673 to 832 were generated from the SCC12B2, SCC12F2 and SCC13 cell lines and a panel of fifteen human genomic DNAs using primers B1.9 and B1.10. The T188I substitution was not found in any of the other cell lines or individuals examined. All
of the sequence changes found map to the A domain of the β subunit. No changes were found in the cytoplasmic, transmembrane or stalk domains.

In an attempt to determine whether the T188I mutation occurred in the tumour or was carried by the patient DNA I isolated DNA from fibroblast like cells (provided by J.Rheinwald) grown out of an early (passage 2) frozen stock of SCC4 cells in fibroblast selective medium. I hoped that these represented dermal fibroblasts that were present in the original tumour sample and were representative of the genotype of the patient. When sequenced DNA isolated from these cells was found to be mix of WT and T188I clones, but the T188I clones were at a lower frequency than those found in the pure SCC4 DNA (1/6 WT). No other material was available for further analysis.
3.3 Discussion

The SCC4 line appears to be unique in that it responds to infection with WT by differentiating. Normal keratinocytes do not differentiate upon infection with WT $\beta_1$ although expression of high levels of a CD8 extracellular domain, $\beta_1$ cytoplasmic domain chimera can increase the rate of differentiation due to a decrease in MAPK signalling (Zhu et al., 1999). Previous work in the lab (Levy et al., 2000) had shown that to induce differentiation the infected $\alpha$ integrin construct had to have a functional ligand binding site, but that the ability of the integrin to be cluster in focal adhesions was not required.

There were two initial possibilities to explain the induction of differentiation by the c$\beta_1$ WT integrin. Firstly, the lack of differentiation could be due to a fault in the integrin signalling pathway controlling differentiation that was overcome by the presence of the c$\beta_1$ WT integrin. Alternatively there may be a fault in the endogenous $\beta_1$ integrin itself preventing correct signalling. I planned to investigate the first possibility by clustering the endogenous human integrin or the introduced chick subunits and comparing the pathways stimulated. However similar experiments with primary keratinocyte lines failed to show differences between signalling from endogenous human and introduced chick $\beta_1$ subunits even when functionally dead chick integrin constructs were used (personal communication, H. Abedi). Immunofluorescence staining of cells expressing chick integrins clustered on blocked anti-chick integrin antibody coated plates showed that the endogenous $\beta_1$ integrin tended to co-cluster with the chick construct explaining why the "dead" integrin
constructs appeared to signal. This co-clustering made it difficult to draw conclusions from the clustering assays and so I turned to the second hypothesis.

I have found three amino acid changes from the published human β1 sequence in the SCC4 cell line that could cause a change in the function of the subunit. The presence of multiple changes from the published sequence of β1 is interesting although of the three changes only one is likely to have any function. H92T may be a common polymorphism rather than an error in the database but T195S is almost certainly an error in the original submission. We have found no evidence of a serine at this point in any sample sequenced and in over 100 tumours (Chapter 6) T195S was found in every sequence. Furthermore the β1 sequence from every other species that is in the NCBI database contains a serine at this point. This shows that a database “WT” sequences can not always be taken as absolute.

To try and establish if the T188I mutation arose as a result of genetic instability on the tumour or was present in the patient I tried to obtain non-tumour material. The only available source was a fibroblast like cell type that was grown out of an early passage of SCC4 under selective conditions. The lower frequency of T188I clones in these fibroblasts does not support any conclusions as to the origin of the tumour. It is possible that this lower frequency represents WT/WT fibroblasts contaminated with WT/T188I SCC4 cells; however with the material available it is not possible to make a statistically safe statement either way. It is also possible that the cells are not dermal fibroblasts as suspected but are, in fact, a keratinocyte clone from the original tumour that is capable of growing in fibroblast selective medium. Further non-
tumour tissue from the patient is not available so it is impossible to be certain whether the T188I substitution is germline or appeared in the tumour.

The position of the T188I mutation makes it a good candidate to have functional significance. The importance of the small C-C loop upon which T188I lies in integrin function has been shown many times and is part of a larger structure referred to as the specificity loop (Takagi et al., 1997). It has been shown that both the ligand and the signalling properties (Miao et al., 2002) of the αvβ3 integrin could be changed by substitution of this loop. The region is also important for folding of the β subunit (Takagi et al., 2002).

The C-C loop is on one of only four areas of the ligand binding domain where there is not a high degree of homology when the β1 is compared to the other known human β subunits (Fig 3.7). When these sites are mapped onto the surface of the RGD bound β3 crystal (Xiong et al., 2002) three of the sites map to the top surface of the protein, two of which lie adjacent to the residues that are close (<4 Å) to the RGD ligand itself. These three sites lie in a line across the top of the domain and while the orientation of the RGD makes it unlikely that they contact that section of the ligand it is possible that they may come into contact with sections of ligand either side of the RGD site. The fourth site lies on the base of the domain away from the ligand binding site and has been identified as the binding site of the TS2/16 antibody, a mAb that can cause activation of β1 integrins (van de Wiel-van Kemenade et al., 1992).
Chapter 3  Analysis of the SCC4 phenotype

Integrin mutations have been found to be the cause of several diseases (reviewed in (Hogg and Bates, 2000)). Mutations in the α6β4 integrin can cause a skin blistering disease known as junctional epidermolysis bullosa (EB) (Pulkkinen et al., 1998a; Pulkkinen et al., 1998b; Pulkkinen et al., 1998c), due to a loss of hemidesmosome function resulting in poor adhesion between the basal keratinocytes and the basement membrane.

Leucocyte adhesion deficiency type 1 (LAD1). Is characterised by a lack of leucocyte function due to loss of adhesion through the β2 family of integrins. LAD1 is usually caused by mutations causing loss of expression (Lipnick et al., 1996; Kijas et al., 1999), although mutations causing expressed but poorly functioning subunits have also been characterised (Wardlaw et al., 1990; Hogg et al., 1999; Mathew et al., 2000; Shaw et al., 2001). The severity of the condition varies depending on the alleles present and range from a sub-clinical condition due to a single partly functioning mutation to a severe life-threatening condition where both alleles are non-functional (Kishimoto et al., 1989; Mathew et al., 2000).

Glanzmann’s thrombasthenia (GT) is characterised by a failure of blood clotting due to a lack of platelet crosslinking and is caused by defects in either the αIIb or β3 genes. Again the severity of the disease depends on the number and type of mutations present. Most GT mutations are loss of function (Dong et al., 2000; Tadokoro et al., 2002; Watkins et al., 2002) although two cases have been identified where the integrin was locked in the high affinity state (Fullard et al., 2001; Ruiz et al., 2001).
Chapter 3  Analysis of the SCC4 phenotype

Integrin mutations have also been found to contribute to other diseases including heart disease (αvβ3)(Undas et al., 2001; Douglas et al., 2002), isolated giant platelet disorder (αIIbβ3)(Kunishima et al., 2001) and certain forms of muscular dystrophy (α7 (Mayer et al., 1997; Pegoraro et al., 2002).

The presence of a mutation in the β1 gene of the SCC4 provides a possible mechanism by which infection with WT cβ1 could restore differentiation. In most cell types the β1 integrin subunit is present in excess, while the levels of α subunits are limiting and control the amount of β1 expressed on the surface. It has been previously shown that infection with the WT cβ1 does not influence the levels of α subunits expressed by the cell (Levy et al., 2000). If the mutant β1 subunit is responsible for the lack of differentiation in the SCC4 line then infection with the WT cβ1 will cause a dilution of the mutant integrin in the cytoplasmic pool of available β1 subunits, thereby reducing the number of mutant integrins on the surface of the cell.
Fig 3.7 ClustalW alignment of the A domains of all eight known human β subunits. All the subunits have a high degree of homology except at four sites (marked) where the subunits have no similarity. T188 (*) lies within one of these sites on a small cysteine bridged loop. When mapped to the surface of the β3 1 domain crystal structure three of the sites form a line across the ligand binding site (marked in light blue). The fourth site lies on the bottom of the domain.
Chapter 4

Analysis of the Effect of the T188I Mutation on ligand binding by the β1 Integrin Subunit

4.1 Introduction

The majority of integrin mutations that have been characterised in human disease cause inactivation or loss of function of the affected subunit (reviewed in (Hogg and Bates, 2000)). In this chapter I set out to discover if the T188I mutation affected integrin function in a similar way. Initially I used a cell free integrin assay system to measure the binding of the T188I subunit to its ligand (Stephens et al., 2000). This system uses the extra cellular domains of the integrin subunits fused to the Fc regions of IgG, combined with a “knob and hole” approach to ensure α/β dimer formation. This allows ELISA style assays of integrin/ligand interactions to be performed in a cell free environment, removing the avidity issues seen in cell based systems. The assay is commonly used to screen libraries of small molecule inhibitors of integrin function.

To confirm the results obtained with recombinant integrins I expressed both the T188I and WT cβ1 in β1 null fibroblasts and performed adhesion assays in a variety of conditions.
Chapter 4  Analysis of T188I Integrin Function

4.2 Results

4.2.1 Analysis of the effect of the T188I mutation on integrin affinity in cell free assays.

To investigate the effect of the T188I mutation on individual \( \alpha/\beta \) integrin heterodimers the T188I mutation and WT \( \beta 1 \) subunits were produced as secreted Fc fusion proteins and used to perform ELISA style assays to measure the binding of the \( \alpha v \) and \( \alpha 5\beta 1 \) dimers to 10 \( \mu g/ml \) Fn coated surfaces. Titration curves were performed for WT \( \alpha v \) and \( \alpha 5\alpha 1 \) and the single concentration points selected for comparison with the mutant corresponded to the concentration of half maximal binding. Changes in observed binding at this point are therefore indicative of changes in affinity. This was performed by Drs A. Henry and V. Perkins at CellTech Chiroscience PLC, Slough UK.

*Binding of \( \alpha 5\)-Fc/ \( \beta 1 \) -Fc fusion proteins to fibronectin.*

It was found that the \( \alpha 5\beta 1 \) T188I subunit combination bound fibronectin with an affinity 20% higher than the WT in the presence of Mg2+ (Fig 4.1A). Addition of either Mn2+ or TS2/16 as activating factors (van de Wiel-van Kemenade *et al.*, 1992) caused the binding of the WT to increase 20 % to that of T188I. There was no observed increase in the binding of T188I upon activation. This suggested that for \( \alpha 5\beta 1 \) the T188I mutation causes a shift to the activated conformation as promoted by Mn2+ even in the absence of the activating cation.
Chapter 4  Analysis of T188I Integrin Function

*Binding of αv-Fc/β1-Fc fusion proteins to fibronectin.*

αv-Fc/β1-Fc T188I dimers bound Fn with an affinity 80% higher than that of the WT in the presence of Mg2+ (Fig 4.1B). On addition of TS2/16 the affinity of both the WT and T188I increased considerably (over 6 fold). In this condition the T188I was still more adhesive than the WT, exhibiting 7.8 times the affinity of the WT non-activated protein compared to 6.3 times for the activated WT.
Chapter 4 Analysis of T188I Integrin Function

Fig 4.1: The T188I mutant βI subunit exhibits a greater affinity for fibronectin coated surfaces than the WT subunit when expressed as a Fc fusion protein with either the α5 (A,C) or αv (B,D) subunit. The degree of binding was influenced by the presence of the cations Mg2+ and Mn2+ as well as the activating anti-βI antibody TS2/16. Mean of triplicate wells±S.D. Data representative of three experiments. (White Bars=WT, Black bars=T188I)
4.2.2 Construction of chick β1 T188I equivalent retroviral construct

In order to express the T188I mutation in human cells and be able to distinguish it from the endogenous protein the equivalent chick mutant subunit was made. The cβ1 subunit is 80% homologous to the human subunit and shows complete conservation of the cytoplasmic domain. WT cβ1 cDNA pBabe-puro retrovirus vector was used as a template for site-directed-mutagenesis by the quickchange protocol (Fig 4.2). Primers T188I+ and T188I- were used.

**Fig 4.2:** pBabe-puro retroviral vector showing position of cβ1 integrin cDNA and the position of the bases corresponding to mutation T188I.
4.2.3 Expression of chick T188I in β1 null fibroblasts

To allow analysis of the effect of the T188I mutation on β1 integrin function in the absence of any WT β1 subunits I used a cell line that lacks β1 integrin, the GD25 cell line (Wennerberg et al., 1996). GD25 cells are immortalised fibroblasts derived from β1 null mouse embryonic stem cells. When the β1 subunit is reintroduced the cells express α3β1, α5β1 and α6β1 on their surface (Wennerberg et al., 1996).

GD25 were induced to express either empty vector pBabe-puro, cβ1 WT or cβ1 T188I by retroviral infection using Phoenix ecotrophic packaging lines. The infected cells were then FACSorted to produce pure populations with high levels of surface expression (Fig 4.3A).

_T188I integrin is recruited to focal adhesions._

GD25 cells infected with either the WT or T188I β1 integrin subunit were grown on coverslips overnight and the stained for cβ1 integrin using the simultaneous fix and permeabilization method. It was found that the infected GD25 cells form focal adhesions containing the introduced chick subunits (Fig 4.3B). There was no obvious difference in size or distribution between the focal adhesions formed from WT or T188I cβ1.
Fig 4.3 The T188I mutant subunit forms is expressed on the surface of the cell and is capable of forming focal adhesions. (A) GD25 cells were infected with either the T188I or WT cβ1 subunit and sorted to give a pure population with equal high expression. Cells stained with JG22 (anti cβ1, Black profile), Grey profile – uninfected cells. (B) The introduced subunits were both able to form focal adhesions when plated on 10 µg/ml Fn. Green: V2E9 - anti cβ1 mAb, Red – Phalloidin, Blue – Hoescht.
4.2.4 Adhesion of GD25 cells expressing WT and T188I integrin subunits

*T188I causes increased adhesion of GD25 cells to fibronectin and laminin but not vitronectin relative to WT controls.*

In order to confirm the results of the cell free integrin binding experiments the adhesion of empty vector, chick β1 WT and chick β1 T188I infected GD25 cells to surfaces coated with various ECM proteins was assayed. Initial assays using serum free E4 as a buffer were performed to determine the optimum concentrations of ligand to use. 5 µg/ml FN represented the point at which 50% of the WT cells attached during the duration of the assay. Cells were then plated on 5 µg/ml Fn in TBS in the presence of 1 mM and 10 mM concentrations of Mg2+, Mn2+ or Ca2+ and were allowed to adhere for 15 minutes at 37 °C. When the cells were plated in TBS in the presence 1 mM of Mg2+ all three cell types adhered poorly (Fig 4.3A). In higher concentrations of Mg2+ (10 mM) and in 1 mM Mn2+ the cells expressing the mutant subunit were found to bind in higher numbers (approximately 50% more in Mg2+ and approximately double in Mn2+) than those expressing the WT. A higher 10 mM concentration of Mn2+ appeared to be toxic to the cells and was not used for further experiments. An activating effect was also observed in the presence of 1 mM Ca2+ but fewer cells adhered than in Mn2+. 10 mM Ca2+ did not support binding as effectively as 1 mM and was not used for further experiments. The empty vector cells adhered poorly in all cations and concentrations.
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Fig 4.4: Adhesion of GD25 cells expressing WT (GD25WT), T188I cβ1 (GD25 T188I) or empty vector (GD25) to ECM proteins. (A) Cells expressing the T188I subunit show a greater affinity for Fn in the presence of Mg2+, Mn2+ and Ca2+. (B) When adhering to Ln an increase in adhesion is only seen in the presence of Mn2+ and is smaller than that seen on Fn. (C) Neither β1 subunit increases adhesion to Vn.

*: p<0.05 that T188I=WT. Error bars are S.D. of triplicate wells. Data representative of four experiments on Fn, three on Ln, two on Vn.
4.2.5 Adhesion of SCC4 cells to ECM proteins.

*SCC4 cells shows increased adhesion to fibronectin and collagen coated surfaces when compared to a similarly non-differentiated SCC WT/WT cell line.*

To have an effect on the behaviour of the SCC4 cells the T188I mutation must be able to influence integrin function when present as a heterozygous allele. The binding of SCC4 cells to surfaces coated in either 10 μg/ml Fn or Col was measured as for GD25 cells and compared to that of SCC13, a SCC line with a similar degree of differentiation (Rheinwald and Beckett, 1981) known to be WT/WT for the β1 subunit. It was found that the SCC4 cells exhibited greater adhesion to both matrix proteins than the SCC13 cells in all conditions assayed, although the difference between the cell types was smaller than that seen in the GD25 WT/T188I comparison presumably due to the heterozygous nature of the mutation (Fig 4.5A,B). In the presence of 10 mM Mg2+ the SCC4 showed approximately 30 % increase in cells adhering to both Fn than SCC13. In the presence of 1 mM Mn2+ the SCC4 cells showed an increase in adhesion of around 50-60 %. The pattern of adhesion was similar on Col, with a 50-75 % increase in adhesion in either cation.

*SCC4 can be induced to express the HUTS-21 epitope but normal keratinocytes cannot.*

The activation state of integrin dimers can be assayed by the binding of reporter antibodies. These bind to sites on the dimer that are only accessible when the integrin is in its high affinity or ligand bound state (Bazzoni *et al.*, 1995; Luque *et al.*, 1996).
Chapter 4  Analysis of T188I Integrin Function

Normal keratinocytes and SCC4 cells were harvested, resuspended in either TBS+Mg2+ (10 mM) or TBS+Mn2+ (1 mM) and then stained with the activated β1 integrin reporter antibody HUTS-21. No HUTS-21 binding was detected on normal keratinocytes in either cation, consistent with previous work suggesting that keratinocyte integrins are normally in a low affinity state unless ligand bound (Bishop et al., 1998). The SCC4 cells showed the same lack of HUTS-21 binding in TBS+Mg2+ as normal keratinocytes but when in the presence of Mn2+ the mean fluorescence increased twofold indicating that some of the integrins present on the SCC4 cells were now in an active conformation (Fig 4.5C).
Fig 4.5: SCC4 shows greater adhesion to (A) FN and (B) Col than SCC13, a WT/WT undifferentiated cell line. Means of triplicate wells±S.D. Data representative of three experiments (C) The HUTS21 β1 integrin activation reporter epitope is expressed on SCC4 in the presence of Mn2+. It is also present on the positive control line MOLT4 but not on normal keratinocytes. Grey trace HUTS21 staining in presence of Mg2+, Black trace HUTS21 in presence of Mn2+. († p<0.05)
4.2.6 Homology modelling of the β1 integrin A domain

In order to better understand the T188I mutation, a model of the β1 A domain was devised. This was possible due to the publication of the crystal structure of the αvβ3 integrin (Xiong et al., 2001). The residues corresponding to the A domain of the β1 integrin were found by alignment of the β3 A domain sequence in MacVector sequence analysis software. This sequence and the corresponding T188I sequence were then fitted to the crystal structure of the ligand bound (1L5GB, (Xiong et al., 2001)) and the ligand unbound αvβ3 (1JV2B (Xiong et al., 2002)) by the SWISS-Model automated structure server (Guex and Peitsch, 1997). The resulting file was then adjusted in SwissPDB viewer to improve the fit and the file optimised by the SWISS-Model server to produce the final models (Fig 4.6, 4.7A). The β1 A domain has a high degree of homology to corresponding β3 domain and the resulting models are all similar to those of the β3 crystals. It was found that the T188I residue projected upwards from the C-C loop which itself projects from the upper surface of the main body of the domain.

The presence of the isoleucine caused a change in the position of the C-C loop in both models however change in the body of the structure was seen. While there are fewer major structural changes in the β A domain upon activation than in the α I domain it is likely that this lack of change is due to the method used to prepare the model which always attempts to fit the modelled sequence as closely as possible to equivalent region of the template. This will tend to minimise the effects of changes in sequence that are not immediately proximal to the mutated region.
When compared to the β1 A domain models of You et al. (You et al., 2002) and Rava Devi and Tajne the model presented here differs in the presence of the disulphide bridge in the C-C loop (Fig 4.7 B-D). You et al. state that to include the C-C bond forces the loop into unnatural kink. While the loop is in a tight hairpin SWISS-MODEL does not remove the C-C loop when the structure is optimised and energy minimised.
Fig 4.6 The C-C loop (red) bearing T188I (white) lies adjacent to the ligand binding site (light blue) on the surface of the β1 A domain. Model based on the crystal structure of αvβ3 in presence of RGD peptide (shown as trace (Xiong et al., 2002). MIDAS and ADMIDAS cations shown in orange. Light blue surface denotes residues within 4 Å of ligand. The presence of the mutation causes a change in the position of the C-C loop in the model. The isoleucine occupies a greater area on the surface of the protein (T188I) than the threonine (WT). Model produced using SWISS-MODEL and rendered using SWISS-PDBviewer and winPOV.
Fig 4.7: (A) Theoretical model of αvβ1 β propeller and β subunit A domain bound to an RGD peptide (produced by SWISS-MODEL based on 1L5GB). Comparison between the model of the β1 A domain produced by SWISS-MODEL based on αvβ3 in absence of ligand (1JV2B) (B,D) and the theoretical model of S. Rama Devi and S. Tajne (1LHA, PDB) (C,E) Helixes shown in red, β-sheets in yellow and random coil in grey. RGD peptide in green. The C-C loop is highlighted in blue and its cysteines in yellow. MIDAS and ADMIDAS cations in blue.
4.3 Discussion

It is clear from the results presented in this chapter that the T188I mutation has an effect on the function of the β1 subunit and that this is to cause an increased affinity for ligand. Prior to the solution of the αvβ3 crystal structure (Xiong et al., 2001) a mechanism for this activation would have been hard to envisage. With the benefit of the structure it is apparent that the equivalent residue in β3 lies on the top of the β3 A domain, close to the MIDAS site and plays some role in the structural rearrangements caused by ligand binding.

The cell free adhesion assays show that the unstimulated conformation of the T188I subunit has a similar affinity to that of the stimulated conformation of the WT. In the case of α5β1 the T188I Mg2+ condition shows the same level of FN binding as the WT Mn2+ state although this represents only a small shift in the affinity of dimer. This suggests that the T188I is permanently in either the high affinity Mn2+ induced state or a similar activated conformation.

The cell based assays show increases in adhesion of the T188I expressing cells in both the Mg2+ and the Mn2+ bound state suggesting a conformational change in the structure exposing the ligand-binding site more in both the low and high affinity conditions. The cell based assays have the disadvantage of measuring the adhesion of the cell to a ligand coated surface rather than the binding of an individual receptor to its ligand. Because of this the assay really measures the avidity of the cell, as the cell's adhesion is affected by the rate at which the many thousands of individual
integrins attach and detach from the surface. The cell based assays have the advantage of working with intact full length integrins.

The comparison between the cell based and cell free systems shows some interesting differences in activation patterns. The WT is activated by around 20% by Mn2+ or TS2/16 in the cell free system. This is also seen in the cell based system. The T188I subunit has the same affinity in Mg2+ as the WT in Mn2+ in both assays systems. However in the case of the α5β1 cell free assay no further activation is seen in the T188I subunit after treatment with Mn2+ or TS2/16. In the cell based assays a large increase in binding can be seen when the T188I subunit is placed in Mn2+. This suggests that the replacement of the integrin cytoplasmic domains with the Fc domain in the cell free system may be somehow preventing maximal activation being achieved.

While the α5β1/Fn cell based assay showed that the mutation caused an increase in both the Mg2+ bound and Mn2+ bound state, the α3/α6β1/Ln assays showed no difference in the low affinity Mg2+ bound state but did show a difference in the Mn2+ state. The cell free assays also showed differences in patterns of activation depending on the α subunit present. This shows that integrin activation is not purely a β subunit event and that the α subunit plays some role. While the structure does not suggest that the C-C loop or T188 itself interacts with the α subunit perhaps there is some difference in the tertiary changes upon ligand binding in the β1 A domain structure depending on the α subunit it is paired with. Alternatively as the recognition sequence in Ln differs from the RGD motif found in Fn perhaps there are
different conformational changes on ligand binding. It is clear that the T188I mutation is capable of activating a range of different \( \alpha \) subunits.

The increased adhesion of the SCC4 cells to collagen shows that the conformational change can be transmitted from the \( \beta \) A domain to the \( \alpha \) subunit I domain and influence the affinity of that ligand binding site. This is consistent with the mechanism recently proposed by Alonso \textit{et al.}(Alonso \textit{et al.}, 2002).

The \( \beta 1 \) integrin found on keratinocytes is usually in a low affinity state, except at points where the integrin is bound to ligand. In culture this is observed in focal adhesions or the "o" ring of integrin seen around the periphery of the cell. \textit{In vivo} the high affinity form is seen at the basement membrane (Bishop \textit{et al.}, 1998). Active integrins are not found in cell-cell junctions (Kim and Yamada, 1997). The increased exposure of the HUTS21 epitope indicates that at least some of the integrin on the SCC4 is in the active, extended form. The lower signal from the SCC4 compared to the MOLT4 line may be indicative of the heterozygous nature of the mutation. The HUTS21 epitope has been mapped to a region on the stalk of the \( \beta 1 \) subunit (Luque \textit{et al.}, 1996). That this epitope is exposed in SCC4 cells in suspension in the absence of ligand shows that the T188I mutation can activate not only the \( \beta \) A domain, but can also trigger the large scale rearrangements typical of integrin activation. The lack of any inducible epitope on normal keratinocytes is also interesting as in many other cell lines, such as MOLT4, it is possible to induce activation of the \( \beta 1 \) integrin using Mn2+ treatment. It is possible that there is an inhibitory inside-out signalling pathway present in keratinocytes and that the mutation, acting locally on the \( \beta \) subunit A domain, overrides this effect.
Chapter 4 | Analysis of T188I Integrin Function

Activation of integrins by mutation is a rare occurrence. To date only two naturally occurring activating mutations have been characterised, both of which are in the β3 subunit (Fullard et al., 2001; Ruiz et al., 2001). One of these, C560R maps to the stalk of the subunit, the other, V193M, the A domain. V193M lies close to the position of T188I, however it is outside the C-C loop although it is within the larger area known as the specificity loop. In addition several mutated subunits have been designed in the laboratory with high affinity conformations. Deletion of the I domain from the αL Subunit of αLβ2 cause the subunit to adopt an active conformation as detected by activation reporter antibodies (Leitinger and Hogg, 2000). α subunit I domains have been locked into the active conformation by locking the α7 helix into its active position (See Fig 1.6) (Shimaoka et al., 2001). Swapping the C-C loop of β2 to the equivalent from β3 has also been shown to activate the integrin αLβ2 (Kamata et al., 2002).

Mutation of the threonine to isoleucine may cause the activation of the integrin in several ways. Movement of the specificity loop towards the cation at the MIDAS site appears to be a significant part of the conformational change in integrin activation (Xiong et al., 2002). The activating effect of the T188I mutation is likely to be due to some conformational change in the structure around this region. T188 may form a bond to another residue in the area that stabilises the low affinity form, loss of this bond leading to a constitutive high affinity state. Alternatively the presence of the large hydrophobic isoleucine side chain may cause a change in the position of the C-C loop causing the integrin to remain in a fully or partially active state rather than a low affinity state. This shift could be caused by the increased size of the side chain...
forcing some rearrangement of the neighbouring amino acids or, as T188 is partially exposed to solvent, caused by the hydrophobic isoleucine sidechain moving away from the surface of the protein.

When the β1 A domain is modelled based on the β3 structure the T188 residue faces upwards from the C-C loop and lies along the top of the main body of the domain towards the ligand. The isoleucine side chain would lie along the surface in the direction in which the C-C loop is thought to move. This may interfere in some way with the correct movement of the loop. The position of the C-C loop does not seem to allow it to be in contact with the α subunit or be part of the RGD binding site (Fig 4.6A). This is similar to the original αvβ3 crystals (Xiong et al., 2001; Xiong et al., 2002) where there is no contact between the subunits or the ligand at this point. However as the C-C loop is positioned just outside the ligand binding site it is probable that the loop is involved in the conformational change upon ligand binding in a similar manner to that seen in the αvβ3 ligand bound crystal. In the αvβ3-RGD crystal the ligand is represented only by a small peptide and it is possible that another part of a larger ligand, such as intact Fn, may come into contact with the C-C loop. This interpretation is supported by photo-cross-linking experiments (Bitan et al., 2000; Yahalom et al., 2002). These studies showed that an RGD peptide with a C-terminal photo-cross-linking group would become covalently attached to the integrin. Subsequent proteolytic sectioning of the integrin showed the RGD peptide bound to a fragment that included the C-C loop.
The Effect of Integrin Activation on Cell Behaviour

5.1 Introduction

The finding that T188I causes an increase in the affinity of the β1 subunit for its ligand led me to investigate the effect of integrin activation on cell behaviour. I investigated this using GD25 β1 null fibroblasts, the SCC4 cell line and normal keratinocytes. To cause activation of the integrin in these cell types I used two methods, retroviral infection with the T188I chick β1 integrin or incubation with an activating anti human β1 integrin mAb TS2/16.
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5.2 Results

5.2.1 Analysis of the effect of the T188I mutation on spreading, migration and invasion.

GD25 spread more quickly on FN when infected with the T188I cβ1 than WT cβ1 integrin subunit.

GD25 cells expressing either empty vector pBabe-puro, the WT cβ1 or T188I cβ1 constructs were plated onto coverslip bottomed cell culture dishes coated with 10 μg/ml fibronectin and allowed to adhere for 5 min. Fields containing 35-40 cells were filmed for 24 hr. The number of spread cells was then counted and plotted against time to determine the rate at which the cells spread. A spread cell was defined as any cell that was no longer round and refractile. Any cell that did not spread within the length of the recording was not included in the analysis on the assumption that it was non-viable.

It was found that the WT expressing cells spread at a rate approximately half that of the T188I expressing cells. Half the WT cells were spread in about 30 minutes compared to approximately 10 minutes for the T188I expressing cells. The empty vector cells spread poorly and took in excess of 90 minutes for 50 % to spread (Fig 5.1).

Once the cells had been plated for 5 hours and had fully spread state their morphology was compared. The WT and T188I infected cells were spread to a greater extent than the parental cells. No differences between the WT and T188I cells
Chapter 5  The Effects of Integrin Activation

were observed that would indicate differences in Rac/Rho/Cdc42 signalling and there
was no difference in the final degree of spreading observed (Fig 5.1 B).

The movement of cells expressing the WT and T188I cβ1 subunits was tracked for
24 hr and quantitated. 40 cells were analysed in each experiment. It was found that
the average speed of the uninfected GD25 was $3.55 \pm 0.045 \text{ µm}/25 \text{min}$ compared to
$9.84 \pm 0.09 \text{ µm}/25 \text{ minutes}$ for the T188I cells and $7.91 \pm 0.08 \text{ µm}/25 \text{ minutes}$ for the
WT. The speeds of cells expressing the WT and T188I β1 are not statistically
different at the $p<0.05$ level (Fig 5.2).
Chapter 5  The Effects of Integrin Activation

A

![Graph showing cellular spread over time](image)

B

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<th>Time</th>
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Fig 5.1: Expression of T188I cβ1 causes an increase in the rate of spreading of GD25 cells. (A) GD25 cells expressing the T188I subunit (GD25 T188I) spread more quickly on 10 μg/ml Fn than those infected with the WT subunit (GD25 WT) or empty vector (GD25). At each time point the number of cells that had begun to spread was counted. Cells which did not spread over the course of the whole film (20 hrs) were discounted as non-viable. This represented fewer than 5 cells in any sample. (B) GD25 cells expressing the WT or T188I cβ1 or empty vector cells 20, 40 or 300 minutes after plating. Bar= 100μm. Data representative of three experiments.
Fig 5.2: The effect of WT or T188I cβ1 on the movement of β1 null fibroblasts. The migration of GD25 cells expressing empty vector (GD25), WT (GD25 WT) or T188I (GD25 T188I) chick β1 subunits on 10 μg/ml Fn was measured over 20 hr. (A) Data plotted is the average speed of the cells±S.D. at each time point. (B) The average speed of each type of cell±standard error over the whole 20 hr. 40 cells tracked for each cell type, data representative of 3 experiments.
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GD25 cells expressing WT β1 integrin invade through Matrigel at a higher rate than those expressing T188I or empty vector.

Invasion through the surrounding extracellular matrix is an important factor in tumorigenesis (Boyd, 1996). In order to determine whether the T188I β1 subunit would promote invasion, Matrigel assays were performed. In this assay the cells are placed above a gel made from a mixture of ECM proteins above a porous membrane. A chemoattractant is placed in the compartment below the gel and over several days the cells invade through the gel and the membrane to reach the lower (Thomas et al., 2001). The rate of invasion is controlled by several factors including the ability of the invading cells to produce proteases to break down the gel (Del Rosso et al., 2002), their responsiveness to the chemoattractant and their integrin expression (Thomas et al., 2001).

Initial experiments where cells were allowed to invade through a gel formed from 1:1 Matrigel:medium were found to give poor results as GD25 cells did not spread well on this concentration of gel. This resulted in clumps of cells forming above the gel and when cells did migrate through the gel they tended to move as clumps (Fig 5.3A). In addition, the 3 day incubation used allowed these cells to start to divide on the lower side of the membrane and produce monolayers. To rectify these problems a 1:2 Matrigel:medium gel was used and the incubation period reduced to 48 hours. This improved the spreading of the cells on the surface of the gel and caused them to migrate individually (Fig 5.3A). The reduced time period also reduced the problem of growth on the lower side of the membrane.
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After the cells had been allowed to invade the membranes were fixed in methanol, stained with crystal violet and photographed at 2.5x using a microscope equipped with a digital camera (Fig 5.3B). The resulting photos were then converted to a black and white image and the number of black pixels (representing cells) measured by image analysis software (Fig 5.3C). This number was then converted to cells/mm² by comparison with the average number of pixels occupied by single cells. It was found that cells transduced with the empty vector migrated slowly and few were found on the lower side of the membrane. Few T188I expressing cells were found to have invaded although there was a statistically significant increase in the number of the T188I cells present below the membrane when compared to the empty vector. The WT cells migrated significantly more quickly with at least three times as many cells present on the bottom of the membrane than the T188I expressing cells (Fig 5.3D). I concluded the T188I subunit does not promote invasion.

GD25 expressing T188I migrate toward serum more slowly when infected with WT β1 subunit.

As cells expressing the T188I cβ1 subunit were less invasive than those expressing the WT subunit (Fig 5.3D) but there was no difference in the rate of random migration (Fig 5.2) it was important to determine whether there was any change in the attraction of these cells to serum. This assay was performed by measuring the number of cells that moved through a transwell filter from a low serum to a high serum environment. It was found that the number of T188I expressing cells that migrated through the membrane was lower than that of the WT. Approximately 15% of the T188I cells compared to 22% of the WT cells migrated through the membrane in 6 hr (Fig 5.3E). This result mirrors the difference in the rate of invasion through
matrigel in that expression of the WT cβ1 promoted movement towards serum more efficiently than the mutant. This suggested that the lack of invasion through Matrigel of the T188I expressing cells may be partly due to a lack of attraction towards the serum in the lower chamber rather than simply an inability to penetrate the Matrigel layer.
Fig 5.3: The effect of WT or T188I cβ1 expression on GD25 movement through transwells in the presence or absence of matrigel. (A) The lower surface of a transwell stained with crystal violet showing clumps of cells when a 1:1 Matrigel:medium gel was used with a 72 hr incubation period (left) or cells more evenly distributed across the membrane with a 1:2 gel and 48 hr incubation. (B) Lower surface of a transwell membrane imaged. Bar=300 μm. (C) Black and white image of B as used for quantitation of cell number. (D) Infection with the WT cβ1 promotes invasion more effectively than T18I cβ1. Mean number of cells/mm² on the lower surface of the transwell after 48 hours migration±S.D. of triplicate wells. (E) Expression of either the T188I and WT supports migration through transwells in the absence of Matrigel but fewer cells expressing the T188I construct are found on the lower side of the transwell. % of cells on the lower surface of the transwell±S.D. of triplicate wells after 6 hours migration.
5.2.2 The effect of integrin activation on cell signalling

GD25 expressing T188I show increased phosphorylation of ERK1/2 in response to adhesion to fibronectin when compared to cells expressing WT cβ1

Integrin signalling, particularly the MAPK pathway (Zhu et al., 1999), plays an important role in the regulation of terminal differentiation. To investigate whether integrin activation could alter integrin signalling I used the GD25 cells to assay MAPK activation upon spreading on ECM. GD25 cells expressing either the WT or the T188I construct were trypsinised, washed in serum containing medium then twice in serum free medium and plated onto 10 µg/ml Fn. Lysates were made at various time points. When the levels of ERK1/2 phosphorylation in the absence of EGF was quantified by western blotting both WT and T188I expressing cells showed an increase in activation for the first 7 minutes which then fell to very low levels of activation until at least 60 minutes after plating (Zhu et al., 1999). However the T188I expressing cells showed a greater activation than the WT with phosphorylated p44 ERK being easily detectable 7 minutes after plating (Fig 5.4).

When the experiment was repeated in the presence of 10 ng/ml EGF the levels of ERK1/2 phosphorylation were found to be much higher and with little difference between the WT and T188I at the 7 minutes time point. However the high level of phosphorylation was found to be maintained for a longer period in the T188I expressing cells with high levels of phospho-ERK still detectable at 40 minutes. It was also observed that there was a slight increase in the level of phospho-ERK in the presence of EGF in T188I expressing cells held in suspension for 60 minutes when compared to the WT (Fig 5.4). These result are interesting as the duration of ERK
activation in the WT expressing cells closely matches the rate of spreading, whereas in the T188I expressing cells MAPK activation continues long after the cells are spread (see Fig 5.1)

Fig 5.4: ERK phosphorylation in β1 WT and T188I expressing GD25 cells. Cells were plated on 10 μg/ml Fn in the presence or absence of 10 ng/ml EGF. ERK phosphorylation is maintained longer in the cells expressing T188Icβ1 than those expressing WT integrin. S = 60 minutes in suspension.
5.2.3 Effect of integrin activation on SCC and normal keratinocytes.

Infection of SCC4 with T188I does not impede growth relative to the WT

As increasing the adhesion of cells can, in some cases, lead to a reduction in growth the growth rate of cultures of SCC4 infected with empty vector, WT or T188I chick integrin was determined. 1x10⁵ cells were plated into each well of 24 well tissue culture dishes and over the space of 17 days wells were harvested and the number of cells counted. It was found that infection with the WT reduced the growth rate of the keratinocytes, the growth curve shifting slightly to the right (Fig 5.5). The presence of the activated integrin did not cause any further reduction. For most of the time-course the growth curves of the WT and the T188I cells were less than one standard deviation apart.

![Graph A](image1.png)  ![Graph B](image2.png)

**Fig 5.5** (A) Expression of the T188I cβ1 does not inhibit growth more than the WT. 1x10⁵ SCC4 cells expressing the WT cβ1, T188I subunit or the empty vector were plated in 24 well plate wells on day 0 and harvested at intervals over the next 18 days. The number of cells per well was counted. Points are the mean number of cells in triplicate wells±S.D. of triplicate. Data representative of three experiments. (B) Expression of the WT and T188I constructs was equivelant. (grey trace - non specific control, Black - JG22, anti cβ1)
Infection of SCC4 with T188I cβ1 subunit does not induce differentiation whereas infection with WT does.

In order to determine whether the T188I mutation was responsible for the lack of differentiation in the SCC4 line SCC4 cells transduced with the empty vector, WT cβ1 or T188I cβ1 subunits were co-cultured with J2F feeder cells and allowed to grow to 75% confluence. The cultures were then disaggregated and stained for the differentiation markers involucrin and cornifin. The presence of the WT integrin subunit induced both involucrin (Fig 5.6) and cornifin (Fig 5.7), to a similar extent as seen before (15-20% for each marker)(Levy et al., 2000). Neither the empty vector or the T188I subunit caused induction of either differentiation marker (<4% positive for either marker).
Fig 5.6: Involucrin expression in SCC4 cells retrovirally infected with the WT, T188I cβ1 subunits or empty vector. (A) Dissaggregated cultures of SCC4 cells infected with either the WT cβ1, T188I cβ1 or the empty vector were dried onto coverslips and stained with SY-5 (mAb to human involucrin). (B) Quantitation of involucrin staining. Triplicate slides were counted for each cell type and the mean±S.D. plotted. At least 300 cells per slide were counted. Data representative of four experiments.
Fig 5.7: Cornifin expression in SCC4 cells retrovirally infected with the WT or T188I cβ1 subunits or empty vector. (A) Dissaggregated cultures of SCC4 cells infected with either the WT cβ1, T188I cβ1 or the empty vector were dried onto coverslips and stained with SQ37C (pAb to human cornifin). (B) Quantitation of cornifin staining. Triplicate slides were counted for each cell type and the mean±S.D. plotted. At least 300 cells per slide were counted. Data representative of five experiments.
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Infection of SCC12B2 or up with T188I does not effect differentiation.

In order to examine whether the T188I mutation suppressed differentiation, failed to induce or was simply inactive in influencing differentiation I expressed the T188I and WT integrin in two keratinocyte derived cell lines with WT endogenous β1 subunits. SCC12B2 is a cell line similar to the SCC4 (Rheinwald and Beckett, 1981). up is a HPV transformed keratinocyte cell line (Hodivala et al., 1994). These were chosen as they are both transformed keratinocyte lines that behave in a similar manner to the SCC4 but express higher levels of differentiation markers. In both cases high levels of cβ1 expression were obtained but in each case the levels of differentiation in the culture was too low to detect any reduction in the presence of the T188I subunit. The WT integrin did not induce differentiation in either case (Fig 5.8). Therefore I could not conclude whether the T188I mutation was able to actively suppress differentiation.

Several attempts to retrovirally express the T188I cβ1 in normal, untransformed keratinocytes did not produce any cells expressing the construct.
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Fig 5.8: Expression of the WT or T188I cβ1 constructs does not induce differentiation in either up or SCC12B2. (A) Involucrin staining (SY-5) of disagggregated cultures of up cells expressing pBp (Up), cβ1 WT (WT) or cβ1 T188I (T188I). (B) Quantitation of involucrin positive cells in cultures of Up and SCC12B2.

<table>
<thead>
<tr>
<th>Cell Type / Construct</th>
<th>% expressing involucrin</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>up pBp</td>
<td>2.23</td>
<td>1.66</td>
</tr>
<tr>
<td>up WT</td>
<td>0.20</td>
<td>0.44</td>
</tr>
<tr>
<td>up T188I</td>
<td>1.38</td>
<td>1.44</td>
</tr>
<tr>
<td>SCC12B2 pBp</td>
<td>5.49</td>
<td>1.19</td>
</tr>
<tr>
<td>SCC12B2 cβ1 WT</td>
<td>6.57</td>
<td>1.31</td>
</tr>
<tr>
<td>SCC12B2 cβ1 T188I</td>
<td>6.70</td>
<td>1.03</td>
</tr>
</tbody>
</table>
5.2.4 The activating anti-β1 antibody TS2/16 can effect normal keratinocyte differentiation

*Incubation of normal adherent keratinocytes with TS2/16 causes a suppression of differentiation.*

To confirm that activation of the β1 integrin subunit is capable of affecting the differentiation of primary keratinocytes, normal keratinocytes were cultured to confluence in the presence of various activating and non-activating antibodies for 48 hr. To avoid affecting plating efficiency by the use of the activating antibodies, the cultures were set up 48 hr before the antibodies were added. Four different monoclonal antibodies to the hβ1 subunit were used, each at 20 μg/ml. In addition, a culture with no antibody and a culture with an antibody to the cβ1 subunit was used as a negative control. After 48 hrs the cultures were washed in versene to remove the feeder cells, disaggregated and stained for differentiation markers.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Specificity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5D2</td>
<td>human β1</td>
<td>Blocks adhesion</td>
</tr>
<tr>
<td>A2B2</td>
<td>human β1</td>
<td>Blocks adhesion</td>
</tr>
<tr>
<td>9EG7</td>
<td>human/mouse β1</td>
<td>Recognises ligand bound conformation</td>
</tr>
<tr>
<td>TS2/16</td>
<td>human β1</td>
<td>Induces active conformation</td>
</tr>
<tr>
<td>W1B10</td>
<td>chick β1</td>
<td>Irrelevant antibody control</td>
</tr>
</tbody>
</table>

*Table 5.1* Antibodies screened for an ability to influence differentiation in cultured keratinocytes.
Incubation of keratinocytes with 20 μg/ml of the non-activating antibodies was found to have no effect on the keratinocytes expression of involucrin or cornifin. However, the cultures incubated with the TS2/16 were found to contain 40% fewer involucrin positive cells than the others. The percentage of cells expressing the differentiation markers was found to vary between experiments with the passage number of the parental cultures (approximately 10% involucrin positive at passage 3 to 25% at passage 6) but in all cases treatment with TS2/16 reduced this by around 40% (Fig 5.9). None of the antibodies caused any affect on cell spreading under these assay conditions.
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Fig 5.9: The activating mAb TS2/16 caused a decrease in the number of involucrin positive keratinocytes in adherent cultures after incubation for 48 hr. (A) No change in the number of involucrin positive cells was found when keratinocytes were treated with an irrelevant antibody control (W1B10), two other non activating mAbs (A2B2, P5D2) or an activation reporter (9EG7). Incubation with the activating antibody TS2/16 suppressed differentiation by approximately 40%. (B) Triplicate dissociated cultures of keratinocytes incubated with A2B2, TS2/16 or untreated. Stained with DH-1, a polyclonal antibody to human involucrin. Triplicate wells were analysed ± S.D.. Data representative of three experiments.
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*TS2/16 is capable of suppressing keratinocyte differentiation in suspension*

It has been previously shown that suspension induced differentiation of keratinocytes can be suppressed by incubation with fibronectin or by certain adhesion blocking anti-integrin antibodies (Adams and Watt, 1989, Watt et al., 1993). It has also been shown that restoration of integrin adhesion by using the activating anti β1 antibody 8A2 does not prevent differentiation in keratinocytes previously placed in suspension (Hotchin et al., 1993). In order to examine whether the activating antibody TS2/16 could enhance the suppressing effect of fibronectin suspension experiments were performed with keratinocytes in the presence of fibronectin (25 μg/ml) TS2/16 (20 μg/ml) or both. It was found that fibronectin produced a suppression of differentiation of 20-30%. TS2/16 produced a similar inhibition (Fig 5.10). The combination of both treatments was found to be no more effective than either component alone.

![Graph](image)

**Fig 5.10** Incubation of keratinocytes in suspension with TS2/16 reduces suspension induced involucrin expression by the same degree as Fn. The combination of both treatments was no more effective than either alone. Cells placed in suspension overnight with 25 μg/ml Fn and/or 20 μg/ml TS2/16.
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5.2.5 Discussion

The results of the previous chapter showed that the T188I mutation had an activating effect on the function of the β1 integrin. In this chapter I have extended my investigation by testing additional aspects of cell behaviour. I have shown that the activation of the integrin causes effects on the behaviour of the cell carrying the mutation. I found that expression of the T188I integrin caused various changes in behaviour of the GD25 line when compared to the WT infected cells. This included an increase in the rate of spreading and changes in ERK activation but a lack of promotion of invasion through Matrigel and migration towards serum. Keratinocytes also show changes in behaviour on integrin activation. SCC4 was found not to differentiate in response to infection with the mutant subunit while the WT caused differentiation as before. Incubation of normal keratinocytes with TS2/16 was found to reduce the number of differentiated cells in culture and suppressed differentiation in suspension.

The increased rate of spreading in GD25 cells expressing the T188I cβ1 subunit is likely to be a direct result of the increased integrin affinity. The lack of spreading observed in the cells infected with the empty vector is likewise probably due to the lack of integrin on the cell surface. Spreading of cells is linked to various events such as MAPK phosphorylation and differentiation in keratinocytes (Watt et al., 1988; Zhu et al., 1999).

Increased adhesion has been linked to an inability to migrate (Kuijpers et al., 1993). It is clear that in the case of GD25 cells infected with the T188I cβ1 subunit the
increase in adhesion is not of an order to preclude cell movement as the T188I promoted migration to the same extent as the WT. It is possible that on very high or very low concentrations of Fn a greater difference in migration may have been observed as high affinity for ligand tends to reduce migration on high densities of ligand (Palecek et al., 1997). It can also be seen that a lack of adhesion due to a lack of integrin precludes cell motility as the cells infected with the empty vector showed little ability to migrate. These results are consistent with the results of Rose et al. (Rose et al., 2001) in which cells with an inability to effectively activate their integrin had a reduced ability to migrate.

The invasion assay system used here, comprising a transwell chamber with a layer of semi-permeable ECM gel and a serum gradient, is a crude attempt to mimic the in vivo process of a tumour cell migrating away from the primary tumour, through the surrounding matrix and towards the vasculature. In order to move into the lower portion of the chamber the cells must be able to migrate effectively, produce appropriate MMPs to penetrate through the gel and also be attracted to the serum in the lower chamber. The link between integrin signalling and MMP production has been shown in numerous cases and involves signalling via proteins including PKC (Niu et al., 1998) and tetraspanins (Sugiura and Berditchevski, 1999). Integrin subunits implicated in these systems include α2 (Dumin et al., 2001), α3 (Sugiura and Berditchevski, 1999), αvβ3 (Brooks et al., 1996) and αvβ6 (Thomas et al., 2001), (Niu et al., 1998). In several cases ligation of the integrin not only induces expression of the MMP but also allows the MMP to directly associate with the integrin to form a ternary adhesion/ECM degradation complex (Dumin et al., 2001).

In the case of the GD25 cells the lack of invasion of the uninfected cells may well be
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due to a lack of MMP production caused by a lack of integrin signalling.
Alternatively without the presence of integrin $\alpha$ subunits on the cell surface the
MMP may not be able to localise to the correct part of the membrane to assist
migration (Brooks et al., 1996; Dumin et al., 2001). The increase in invasion on
infection with the WT c$\beta$1 could be explained by a possible induction of MMPs by
the $\alpha$3 subunit now present on the surface. However the lack of invasion by GD25
cells expressing the T188I c$\beta$1 subunit is hard to explain in terms of MMP
production.

Palecek et al propose that the optimum ligand concentration for cell migration is
inversely proportional to the affinity of the cell for its ligand (Palecek et al., 1997).
As the matrigel presents the cell with a very high effective concentration of ligand it
is possible that this is causing inhibition of migration through the gel. In an attempt
to answer this question we performed similar assay in the absence of a matrigel layer.
In these assays the GD25 cells failed to migrate. This is likely to be due to the
absence of integrin on their surface as was seen in the random migration assays. The
number of WT cells that migrated through the membrane was higher than the number
of MT cells, although the difference was not as marked as for the matrigel assays.
This suggests that the T188I subunit fails to promote directional movement towards
serum as effectively as the WT expressing cells and this may be a contributory factor
in the lack of invasion of the T188I cells. The interplay between integrin signalling
and growth factor receptor signalling is well known (Moro et al., 1998). It is possible
that over activation of one pathway can support signalling in both when the second
stimulus is at a sub optimal level. In this situation increased signalling from the
integrin may be compensating for the low growth factor signalling in the low serum
environment above the membrane resulting in reduced migration into the high serum medium below the membrane.

The ERK1/2 pathway has been shown to be capable of influencing keratinocytes behaviour, in particular the ability of the cell to differentiate (Zhu et al., 1999). Two pathways that can lead to ERK are signalling from the EGF receptor (reviewed in Yarden and Sliwkowski, 2001) and from integrin ligation to ECM (Chen et al., 1994). The signalling pathways from these two sources share many common components and for maximal activation of ERK both signals are required. The strength of ERK signalling from β1 integrin ligation has been shown to be proportional to the number of ligated integrin receptors (Asthagiri et al., 1999) and a correlation between the strength of adhesion and the strength of ERK signalling has been observed (Ishida et al., 1996). An increase in ERK signalling due to a increase in the number of ligated integrin subunits due to the increased affinity of T188I is likely to explain the increased signalling at the initial time points in the absence of EGF and the increased duration of ERK signalling in the presence of EGF.

The signal from the integrin is often described as being “permissive”, for the more important EGF signalling event (Miyamoto et al., 1996). In both the WT and T188I expressing cells this can be seen as ERK activation drops to low levels in the absence of integrin ligation even in the presence of EGF. This interplay between ERK activation and integrin ligation is linked to the initiation of cell spreading due to changes in the cytoskeleton (Asthagiri et al., 1999). This is seen in the WT cβ1 expressing GD25 cells where ERK activation drops back to basal levels once the cells are spread at 40 to 60 min. However the T188I cβ1 expressing GD25 show
strong activation of ERK at 40 minutes, at a point where most of the cells have spread suggesting the mutation can affect integrin signalling independently of its effects on spreading.

The absence of any induction of differentiation in the SCC4 cells expressing the T188I subunit suggests that the mutant integrin is, in some way, involved in the differentiation phenotype seen in the SCC4 cell line. I have not been able to express this construct in normal keratinocytes or any keratinocyte line that differentiates at levels high enough to see any suppressive effect so I cannot determine whether the construct simply fails to induce differentiation or actively suppresses it. The results of the experiments using TS2/16 to activate the endogenous integrin on normal keratinocytes suggest that raising the affinity of the β1 integrin does, in fact, actively suppress differentiation. TS2/16 and T188I both cause integrins to switch into a higher affinity state, increasing the affinity of the cell for extracellular matrix and promoting ligand binding. It is probable that this increase in ligand binding causes the decrease in the number of differentiated cells. This is consistent with previous work (Adams and Watt, 1989; Levy et al., 2000) where it was shown that β1 integrin binding to ligand sends a "do not differentiate" signal. This would also explain the restoration of differentiation seen after infection with the WT cβ1 subunit. The WT subunit competes out the effect of the T188I by diluting the number of T188I integrins, reducing the strength of the "do not differentiate" signal and restoring differentiation.

The effect of TS2/16 in suspension is harder to reconcile with the previous study using a different activating mAb, 8A2 (Hotchin et al., 1993). 8A2 was not found to
supress suspension induced differentiation either as whole antibody or as Fab fragments. It is possible that the whole antibodies used in the TS2/16 experiments were simply crosslinking the β1 subunits and thereby activating signalling. However if this was the case the whole 8A2 antibodies would have been likely to be equally effective. It is clear from the results from Chapter 4 that there are a range of ways of stimulating a high affinity state (cations, antibodies, mutation) and that they do not necessarily have identical effects on adhesion (Ortlepp et al., 1995). It has also been shown that different functional forms of integrins are often found on cells (Chan and Hemler, 1993) and that different conformations of the extracellular domains can cause different signalling events (Miao et al., 2002). The TS2/16 epitope lies close to the site of the T188I mutation in the A domain of the β subunit. The epitope of 8A2 is not known. It is possible that the TS2/16 activation occurs in a way that is sufficiently different to that of 8A2 that the signalling that results differs. This would also explain why 9EG7, a anti hβ1 antibody that has been reported to activate integrins in certain situations also did not affect differentiation (Lenter et al., 1993; Bazzoni et al., 1995).

Modulation of integrin affinity has been shown to influence cell fate in other cell systems. Stem Cell Factor has been shown to cause a transient increase in integrin mediated cell adhesion in MO7E cells and that this correlated with mobilisation of haemopoetic cells from the bone marrow in vivo (Kovach et al., 1995). Myoblast development is also influenced by integrin signalling. Cell cycle withdrawal is suppressed by integrin signalling through FAK and ERK, and perturbation of integrin function results in faults in muscle differentiation (Sastry et al., 1999). Activation of α5β1 has been shown to disrupt myotube morphogensis although not
to prevent differentiation (Boettiger et al., 1995). My results show that modulation of integrin affinity can similarly affect the fate of keratinocytes.
Chapter 6

Screening for Further Integrin Mutations

6.1 Introduction

There have been many previous studies published comparing integrin expression patterns on different types of tumour, relating this to tumour grade or prognosis (Peltonen et al., 1989; Jones et al., 1993; Jones et al., 1997; Bagutti et al., 1998, Van Waes et al., 1991). To date there has been no previous report of integrin mutations having an effect in tumour development and no published studies of β1 integrin mutations or polymorphisms in tumours exist. In order to investigate the frequency of β1 mutations we screened other oral SCCs from the tumour collection of St Thomas's Hospital (London) for other integrin mutations.
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Screening for Further Mutations

6.2 Results

Frozen sections of 124 human SCCs were provided by Dr J. Jones, UMDS (London) from the histopathology collection of St. Thomas's hospital, London. For each of these tumour blocks a matched specimen of normal tissue or blood was available. Genomic DNA was extracted from the tumour samples by proteinase K digestion, phenol/chloroform extraction and ethanol/sodium acetate precipitation. Sequencing of all the exons of the β1 was not feasible due to limits on access to the equipment. Therefore I examined exons 3-7, the region surrounding the location of the T188I mutation. Each exon was analysed by SSCP analysis and where changes from the WT trace were found the presence of a mutation was confirmed by sequencing (example traces shown in Fig 6.1). SSCP and sequencing were carried out at the Cancer Research UK mutation detection facility (Leeds) by Dr Claire Taylor.

<table>
<thead>
<tr>
<th>Sequence Change</th>
<th>Amino Acid effect</th>
<th>Number of tumours</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS2-36 C&gt;A</td>
<td>N/A</td>
<td>43</td>
<td>34.9 %</td>
</tr>
<tr>
<td>459 C&gt;T</td>
<td>No change</td>
<td>29</td>
<td>23.5 %</td>
</tr>
<tr>
<td>471 C&gt;T</td>
<td>No change</td>
<td>2</td>
<td>1.6 %</td>
</tr>
<tr>
<td>776 C&gt;T</td>
<td>A239V</td>
<td>1</td>
<td>0.8 %</td>
</tr>
<tr>
<td>783 T&gt;C</td>
<td>No change</td>
<td>30</td>
<td>24.3 %</td>
</tr>
<tr>
<td>IVS6+8 C&gt;T</td>
<td>N/A</td>
<td>2</td>
<td>1.6 %</td>
</tr>
</tbody>
</table>

Table 6.1 Summary of sequence changes found. (IVS – Intron Variable Sequence)

Sections of tumour positive for the 471 C>T, 776 C>T A239V and IVS2-36 C>A variants were stained with antibodies to the β1 subunit. All tumours showed expression of the β1 subunit. In addition the 776 C>T A239V was stained with the HUTS21 activation reporter antibody (Fig 6.2). This was performed by Dr J. Jones,
UMDS (London). Strong staining for the β1 subunit was found in tumours carrying each mutation indicating that they did not affect the expression of the subunit. HUTS21 staining, indicating high affinity or ligand bound β1, in the 776 C>T A239V was only seen in the layers of tumour adjacent to a basement membrane (Bishop et al., 1998)

Normal tissue was analysed from patients expressing the 776 C>T A239V, IVS2-36 C>A and 471 C>T variants. All three sequence changes were found in normal tissue and were therefore not tumour acquired.
Fig 6.1: (A) Example of SSCP traces. Upper trace is sample 51 carrying the 776 C>T A239V Mutation. Lower trace is WT control. The extra peak (arrowhead) shows the presence of a sequence change, which was then determined by sequencing. (B) Sequencing results of the same samples. Upper trace shows 776 C>T A239V heterozygous mutation, lower trace is WT.
Fig 6.2: Staining of β1 subunit in SCC samples with the (A) 776 C>T A239V, (B) 471 C>T and (C) IVS2-36 C>A mutations. β1 expression is not perturbed by any of these sequence changes. (D) HUTS-21 staining of the SCC containing the 776 C>T A239V mutation. Bar = 500 μm.
6.3 Discussion

The results of this chapter suggest that, although a second mutation causing a change in amino acid sequence was found, amino acid mutations in the β1 integrin subunit in human SCC are not a common occurrence. The single amino acid change found, A239V, is in a highly conserved region of the A domain (see Fig 3.7). Based on the model presented in Chapter four (fig 4.7A) it lies in the α3 helix facing into the centre of the domain. While an alanine to valine change is a significant change in side chain character (a small -CH₃ group to a larger hydrophobic -CH(CH₃)₂) there appears to be sufficient space to accept the larger side chain without any clashes with the surrounding amino acids. The surrounding residues are already mostly hydrophobic so this change would not appear to alter the surrounding environment. As A239V was also found in normal tissue from this patient and is heterozygous in both tumour and patient I conclude that this mutation is not tumour derived and is unlikely to affect integrin function or the characteristics of the tumour.

Several changes in sequence that do not change the amino acid sequence were also found. Two of these were found at a high frequency (almost 1 in 4 tumours). While these will not affect integrin function this does show that the database WT sequence does not represent the only “normal” β1 subunit sequence.

Two sequence changes were found in the non-coding intron sequences between exons. Mutations in certain areas of the intron can lead to aberrant splicing of mRNA. However the IVS6+8 C>T mutation is not in a region thought to influence splicing. IVS2-36 C>A is in an area that could be part of a branch site consensus
sequence although this could only be confirmed experimentally ((Hertel et al., 1997) and personal communication C. Taylor). However tumours containing this mutation have normal β1 integrin staining making a change in splicing of the subunit seem unlikely. Three inherited human diseases are thought to be caused by mutations in branch sites. Familial hypercholesterolaemia is caused by an intron mutation 30 bp upstream of the start of exon 10 in the low density lipoprotein receptor gene (Webb et al., 1996). Fish eye disease, a condition involving clouding of the cornea and atherosclerosis, is caused by a mutation 22 bp upstream of exon 4 of the lecithin:cholesterol acyltransferase gene (Kuivenhoven et al., 1996; Li et al., 1998). Glycogenosis type II is an inherited disorder caused by mutations in intron 1 of the acid maltose gene (Raben et al., 1996).

In this work I examined only exons 3 to 7 as the primary aim was to find mutations that acted on the A domain in a similar manner to the T188I. It may be of use to screen other parts of the gene. Naturally occurring mutations in the stalk region have been shown to activate αIIbβ3 in GT (Fullard et al., 2001; Ruiz et al., 2001). The cytoplasmic domains have been shown play vital roles in the regulation of integrin activation so both of these areas may prove interesting.

In summary while the results from the previous chapter show alterations in integrin affinity can modulate tumour and keratinocyte function in vitro, this may be a rare event in SCC development.
Chapter 7

General Discussion

In this chapter I will summarise the main findings of this thesis and suggest directions for further research.

7.1 The T188I mutation activates the β1 subunit.

The T188I mutant integrin subunit causes an increased rate of binding to integrin ligands, both in cell free and cell based systems (Chapter 4). The degree of activation observed varies according to the type of assay, the partner α subunit and the ligand used. The T188I mutation lies on a small cysteine bridged structure previously shown to be important in regulation of integrin function.

In this thesis I have compared the binding of the WT integrin to the T188I mutant. In order to discover more about the importance of the loop on which T188I lies further A domain mutants could be made. Mutation of the threonine to other residues may show whether it is the presence of the isoleucine or absence of the threonine that is the cause of the activation. It would also be very interesting to look at the effect of complete substitution of the C-C loop for the equivalent region of other subunits, as in the work of Takagi. This is a substitution that has been shown to cause alterations in both signalling and ligand binding properties and thus has the potential to alter keratinocyte behaviour (Takagi et al., 1997).

Ultimately deeper understanding of the process of activation of the A domain and the β subunit as a whole will most likely require the solution of further crystal structures.
Specifically, solution of a β1 subunit containing integrin both in a ligand bound and ligand unbound state would provide a great deal of information as to the mechanics of integrin function. Solution of αvβ1 would also allow comparison of a dimer containing β1 with β3 and give more information on how structures are conserved between the various β subunits.

Two of the constructs in Levy et al. (Levy et al., 2000), containing deletions of regions of the cytoplasmic domain and were judged to be effectively non-functional as they lacked the ability to regulate differentiation or cell attachment. These subunits have intact ligand binding domains and there is some evidence that in CD8/β1 cytoplasmic domain chimera these deletions cause gain of function (personal communication D. Campbell). It may be worth expressing the full length cβ1 constructs in GD25 cells to determine if, although they cannot support cell attachment, their ligand binding domains are constitutively active.

7.2 Integrin activation affects different aspects of β1 null fibroblast behaviour.

Integrin activation causes changes in the behaviour of GD25 cells. Integrin activation due to T188I promotes attachment to cell attachment matrix coated surfaces and subsequent spreading (Fig 4.4, 5.1). T188I expression appears to have no effect on random cell migration across matrix coated surfaces when compared to WT (Fig 5.2) but T188I does not promote invasion of GD25 cells through Matrigel as effectively as the WT (Fig 5.3). The discrepancy between the observed effects on random migration and invasion may be resolvable with further experimentation. Palecek et
General Discussion

al. (Palecek et al., 1997) suggest that the optimum concentration for efficient cell migration is related to the affinity of the cell for the matrix. Therefore it is likely that the lack of observed difference between the migration rate of the T188I and WT expressing cells is due to the concentration of the matrix protein used in these experiments. It is possible that higher concentrations would show a reduction in the rate of movement of the T188I relative to the WT while lower concentrations may promote migration of the activated mutant. The lack of invasion of T188I expressing cells through Matrigel may be due to differences in MMP expression. This could be investigated by gelatin gel zymography to screen for changes in MMP activity induced by expression of the T188I or WT integrin subunits.

7.3 Integrin activation by expression of the T188I subunit or treatment with TS2/16 influences keratinocyte differentiation.

Infection with the T188I subunit does not induce differentiation of the SCC4 cell line, whereas infection with the WT does (Fig 5.6, 5.7). In a similar manner activation of the endogenous β1 subunit on normal keratinocytes reduced the number of differentiated cells (Fig 5.9, 5.10).

There are several further experiments that could be performed. Expression of the T188I subunit in normal keratinocytes should lead to a decrease in differentiated cells. This experiment has been attempted several times but no normal keratinocytes expressing T188I have been generated in four attempts. The retroviral producer cells
are capable of infecting other cell lines as GD25 and SCC lines were generated using these producers. Concentration of the T188I virus did not result in successful infection. The WT β1 subunit can be infected easily into normal keratinocytes.

Other β subunit cytoplasmic domain mutations have been shown to cause activation of the subunit. Expression of the equivalent β1 mutation in keratinocytes may provide more evidence of the effect of activation of the subunit. One of these, D764A, is thought to function by removal of a salt bridge between the α and β cytoplasmic tails (Hughes et al., 1996). I have attempted to express this subunit in human keratinocytes without success, as have other members of the laboratory.

It would also be interesting to look at the behaviour of SCC4 cells expressing the WT and T188I subunits in tumour formation experiments in vivo, for example by injection into nude mice. Blockade of integrins by peptide ligands has been shown to reduce the formation of tumours in the lungs of mice injected with mouse malignant melanoma (Humphries et al., 1986b). It would be interesting to see if an increase in integrin function could promote this process.

7.4 Mutation of the A domain of the β1 subunit is not a common event in human SCC.

I have found little evidence that mutations of the β1 subunit are common in human SCC. Including the T188I mutation only two mutations that result in amino acid mutations have been found in 125 tumours. While I have found only one other sequence change in the area surrounding T188I it is possible that if the entire gene was sequenced in the each patient other sequence changes may be found.
It may be informative to compare the frequency of the sequence changes found with their frequency in the general population as this may show whether any of the polymorphisms result in a predisposition to cancer. It may also be worth examining other types of tumour.

It would also be interesting to look for other events that could lead to changes in integrin activity. PKCα signalling has been implicated in integrin function (Ng et al., 1999). It may be useful to look for changes in this pathway that may influence integrin affinity.

7.5 General Discussion

The initial idea that a mutation that causes an increase in cell adhesion can affect a tumour in such a way as to make it less differentiated seems to be counter-intuitive as transformation of cell lines is normally associated with a loss of adhesion and a down regulation of ECM proteins (e.g. fibronectin) (Hynes et al., 1978; Hynes et al., 1979). However, the T188I mutation is not acting as a transforming mutation in the manner of an oncogene, rather it is acting on an already tumorigenic cell line to cause changes in the cells behaviour. In fact there may be a place for increased adhesion in the tumorigenic process. While fibronectin is initially downregulated in most tumours there is evidence that in cells isolated from metastases fibronectin and other ECM genes are upregulated (Clark et al., 2000). This production of matrix by the tumour cells allows the metastatic cells to ensure that they receive survival
signals from the matrix surrounding them. It is possible that activating integrin mutations could function in a similar manner, promoting the survival signals provided by the ECM present around the tumour. My work shows that it is not just the up or down regulation of integrin subunits that is relevant to tumour behaviour, mutation of integrins can also play an important role.

The existing model of regulation of differentiation in cultured human keratinocytes revolves around the ligation of the β1 integrin subunit being the prime source of a suppression of differentiation signal ((Adams and Watt, 1989),(Watt et al., 1993), (Levy et al., 2000)). The induction and commitment to terminal differentiation is triggered by the down regulation of the affinity of the β1 subunit and subsequent withdrawal from the surface (Adams and Watt, 1990) as this removes the "do not differentiate" signal. Proliferative potential is observed to correlate with integrin expression in the epidermis. Cells with the highest levels of β1 exhibit stem like behaviour and the lowest levels of differentiation (Jensen et al., 1999). Other results showing the importance of adhesion in regulating differentiation were provided by Zhu et al (Zhu et al., 1999). Reduction of cell/matrix adhesion by expression of a dominant negative CD8β1 chimera caused a reduction in the levels of MAPK signalling and resulted in premature differentiation. This affinity/differentiation correlation is seen again in cells taken from the K5β1 null mouse (Grose et al., 2002). In this situation mouse keratinocytes lacking β1 show greatly reduced adhesion to ECM components and show massively increased differentiation.

When added to the data presented in this thesis it is clear that the strength of matrix/cell binding is an important factor in the fate of the cell as regards to
differentiation (Fig 7.1). Whether this relationship is linear or whether a certain level of loss integrin ligation is required to trigger differentiation is a question that remains to be determined.

**Fig 7.1** The inverse relationship between adhesion and differentiation observed in keratinocytes. As adheriveness and ligand binding increases differentiation is seen to decrease. Whether the relationship is linear (A, solid line) or whether a critical level of ligand binding is required for suppression of differentiation (B, dotted) is unknown.
Appendix 1: Details of \( \beta \)1 mutation screen

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**Summary**

Number of discrete samples (after duplicates/failed samples excluded) – 124

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References


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collagen, and laminin in human skin. Variable expression in basal and squamous cell

Persad, S., Atwell, S., Gray, V., Mawji, N., Deng, J. T., Leung, D., Yan, J.,
B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase

92, 477-94.

function-associated molecule-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) mimicked


can be duplicated by small synthetic fragments of the molecule. *Nature*, 309, 30-3.

heterozygosity for missense (L156P) and nonsense (R554X) mutations in the beta4
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Pulkkinen, L., Rouan, F., Bruckner-Tuderman, L., Wallerstein, R., Garzon, M.,
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