SIGNALLING VIA INTEGRINS AND MITOGEN ACTIVATED PROTEIN KINASE IN HUMAN EPIDERMAL KERATINOCYTES

Ingo Haase

Faculty of Medicine, University College London
and
Keratinocyte Laboratory, Imperial Cancer Research Fund

Internal supervisor: Dr Richard Groves
External supervisor: Dr Fiona M. Watt

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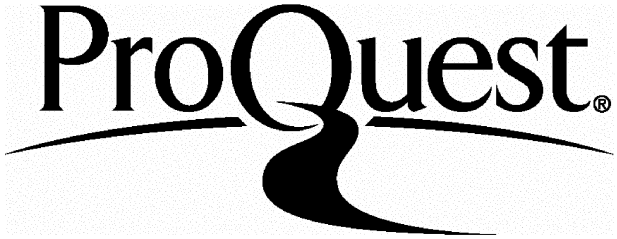

ABSTRACT

The structure of human epidermis is maintained by a balance between proliferation and differentiation of keratinocytes. The source of all epidermal keratinocytes is a population of epidermal stem cells in the basal layer of the epidermis that keeps proliferating through adult life and gives rise to committed daughters, transit amplifying cells. After a few rounds of divisions these daughter cells undergo a maturation process called terminal differentiation. Integrin receptors transduce signals from extracellular matrix molecules into the cell and thereby control both exit from the stem cell compartment and the onset of terminal differentiation. The aim of my PhD project was to investigate signalling pathways by which integrins exert these effects.

I found that integrins of the β1 subfamily can regulate the activity of mitogen activated protein kinase (MAPK) in keratinocytes. Detachment of keratinocytes from culture dishes prevented activation of MAPK. Partial inhibition of integrin function by introduction of a dominant inhibitory β1 integrin mutant caused reduced MAPK activity and decreased proliferative potential. This reduction in proliferative potential was due to inhibition of MAPK signalling since it could be restored by adhesion independent activation of MAPK. Inhibition of MAPK caused decreased proliferative potential and adhesiveness in the absence of the dominant negative integrin.

Modulation of MAPK activity in adherent and suspended keratinocytes and in organotypic cultures revealed that MAPK activity regulated proliferation, migration and terminal differentiation. In reconstituted epidermis in vitro MAPK activation caused histological changes typical of hyperproliferative skin diseases. Using nuclear localization of MAPK as a readout for its activity I found increased MAPK activity in psoriatic lesions of human skin and in healing skin wounds of mice. My results indicate a function of integrin dependent MAPK signalling in the regulation of keratinocyte differentiation and in the development of hyperproliferative skin conditions.

To Marina

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ABBREVIATIONS

AP	ammonium persulphate
BHI	brain heart infusion
bp	base pairs
BPAG	bullous pemphigoid antigen
BSA	bovine serum albumin
CD	cluster of differentiation antigen
CFE	colony foming efficiency
cfu	colony forming units
COL	collagen
DAB	3,3-diaminobenzedene tetrahydrochlodride
DCS	donor calf serum
DED	dead, de-epidermised dermis
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulphoxide
DTT	dithiolthreitol
EB	epidermolysis bullosa
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethyldiaminotetraacetic acid, disodium salt
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorter
FAD	Ham's F12 + adenine + DMEM
FAK	focal adhesion kinase
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FN	fibronectin
FSG	fish skin gelatin
H&E	haematoxylin and eosin staining
HICE	hydrocortisone, insulin, cholera toxin and EGF
HRP	horseradish peroxidase
IL	interleukin
ILK	β1 integrin-linked kinase
kDa	kilo Dalton
KL	keratinocyte extracellular matrix
LDH	lactate dehydrogenase
LN	laminin
MAPK	mitogen activated protein kinase
Mo MuLV	Moloney murine leukemia virus
MOPS	3-[N-Morpholino]propane-sulfonic acid
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS/Tween
pen/strep	penicillin/streptomycin
PI-3 kinase	phosphatidylinositol 3-kinase
PLL	poly-L-lysine

PMSF	phenylmethanesulphonyl fluoride
polyHEMA	poly(2-hydroxyethyl methacrylate)
PVDF	polyvinylidene fluoride
RNase	ribonucleic acid endonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TAE	Tris-acetate-EDTA buffer
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine

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PUBLICATIONS

The data presented in Chapter 3 are part of a publication with a joint first authorship for Alan Zhu and Ingo Haase:

Zhu, A. J., I. Haase and F. M. Watt (1999). "Signaling via beta1 integrins and mitogen-activated protein kinase determines human epidermal stem cell fate in vitro." Proc Natl Acad Sci U S A: 6728-33.

Another manuscript, describing most of the work presented in Chapters 4 and 5, has been submitted to the Journal of Clinical Investigation.

CHAPTER 1
INTRODUCTION

The subject of my PhD project is the function of integrin signalling to mitogen activated protein kinase in the epidermis. In this first chapter I will illustrate the structure and function of this tissue and I will summarise current knowledge of the mechanisms that regulate the epidermal homeostasis. I will also explain what is known about integrins and their functions in skin and give an overview of signal transduction through mitogen activated protein kinase. In the last section of this chapter I will introduce current hypothetical models for the explanation of epidermal wound healing and of skin conditions characterised by disturbances of epidermal proliferation and differentiation.

1.1.Structure and function of the epidermis

In order to form an independent biological unit every individual being needs mechanisms both to demarcate itself from the environment and other individuals and to maintain the integrity of its own organism. To ensure its survival in a potentially hostile environment this organism must also possess structures and mechanisms that protect it from harmful physical, chemical and biological influences of the outside world. In order to fulfil these vital requirements, evolution has produced a highly organised stratified epithelium that covers the body surface as epidermis and forms a mucosa lining the border areas to the gastrointestinal and urogenital tracts. Consisting of the greek prefix „epi“ and the noun „derma“ epidermis means the outermost layer of the skin that lines the whole surface of the body. In plants, insects and lower vertebrates the epidermis consists of a single layer of cells whereas higher vertebrates and humans possess a multilayered, stratified epidermis.

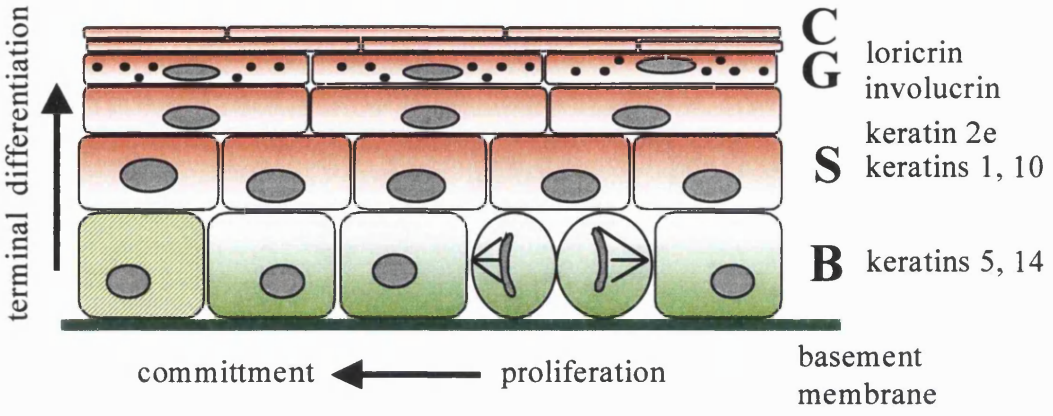


Figure 1.1: Schematic representation of the human epidermis

B: basal layer; S: spinous layer; G: granular layer; C: cornified layer.

The human epidermis consists of 15 to 20 layers of epidermal keratinocytes depending on the region of the body (Holbrook and Odland 1974). The most proximal layer of epidermal keratinocytes is in contact with the epidermal basement membrane, a layer of extracellular matrix proteins which interact to form a meshwork. This layer separates dermis from epidermis and both dermal fibroblasts and epidermal keratinocytes are thought to contribute to its assembly by secreting basement membrane components (Marinkovich et al. 1993). Extracellular matrix proteins that form the basement membrane include: collagen IV (Briggaman et al. 1991), laminins (Carter et al. 1991; Rousselle et al. 1991; Marinkovich et al. 1992), fibronectin (Hynes 1990), entactin/nidogen (Caughman et al. 1987), heparan sulphate and proteoglycans (Kjellen and Lindahl 1991). Co-culture experiments with bovine keratinocytes and human fibroblasts showed a basement membrane was only formed when both cell types were present: keratinocytes contributed collagen IV and VII, laminins 1, 5 and 6 and heparan sulphate as well as proteoglycans, and fibroblasts synthesised laminin 1 and collagens IV and VII (Marinkovich et al. 1993).

On their surface keratinocytes bear receptors that selectively bind to certain basement membrane components and interstitial extracellular matrix (ECM) molecules: the integrins, which are receptors for laminin, collagen and fibronectin, as well as CD44, a

receptor for hyaluronic acid, discoidin domain receptor, a receptor for collagen (Vogel et al. 1997) and the bullous pemphigoid antigen II (BPAG II) (Li et al. 1993).

The multilayered, stratified structure of human epidermis is achieved by a specialised cellular function that is unique to keratinocytes: terminal differentiation. The concept of terminal differentiation describes the migration of keratinocytes from the basal, most proximal layer of the epidermis or mucosa into more distal, suprabasal layers and the structural and biochemical changes that occur during this continuous outwards movement. The different layers of the epidermis are formed by keratinocytes in different stages of the differentiation process. These stages correspond to defined patterns of proliferative behaviour, protein expression and lipid synthesis. There are striking morphological changes that occur to differentiating keratinocytes: the cell body of suprabasal keratinocytes enlarges and increasingly flattens and cells in higher suprabasal strata produce high amounts of keratohyalin granules containing lipids that fill the intercellular spaces in the superficial layers of the epidermis. At the end point of the differentiation program keratinocytes lose their nucleus and become flat squames that form the cornified, outermost layer of the epidermis and are finally shed from the skin surface. Such morphological criteria form the basis for a subdivision of the epidermis into distinguishable layers (see Figure 1): the basal or germinative layer that contains the proliferative cell pool of the epidermis; the spinous layer in which suprabasal keratinocytes start to express cell-cell adhesion molecules giving them the appearance of cells with spines on their surface; the granular layer in which keratohyalin granules are produced; and the cornified layer that is formed of dead, cornified squames representing keratinocytes that have completed the differentiation program.

The high stability and resistance of human epidermis is mainly due to its special architecture that creates a three dimensional network of interconnected keratinocytes which express high amounts of various intermediate filaments of the keratin type. Keratin filaments are approximately 10 nm thick and are formed by heterodimers of different type I (basic) and type II (acidic) keratins. Keratin filaments insert at special sites of cell- matrix (hemidesmosomes) or cell-cell contacts (desmosomes) and run through the cell as tonofilaments or tonofibrils thus providing mechanical stability. The pattern of keratin expression depends on the differentiation state of a keratinocyte. Basal keratinocytes of stratifying epithelia, including basal epidermal cells, express the keratin pair 5 and 14 (Fuchs and Green 1980). As these cells leave the basal layer keratin

transcription and expression switches to a different pair of keratins: 1 and 10 (Fuchs and Green 1980). Filaments built from keratins 1 and 10 are able to aggregate and form tonofibrils which are thicker than the tonofilaments of basal keratinocytes and may therefore enhance mechanical strength of suprabasal cells (Montagna and Lobitz 1964). In more highly differentiated keratinocytes additional keratins are made: keratin 2e (Collin et al. 1992) and, restricted to palmar and plantar skin, keratin 9 (Fuchs and Green 1980).

Numerous mutations in keratin genes have been found that correspond to pathological phenotypes in humans. Mutations in the basal keratin pair 5/14 lead to cytolysis of basal epidermal keratinocytes and cause different types of Epidermolysis bullosa simplex, dependent on the localisation of the respective mutation within the molecule (Lane et al. 1992; Rugg et al. 1994), reviewed in (Fuchs 1997). By analogy, mutations in the suprabasal keratin pair 1/10 lead to a different, more mild disorder caused by the lysis of suprabasal keratinocytes: Epidermolytic hyperkeratosis (Cheng et al. 1992; Chipev et al. 1992; Compton et al. 1992; Rothnagel et al. 1992). Mutations in keratins 2e and 9 have also been found in humans; they cause milder forms of skin diseases: Ichthyosis bullosa Siemens and Epidermolytic palmoplantar keratoderma, respectively (Kremer et al. 1994; McLean et al. 1994; Rothnagel et al. 1994; Torchard et al. 1994). In addition, mutations in the genes for keratins 6, 16 and 17 have been described. Keratin 16 mutations have been found in focal non- epidermolytic palmoplantar keratoderma (Shamsher et al. 1995). Mutations in keratins 6, 16 and 17 also cause Pachyonychia congenita (Bowden et al. 1995; McLean et al. 1995).

In addition to the changes in keratin expression profiles, suprabasal keratinocytes synthesise glutamine- and lysine- rich proteins that are crosslinked in the granular layer to form a cornified envelope that encases differentiated keratinocytes. Such proteins include involucrin (Banks-Schlegel and Green 1981) which is produced early during differentiation, loricrin (Hohl et al. 1991) which is made in late spinous and in granular keratinocytes and cornifin, a small, proline rich protein (Marvin et al. 1992). Two other proteins, envoplakin and periplakin, are also components of the cornified envelope; in addition they belong to the plakin family of desmosomal proteins that are thought to anchor keratin filaments in the desmosomal plaque (Ruhrberg et al. 1996; Ruhrberg et al. 1997; DiColandrea et al. 2000). Crosslinking of these proteins occurs by formation

of epsilon-(gamma-glutamyl) lysine bonds, a reaction which is catalysed by the enzyme epidermal transglutaminase (Buxman and Wuepper 1976; Ogawa and Goldsmith 1976). Since expression of these proteins is part of the keratinocyte terminal differentiation program they are frequently used as markers of the differentiation states to which keratinocytes have progressed.

The cornified envelope provides mechanical stability and resistance to an epidermal keratinocyte. Furthermore proteins in the cornified envelope possess binding sites for lipids that are deposited in the intercellular spaces of the upper epidermis in order to form an unpermeable barrier (Nemes et al. 1999). The cornified envelope can also be a starting point for skin disease. Mutations in the loricrin gene and in epidermal type transglutaminase have been found to cause Vohwinkel's syndrome and Lamellar Ichthyosis, respectively (Huber et al. 1995; Russell et al. 1995; Maestrini et al. 1996). In paraneoplastic pemphigus autoantibodies against envoplakin, periplakin and other plakins are produced (Kiyokawa et al. 1998; Mahoney et al. 1998).

1.2. Epidermal stem cells

The basal epidermal layer contains cycling keratinocytes and most of the proliferation is, under physiological conditions, restricted to this compartment. Basal keratinocytes do not constitute a homogeneous population, however. The basal epidermal layer harbours at least two sorts of dividing keratinocyte: stem cells and transit amplifying cells (Potten 1974;1981). A current model for the structure of the proliferative compartment of the epidermis is depicted in Figure 1.2. Epidermal stem cells divide slowly and give rise to two different types of daughter cell populations: stem cells, in order to conserve the proliferative pool of the epidermis, and transit amplifying cells that cycle rapidly and thus generate more daughter cells in order to populate the epidermis. It is thought that the ratio between stem and transit amplifying cells is regulated on a population basis rather than on the level of individual cell divisions (Watt and Hogan 2000). In comparison to the classical model of asymmetric cell division this hypothesis increases the latitude of possible regulatory mechanisms and takes into consideration that gradual differences between the proliferative potentials of individual cells might exist rather than two distinct cell populations. Evidence for the existence of

stem cells has also been found in another stratifying tissue formed by keratinocytes, the esophageal epithelium, in which asymmetric cell divisions have been described (Seery and Watt 2000).

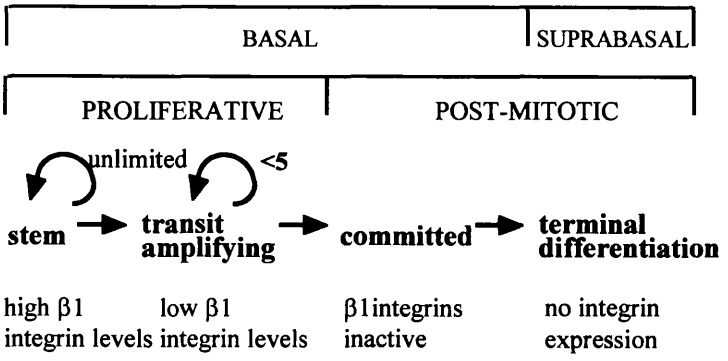


Figure 1.2: Keratinocyte subpopulations in human epidermis. Each subpopulation can be distinguished on the basis of β1 integrin expression or function. From (Zhu et al. 1999).

Epidermal stem cells have a high self renewal capacity and low probability of terminal differentiation whereas their daughters, transit amplifying cells are destined to differentiate after a few rounds of divisions (Hall and Watt 1989). The stem cells are therefore the source of all keratinocytes in the epidermis; this becomes most obvious in regenerating skin where renewal of the epidermis proceeds from only 10% of basal keratinocytes (Withers 1967; Potten and Hendry 1973). The proliferative capacity of keratinocytes can also be demonstrated in culture: keratinocytes with high proliferative potential (stem cells) form large, actively proliferating cell clones that can be serially passaged, whereas transit amplifying cells form smaller, abortive clones that contain a high number of differentiated cells (Barrandon and Green 1987; Jones and Watt 1993). Another typical feature of stem cells is thought to be scarcity of their cell divisions (Lajtha 1979; Watt and Hogan 2000). Therefore, by definition, a population of

keratinocytes should exist within the epidermis that cycles much less frequently than the majority of epidermal keratinocytes. These cells have indeed been identified by experiments in which mice were labelled in vivo with markers for DNA replication (Cotsarelis et al. 1990; Taylor et al. 2000). In these experiments certain keratinocytes were able to retain label for a long time (label retaining cells) as a result of infrequent S phase and cell division. These keratinocytes are preferentially localised in a defined portion of the hair follicle, the bulge region. In mice the bulge region keratinocytes give rise to progenitor cells that form the hair follicle and in a wound healing situation also contribute to the regeneration of interfollicular epidermis (Withers 1967; Potten 1973; Potten and Hendry 1973; Taylor et al. 2000). They are therefore thought to be epidermal stem cells from which keratinocytes of the hair follicle and, at least in regenerating epidermis, keratinocytes of the interfollicular epidermis can originate. Hair follicle keratinocytes with high proliferative potential have also been detected in human scalp hair follicles (Rochat et al. 1994). Human non scalp- skin, however, possesses by far fewer hair follicles than murine skin and there is evidence that stem cells must also exist in the interfollicular epidermis: keratinocytes grafted onto large burn wounds last there for many years without forming hair follicles (Compton et al. 1989).

A third hallmark of stem cells is their ability to differentiate into various types of progenitor cells: they are pluripotential. The origin of cells other than keratinocytes in the epidermis has not been thoroughly studied. It is well known, however, that both keratinocytes in the upper layers of the epidermis and keratinocytes that form the hair shaft are of the same origin. This has been demonstrated by experiments showing that interfollicular epidermis can be repopulated by hair follicle keratinocytes after injury (Al-Barwari and Potten 1976; Taylor et al. 2000) and that hair follicles are formed when interfollicular keratinocytes are brought into contact with hair papilla fibroblasts (Reynolds and Jahoda 1992). In addition, there is recent evidence that epidermal stem cells can give rise to sebaceous gland epithelia (Oshima et al. 2001). Whereas there is no direct evidence for the formation of sweat gland epithelia from epidermal stem cells it has been shown that wounded epidermis can be repopulated by the sweat apparatus (Miller et al. 1998).

1.3.Integrins

1.3.1. Structure and function

Integrins are adhesion receptors for extracellular matrix proteins. They are heterodimeric transmembrane cell surface proteins that consist of one alpha and one beta subunit. Whereas beta subunits can form functional dimers with different alpha chains creating thereby integrin receptors of different specificity, alpha subunits specifically bind to one particular beta chain with the exception of αv that can form dimers with 5 β subunits. For example, the β1 integrin chain can dimerize with an α2 subunit thus forming the α2β1 integrin, a receptor for collagens. Other possible combinations are α3β1, a receptor for laminin, and α5β1, a fibronectin receptor (Hynes 1987;1992).

The extracellular domains of the α and β subunits interact non-covalently to form a globular head structure at their N terminus with a diameter of 70 Å. This head domain is connected to two membrane spanning helix domains by a long, rigid stalk of approximately 100 Å (Erb et al. 1997). Short cytoplasmic domains comprise the C termini of α and β integrin chains with two tails extending into the lipid bilayer in a divalent cation-dependent fashion (Hayashi et al. 1990). The transmembrane and cytoplasmic domains are not required for α– β interactions as truncated integrins that lack the transmembrane and cytoplasmic domains can form functional αβ dimers (Dana et al. 1991). Both subunits contain multiple internal disulfide bridges that contribute to the overall three dimensional structure of the heterodimer (Calvete et al. 1989;1991).

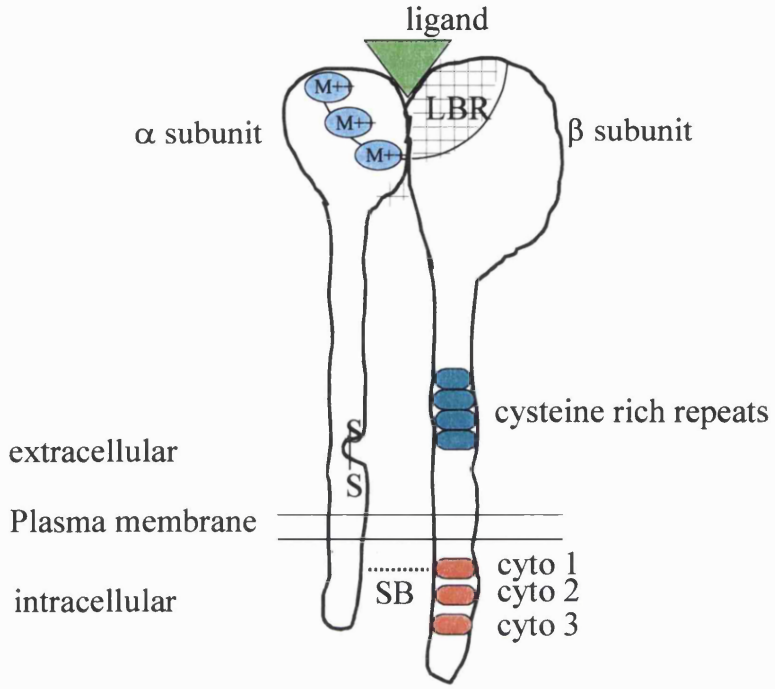


Figure 1.3: Schematic representation of an integrin heterodimer (modified from (Hynes 1992; Hughes et al. 1995)
LBR: ligand binding region; M++: cation binding region; SB: potential salt bridge

It is believed that the ligand binding site for extracellular matrix proteins is formed by the extracellular domains of both integrin α and β subunits (Loftus et al. 1994; Humphries 1996), see Figure 1.3. The α subunits contain divalent cation binding sites, the EF-hand-like motifs (D'Souza et al. 1991; Gulino et al. 1992). Divalent cations are essential for integrin function and the nature of the cation can affect both the affinity and specificity for ligands (Gailit and Ruoslahti 1988; Smith and Cheresh 1991). The inserted (I) domain present in several α integrin subunits contains a metal-ion-dependent adhesion site (MIDAS) motif (Lee et al. 1995). Three loops within this motif on the upper surface of the domain coordinate a metal ion and thus form a binding site for the GFOGER motif of collagen (Emsley et al. 2000).

1.3.2. Keratinocyte integrins

Keratinocytes can express certain integrin combinations and the pattern of available integrin receptors corresponds to different activation states. In resting, healthy,

unwounded epidermis integrins are only found in the basal cell layer. In this context the following set of integrin dimers can be found on the keratinocyte surface (ligands in brackets): α6β4 (laminin), α2β1 (collagen), α3β1 (laminin, collagen), αvβ5 (vitronectin) (Watt and Hertle 1994). In wounded and hyperproliferative epidermis additional integrins are expressed: α5β1 (fibronectin) and αvβ6 (Hertle et al. 1992; Zambruno et al. 1995; Hakkinen et al. 2000). There is also recent evidence for expression of α9 and β8 integrin chains by epidermal keratinocytes (Hakkinen et al. 1999; Stepp 1999).

Adhesion receptors for ECM on the surface of keratinocytes are organised in different ways. The α6β4 integrin localises to specialised structures on the basal surface of basal keratinocytes, the hemidesmosomes. Here the extracellular domains of the α6 and the β4 monomers are in contact with the basement membrane. Unlike β1 integrins the intracellular domain of the β4 integrin is more than 1000 amino acids long and connects to the intracellular keratin filament network (Borradori and Sonnenberg 1996; Rezniczek et al. 1998). Adhesion of basal keratinocytes to laminin via the α6β4 integrin is of major importance for the anchorage of the epidermis to the underlying tissue: mutations in either the receptor (Dowling et al. 1996; Georges-Labouesse et al. 1996; van der Neut et al. 1996) or the ligand (Pulkkinen et al. 1994; Kivirikko et al. 1995) lead to life threatening blistering skin disease which in humans has been called junctional Epidermolysis bullosa.

α6β4 contributes to a high degree to the adhesion strength of the epidermis; there are, however, other adhesion receptors localised in hemidesmosomes contributing to the attachment of epidermal cells, e.g. the bullous pemphigoid antigen II (BPAG II) which is a transmembrane adhesion receptor (Li et al. 1993; Masunaga et al. 1997) and thought to be responsible for the assembly of hemidesmosomes (Fuchs et al. 1997). Mutations in BPAG II lead to a variant of junctional Epidermolysis bullosa (McGrath et al. 1995; McGrath et al. 1996). Apart from mutations in genes involved in epidermal adhesion the function of adhesion receptors can be impaired by autoimmune reactions. In Bullous pemphigoid, a potentially life threatening autoimmune condition autoantibodies against BPAG I and II are produced. BPAG I (bullous pemphigoid antigen I) is an intracellular protein that mediates the insertion of keratin filaments in the hemidesmosomal plaque (Guo et al. 1995). Binding of autoantibodies to these proteins finally results in loss of adhesion of the basal keratinocyte layer to the basement membrane and provokes an

inflammatory reaction. As a consequence blisters form on the skin surface, the roof of which consists of full thickness epidermis. The mechanisms that lead to the destruction of the hemidesmosomal structure and detachment of the epidermis are incompletely understood.

The second large group of keratinocyte adhesion receptors of the integrin class is formed by the β1 integrins. In keratinocytes the β1 subunit pairs with different alpha subunits to form adhesion receptors of different specificity and function. In contrast to the α6β4 integrin which is concentrated at the basal cell surface that is in contact with the basement membrane, all β1 integrins are equally distributed over all parts of the membrane (Hertle et al. 1991). From knock out experiments it is known that both α6β4 and β1 integrins contribute to the attachment of the epidermis to its basement membrane (Raghavan et al. 2000). There are additional functions for β1 integrins which are so far incompletely understood. One function is the regulation of collagenase expression in keratinocytes by α2β1 (Pilcher et al. 1997), another seems to be the organisation of components of the epidermal basement membrane (Raghavan et al. 2000). In addition, keratinocyte β1 integrins seem to be important mediators of dermal-epidermal interactions since epidermis specific β1 deficiency in knock out mice results in severe inflammation and fibrosis within the dermis (Brakebusch et al. 2000). These results provide very important clues about the role of β1 integrins as matrix receptors in skin physiology; they cannot explain, however, why these receptors are not only expressed on the basal membrane surface but also on lateral and apical parts of the cell membrane. Since these are the sites where keratinocytes are connected to neighbouring cells it has been hypothesised that β1 integrins may not only mediate cell- matrix but also cell- cell adhesion (Larjava et al. 1990; Symington et al. 1993). Until now the evidence for this is merely circumstantial and in view of existing evidence against this function (Tenchini et al. 1993) no final conclusions about the function of integrins in keratinocyte cell- cell adhesion can be drawn yet. Another unsolved question is why β1 integrins are expressed in suprabasal layers of hyperproliferating epidermis (Hertle et al. 1992) since the known integrin ligands have not been found in this location. One could argue that here β1 integrins fulfil a so far unknown and possibly ligand independent signalling function; this hypothesis is supported by the finding that forced expression of β1 integrins in suprabasal layers of murine epidermis causes the development of a hyperproliferative skin disease (Carroll et al. 1995).

An important function of β1 integrins in basal keratinocytes seems to be the regulation of events determining the onset of terminal differentiation. If keratinocytes in culture are deprived of their contact to ECM proteins, e.g. in suspension culture, they enter the program of terminal differentiation. This differentiation process can be delayed by addition of a ligand for the α5β1 integrin, fibronectin, at a concentration of 100 μg/ml or fibronectin at 25 μg/ml in combination with two other β1 integrin ligands, collagen IV and laminin (Adams and Watt 1989; Watt et al. 1993). All these ligands occur naturally in the epidermal basement membrane and fibronectin is more abundant in the provisional matrix of healing skin wounds. Terminal differentiation can also be inhibited by addition of antibodies against the β1 integrin subunit or of a combination of antibodies against the α2, α3 and α5 chains to the suspension medium (Adams and Watt 1989; Watt et al. 1993). These data suggest that engagement of β1 integrins in keratinocytes generates signals that prevent the onset of the differentiation program. These signals seem to be effective only when present at an early time point when keratinocytes undergo the first step of initiation of differentiation, commitment (Adams and Watt 1989), see Figure 1.2. Furthermore, signalling pathways that prevent keratinocytes from commitment to differentiation are distinct from pathways involved in β1 integrin-mediated adhesion (Levy et al. 2000). The crucial precondition for generation of such signals is obviously binding of a ligand to the integrin that has to be in an active, ligand binding conformation (Levy et al. 2000). In vitro data show that the loss of this active conformation precedes the loss of integrin receptors from the keratinocyte surface (Adams and Watt 1990) suggesting that integrin binding to ligand can be regulated by additional mechanisms which could be important determinants of differentiation onset. Though there is convincing evidence for a role of β1 integrins in the regulation of differentiation onset, in a recent paper Kaur & Li propose that this function might be solely attributed to the α6β4 integrin (Kaur and Li 2000).

A special feature about the expression profile of β1 integrins in human epidermis is the fact that surface levels of β1 are not homogeneously distributed throughout the basal layer but that there are areas in which keratinocytes have high surface levels (integrin bright) and regions in which integrin levels are relatively low (integrin dull). In non-palm skin the integrin bright patches are situated on top of the dermal papillae whereas the cells in the rete ridges are integrin dull. The dermal papillae are the parts of the dermis that are closest to the skin surface, the rete ridges are the regions of the

epidermis that project deepest into the underlying connective tissue. There is evidence from in vitro experiments that integrin bright keratinocytes have higher proliferative potential than integrin dull cells (Jones and Watt 1993; Jones et al. 1995). In vivo this population of integrin bright cells cycles less frequently than integrin dull cells (Jensen et al. 1999) and therefore corresponds most likely to the epidermal stem cell compartment.

1.3.3. Integrin signalling

In the past 10 years a major field of research interest has been the question of how integrins transduce information from the cell environment into intracellular signals. In contrast to the linear signalling cascades that were proposed several years ago (Schlaepfer et al. 1994) integrin signalling seems to involve the formation of aggregates of multiple, spatially well organised signalling molecules which in cultured cells in vitro appear as focal contacts or focal adhesions (Burridge et al. 1988). Focal adhesions are complexes of transmembrane and intracellular proteins at sites of cell- matrix contact. They are highly dynamic and undergo constant destruction and reassembly when a cell moves. Recent results suggest that „focal adhesion" might be a generic term designating cell matrix contacts of different morphology and function (Katz et al. 2000).

An early event in the formation of a focal adhesion and signal transduction from it seems to be clustering of integrin receptors (Kornberg et al. 1992) which is a function of the cytoplasmic domain of the integrin beta chain (LaFlamme et al. 1992; LaFlamme et al. 1994). Oligomerisation is thought to occur through traction of individual molecules by actin cables that directly or indirectly bind to integrin cytoplasmic domains. Upon dimerisation and oligomerisation of integrins other molecules are recruited into the adhesion complex. Since, unlike many growth factor receptors, integrins do not possess intrinsic tyrosine kinase activity in their intracellular domain it is likely that the primary function of the focal adhesion in signal transduction is the spatial organisation of signalling molecules by providing a scaffold that facilitates contact of these proteins to their upstream and downstream binding partners. This then enables signal transduction along established pathways. One example is the activation of EGF receptor mediated signalling by β1 integrins (Moro et al. 1998; Wang et al. 1998; Yu et al. 2000).

In the process of signal transduction integrin beta chains seem to play an important role. They are highly conserved proteins, especially the intracellular domains which, for example in the case of the β1 chain show 100% sequence conservation on the amino acid level between Xenopus, chicken and humans (Tamkun et al. 1986; Argraves et al. 1987; DeSimone and Hynes 1988). In comparison to other cell surface receptors, for example for growth factors and cytokines, the intracellular domain of β1 integrins is relatively small with a total length of only 46 amino acids (Reszka et al. 1992). Within the intracellular β1 domain there are three subdomains (cyto 1-3; see Figure 1.3) which have defined roles in the binding of intracellular proteins that mediate or modulate integrin function (Reszka et al. 1992). Different research groups have mapped binding sites for various intracellular proteins in the β1 cytoplasmic tail. These proteins are: talin, filamin, α actinin, which bind also actin, the adapter protein paxillin and the protein ICAP-1 (integrin-cytoplasmic-domain-associated protein 1), which both seem to regulate cell spreading, CD98 which is a transmembrane protein possibly regulating the integrin activation status (Fenczik et al. 1997), and Rack1 (receptor for activated protein kinase C) whose functions have not been clarified yet (Liliental and Chang 1998). In contrast to these proteins mentioned there are two binding partners of β1 integrin tails that have kinase activity: Focal adhesion kinase (FAK) (Kornberg et al. 1992; Zachary and Rozengurt 1992) and integrin linked kinase (ILK) (Hannigan et al. 1996). Though it is believed that all these proteins take part in signalling pathways that either originate from or target the β integrin chains their individual functions and impact on cellular behaviour are until now incompletely understood.

Integrin binding proteins that can also associate with actin are thought to be responsible for the connection of the actin cytoskeleton to ECM via integrins. Specific disruption of the talin gene results in a failure of embryonic stem cells to assembly filamentous actin (F-actin) and compromises focal adhesion formation (Priddle et al. 1998).

Filamin can be found along actin filaments and also in focal adhesions (Pavalko et al. 1989). It has also been shown to bind directly to the β1 integrin cytoplasmic domain (Loo et al. 1998). From reconstitution experiments in tumour cells functions for filamin in the regulation of cell shape and migration have been identified (Cunningham et al. 1992) and mutations in filamin have been implicated in the pathogenesis of the human disease Periventricular heterotopia (Fox et al. 1998). In cultured cells α actinin localises

to stress fibres and focal adhesions and competitive inhibition of its binding to integrin tails disturbs their formation (Pavalko and Burridge 1991).

Paxillin, which binds both to β integrin tails and the α4 tail (Schaller et al. 1995; Chen et al. 2000; Liu and Ginsberg 2000), can inhibit cell spreading and enhance migration (Liu et al. 1999) and couples integrins to other intracellular signalling proteins (Turner et al. 1999). ICAP-1 specifically binds to the membrane distal region of the $\beta 1_A$ tail and is phosphorylated in response to integrin binding to fibronectin (Chang et al. 1997; Zhang and Hemler 1999). It also contains phosphorylation sites for different protein kinases, e.g. calcium/calmodulin- dependent protein kinase II and this interaction seems to be important for the regulation of cell spreading and migration (Bouvard et al. 1998). CD98 is a transmembrane protein involved in the regulation of amino acid transport and integrin activation. It can stimulate integrin mediated adhesion upon crosslinking with specific antibodies in vitro (Fenczik et al. 1997).

Proteins that have been reported to bind to β integrins tails and possess enzymatic activities are focal adhesion kinase (FAK), integrin linked kinase (ILK) and Src. For FAK direct binding to the intracellular domain of the β chain is not the only mechanism of association with integrins: there is evidence that FAK can also bind to talin and paxillin which then in turn can associate with integrins (Schaller et al. 1995; Chen et al. 2000). Disruption of the FAK gene in knock out mice does not prevent formation of focal adhesions (Ilic et al. 1995) and phosphorylation of FAK upon integrin engagement is not dependent on focal adhesion formation (Lyman et al. 1997). Cells isolated from FAK knock out mice show, however, impairment of cytoskeletal reorganisation and motility (Ilic et al. 1995; Richardson and Parsons 1996; Cary et al. 1998). Furthermore, FAK has been implicated in an anti- apoptotic pathway since inhibition of its function can stimulate apoptosis (Hungerford et al. 1996; Xu et al. 1996) and expression of an activated version of the molecule is able to prevent anoikis (Frisch et al. 1996). It is possible that this function is mediated by signalling of FAK to PI-3-kinase (Chen and Guan 1994) which is known to regulate apoptosis.

Integrin linked kinase (ILK) was identified in a two hybrid screen for β1 integrin cytoplasmic domain binding proteins (Hannigan et al. 1996). It is a serine-threonine kinase that can phosphorylate the cytoplasmic domain of the β1 integrin subunit and is capable of autophosphorylation. Other targets of ILK activity are PI-3-kinase and glycogen synthase kinase 3 (GSK3) (Delcommenne et al. 1998). ILK localises to focal

adhesions where it interacts with integrins and the adapter protein PINCH which in turn can bind to Nck-2, an adapter protein involved in growth factor signalling cascades (Tu et al. 1998; Tu et al. 1999). The interaction of ILK with several important signalling proteins suggests a central role for this kinase in the co- ordination of signals from integrins and growth factor receptors. Since its discovery ILK has been found to be involved in the regulation of anchorage dependent cell growth, apoptosis, invasion and tumorigenicity. Overexpression of ILK enables epithelial cells to grow in soft agar (Hannigan et al. 1996), suppresses anoikis (Radeva et al. 1997) and increases cell invasion (Novak et al. 1998), possibly by inducing epithelial-mesenchymal transition and loss of cell- cell adhesion (Novak et al. 1998; Wu et al. 1998). Reduced expression of the cell-cell adhesion molecule E- cadherin could be one molecular mechanism responsible for increased invasiveness. ILK overexpression also stimulates nuclear translocation of β catenin which in keratinocytes can regulate proliferative potential (Zhu and Watt 1999) and ILK could therefore be a molecule that mediates crosstalk between integrins, cadherins and the wnt signalling pathway. The suggestion that ILK is selectively expressed in the basal epidermal layer (Xie et al. 1998) is in agreement with a role for ILK in proliferating keratinocytes.

Another kinase that can interact with integrins and functions in integrin mediated signalling pathways is the transforming gene product of the Rous Sarcoma Virus, Src. Upon integrin crosslinking Src translocates to focal adhesions (Kaplan et al. 1995) and this translocation is directed through its SH3 domain and depends on actin cytoskeletal rearrangements (Fincham et al. 2000). In addition, Src activity is apparently required to induce actin cytoskeletal changes since fibroblasts from src -/- mice show a spreading defect (Kaplan et al. 1995) and kinase dead Src acts as a dominant inhibitor of actin remodeling (Fincham et al. 2000). In this context it is interesting that in src -/- fibroblasts integrin mediated activation of MAPK is severely attenuated (Schlaepfer et al. 1997), indicating a function for Src in this signalling pathway.

Whereas β integrin subunits seem to be involved in several signal transduction pathways integrin alpha subunits determine the substrate specificity of the respective integrin dimer and modulate β integrin function. For a long time it was thought that this was their only function, especially because the intracellular domains of α chains are even shorter than those of beta chains. This view has recently been challenged by data

indicating that the transmembrane domain is able to connect to signalling networks in the cell via binding of adapter proteins. In particular, caveolin I is thought to bind directly to the transmembrane portion of α subunits and then connect via the tyrosine kinase Fyn to the adapter protein Shc. Shc associates with the adapter Grb2 and connects this pathway to Ras and subsequently the classical MAP kinase (MAPK) cascade (Wary et al. 1996; Wary et al. 1998). It is thought that activation of MAPK leads to specific cellular responses like cell cycle progression (Mainiero et al. 1997) and thus controls proliferation in keratinocytes. Binding of adapter proteins to the intracellular portions of alpha subunits is apparently highly specific since differential signal transduction has been reported even for different alpha 6 integrin subtypes (Shaw et al. 1995).

1.4. Mitogen activated protein kinase

The MAP kinases comprise a family of signal transducing molecules that are activated by receptors for extracellular mediators like growth factors, cytokines and extracellular matrix molecules. This family is still expanding; all family members are, however, regulated by a characteristic cascade of upstream regulating kinases. The complete MAP kinase cascade consists of a module of three protein kinases acting in a hierarchical order: the MAP kinases (MAPK) which are activated by MAP kinase kinases (MAPKK) through phosphorylation of conserved threonines and tyrosines, within a TXY motif (Canagarajah et al. 1997) and the MAP kinase kinase kinases (MAPKKK) which regulate MAP kinase kinase activity. Phosphorylation both of tyrosine and threonine in MAPK are catalysed by MAPKK which is a dual specific kinase. The principle of MAPK cascades is conserved from yeast to mammals and fundamental cellular functions like proliferation, migration, apoptosis, differentiation and hormone secretion are regulated by MAPK pathways (Marshall 1994;1999).

The most thoroughly studied MAPK cascade is the classical MAPK cascade or ERK cascade. In this system the MAPK is ERK which stands for extracellularly regulated kinase. ERK exists in two isoforms: ERK1 with a molecular weight of 44 kD and ERK2 with 42 kD (Seger et al. 1991). The activity of ERKs is regulated by a MAPKK: MAPKK1 which is also called MEK1. Upon activation MAPKK1 phosphorylates ERKs

at threonine 183 and tyrosine 185 (Rossomando et al. 1992) thus stimulating their kinase activity. This phosphorylation can be visualised using phosphorylation specific antibodies which only detect ERKs when they are phosphorylated at these two residues. There is more than one upstream regulating kinase for MAPKK1, one of them is the MAPKKK Raf1. Raf1 phosphorylates MAPKK1 at two serine residues: Ser 217 and Ser 221 (Alessi et al. 1994). This phosphorylation stimulates kinase activity of MAPKK1.

Raf1 is part of the signalling cascade of the epidermal growth factor receptor (Marshall 1999). It is thought that Raf1 is bound by GTP loaded Ras and thus translocated to the plasma membrane where it is fully activated through phosphorylation by an unknown kinase (Marshall 1999). Knowledge about the sites within MAPKK1 phosphorylated by Raf1 has been used to create mutated versions of MAPKK1 in which these sites are modified. An exchange of both serine residues at 217 and 221 against glutamic acid mimics phosphorylation of MAPKK1 by Raf1 and thus creates a constitutively active molecule which phosphorylates MAPK independently of Raf1 activation. Conversely, mutation of serine 217 to alanine blocks this site from phosphorylation by Raf and thus creates a dominant negative MAPKK1 (Cowley et al. 1994).

The MAPK pathway acts in the transduction of signals from the extracellular milieu into the cell thus enabling the cell to adapt to changing extracellular conditions. Such signals can be mediated by growth factors (e.g. EGF) (Gotoh et al. 1990; Takishima et al. 1991), cytokines (e.g. IL-1) (Saklatvala et al. 1993) and cell-matrix and cell- cell adhesion molecules (e.g. integrins, ICAM-1) (Holland and Owens 1997).

At least three mechanisms have been suggested by which integrins can activate MAPK. In the first model, integrin engagement leads to FAK autophosphorylation through an integrin β subunit cytoplasmic domain, generating a binding site on FAK for the SH2 domain of Src. Src then phosphorylates FAK, which results in the binding of $p130^{CAS}$ and Grb2 to FAK. This then allows membrane localisation of SOS, a guanine nucleotide exchange factor, which in turn promotes GTP loading and activation of Ras. Activated Ras binds and activates Raf-1, which in turn phosphorylates and activates MAPKK. MAPKK then activates MAPK (Clark and Brugge 1995; Vuori et al. 1996; Clark and Hynes 1997). Other results show that there must exist integrin mediated signalling to MAPK without FAK involvement. Overexpression of a constitutively activated FAK in epithelial cells fails to activate MAPK (Frisch et al. 1996) while

overexpression of a dominant negative FAK in fibroblasts blocks FAK activation, but is unable to block MAPK activation (Lin et al. 1997).

An alternative pathway which bypasses FAK is also suggested by experiments showing that caveolin 1, a transmembrane protein can associate with the transmembrane domains of α integrin subunits (Wary et al. 1996; Wary et al. 1998). This leads to subsequent recruitment of the adapter protein Shc into the complex which then extends the signalling cascade to GRB2, SOS and Ras.

A third hypothetical mechanism of MAPK activation by integrins arises from work of (Chen et al. 1996). Membrane localisation of Raf is thought to be caused by integrin engagement and seems to be the key event that triggers signalling (Howe et al. 1998). This pathway is therefore independent of Ras. Similar mechanisms have been implicated in a permissive role of integrins in growth factor signalling: growth factor signals that activate Ras cannot be transduced to signalling molecules further downstream without integrin engagement. The point at which integrins synergise with growth factor signalling cascades has been identified to be Raf (Lin et al. 1997) or MAPKK (Renshaw et al. 1997). In suspended cells growth factor induced MAPK phosphorylation is weak and enhances greatly when cells are brought into contact with ECM proteins. Conversely, when adherent cells kept under conditions of virtual growth factor absence are treated with growth factor containing medium this leads to a strong stimulation of MAPK activity. In fibroblasts devoid of cell- matrix contact growth factor treatment leads to a stimulation of Ras, but not MAPKK1. MAPKK1 is, however, activated when these cells are plated onto fibronectin, the ligand for the α5β1 integrin. This means that the point of crosstalk is situated between Ras and MAPKK1 (Renshaw et al. 1997). This work provides a molecular basis to explain synergistic signalling of integrins and growth factors and is therefore of high biological significance.

Cellular functions of the MAPK cascade are diverse and dependent on the cell type. Several target proteins in the cytoplasm and in the nucleus are phosphorylated by MAPK. Cytoplasmic targets include phospholipase A_2 (PLA_2) (Clark and Hynes 1996) and myosin light chain kinase (MLCK) (Klemke et al. 1997). A significant part of cellular MAPK translocates to the cell nucleus after activation where it targets transcription factors like the AP-1 complex, ELK-1 and Ets (Marshall 1999). Both phosphorylation and translocation to the cell nucleus can therefore be used as activation markers of the MAPK cascade (Lenormand et al. 1998; Brunet et al. 1999).

Whereas MAPK activity seems to stimulate proliferation in many cell types (Brunet et al. 1994; Mansour et al. 1994; Pages et al. 1994; Mainiero et al. 1997; Sebolt-Leopold et al. 1999) its role in the regulation of differentiation is more variable: in MDCK cells features of differentiation are suppressed (Schramek et al. 1997) whereas in PC12 cells MAPK stimulates neurite outgrowth, a marker of neuronal differentiation (Cowley et al. 1994). Interestingly, in skeletal muscle cells MAPK activity inhibits early stages of differentiation but stimulates later phases (Bennett and Tonks 1997). In mammalian cell lines constitutive activation of the MAPK pathway is sufficient for cell transformation (Brunet et al. 1994; Cowley et al. 1994; Mansour et al. 1994).

1.5.Epidermal regulation

1.5.1. Keratinocyte proliferation

The requirement for tight regulation of proliferation within the epidermis arises from its protective function: the epidermis covers the outer surface of the body and is therefore exposed to a multitude of potentially damaging influences from the environment. This requires that there is always a sufficient number of cells present in the epidermis in order to give it a certain thickness that is necessary for optimal protection. In addition, if injury has occurred proliferation must be tuned to the maximum quickly in order to close the defect and regenerate the protective shield. On the other hand, proliferation has to be restrained so that a balance between differentiation and proliferation within the epidermis is achieved. Inefficacy of the mechanisms that restrain keratinocyte growth could be involved in the pathogenesis of hyperproliferative skin diseases, such as Psoriasis, and it is probably not a coincidence that Psoriasis and epidermal wound healing have many common features.

In theory, there are at least two ways of regulating proliferation within the epidermis. One would be to influence cell cycle time: fast cycling keratinocytes can produce more daughter cells. Although there are published data suggesting that cell cycle times of keratinocytes are regulated in culture (Dover and Potten 1983) we do not know whether this actually occurs in vivo and, if it occurs, under which circumstances. A stimulus decreasing cell cycle time would have to affect epidermal stem cells and transit

amplifying cells in the same way since transit amplifying cells have a limited proliferative potential, and a faster cell cycle only of this subpopulation would then lead to a deficiency in numbers of keratinocytes after a relatively short time. This, however, has not been observed in vivo. In addition, there would have to be mechanisms that steer stem cell differentiation into the interfollicular keratinocyte differentiation pathway since de novo hair follicle formation or hyperplasia of sebaceous glands have not been reported so far in the context of hyperproliferative skin diseases though these are very common.

Another, probably more powerful mechanism to regulate cell number in the epidermis would be to influence proliferative potential of transit amplifying cells. If a transit amplifying cell which has been born by division of an epidermal stem cell has a proliferative potential of three rounds of divisions this would result in 8 (2^3) postmitotic keratinocytes which then can start the program of terminal differentiation. If the proliferative potential of these transit amplifying cells could be increased by just one more round of division the number of resulting postmitotic cells would double (2^4).

One hallmark of basal, proliferating keratinocytes is the fact that they are in contact with a layer consisting of different ECM proteins that form the epidermal basement membrane. The requirement of ECM contact for keratinocyte proliferation is most convincingly demonstrated by the fact that upon detachment of cells from their substratum all proliferation stops (Adams and Watt 1989). Keratinocytes are then postmitotic and after a lag of 5 hours enter the program of terminal differentiation (Hotchin et al. 1993). Since detachment of cells releases all receptors for ECM on the keratinocyte surface from their ligands this experiment does not answer the question whether certain receptors provide specific contributions to keratinocyte proliferation. This problem has been tackled by several research groups conducting experiments in which the function of different adhesion receptors was inhibited specifically.

Partial and complete deletions of the gene for the β4 integrin chain have led to contradicting conclusions about the role of the laminin receptor α6β4 in keratinocyte proliferation. In an in vitro study with cultured keratinocytes (Mainiero et al. 1997) have shown that attachment of keratinocytes to ECM via α6β4 supports the activation of MAPK and concluded upon a role of α6β4 signalling to MAPK in the regulation of keratinocyte proliferation. Furthermore, a deletion of the β4 cytoplasmic domain was reported to cause keratinocyte detachment and inhibition of keratinocyte proliferation

(Murgia et al. 1998). In contrast, complete ablation of β4 by two different groups also led to regional detachment of the basal epidermal layer, but not to a marked inhibition of proliferation in the attached keratinocytes (Dowling et al. 1996; van der Neut et al. 1996). The role of α6β4 in the regulation of epidermal proliferation is thus not entirely clear.

For β1 integrins a role in the regulation of cell proliferation in general and of keratinocyte proliferation is somewhat more established. If the β1 chain is ablated specifically in murine epidermis by conditional targeted deletion, a thinner epidermis with fewer proliferating basal keratinocytes as compared to the littermate controls is formed (Raghavan et al. 2000).This is in agreement with earlier results showing that high proliferative potential of keratinocytes correlates with a high surface level of β1 (but not α6β4) integrins (Jones and Watt 1993). Another group, using also conditional knock out technique, has come to different results: murine epidermis null for the β1 integrin gene was thickened and showed a higher number of cycling keratinocytes as assessed by staining with an antibody against the Ki67 proliferation associated antigen (Brakebusch et al. 2000). A major difference between both phenotypes seems to be the presence of an inflammatory infiltrate in the dermis of latter mice which has not been described in the first ones. It is possible that these inflammatory cells by release of growth stimulatory cytokines stimulate epidermal proliferation in a paracrine manner (Szabowski et al. 2000).

A function in the regulation of epidermal proliferation has also been assigned to CD44, a receptor for hyaluronic acid which is very abundant on the keratinocyte surface (Hudson et al. 1995). In transgenic mice prevention of CD44 synthesis by expression of a specific antisense oligonucleotide under the control of the keratin 5 promoter led to an inhibition of keratinocyte proliferation (Kaya et al. 1997).

Other proteins that are expressed in basal epidermal cells and regulate their proliferation are the surface receptors for growth factors that can either be produced in an autocrine way by keratinocytes or in a paracrine fashion, e.g. by dermal fibroblasts. These receptors are the EGF receptor, which binds both epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) (Taylor et al. 1985; Kramer et al. 1994), and receptors for insulin like growth factor I (IGF-1) (Tavakkol et al. 1992), keratinocyte growth factor (KGF) (Werner et al. 1994), Leptin (Frank et al. 2000) and certain interleukins (Turksen et al. 1992; Rauschmayr et al. 1997).

Among these growth factors the most extensively studied are TGFα and KGF. Apart from being strong mitogens these factors have other functions. They are, for example, motogens for keratinocytes (Barrandon and Green 1987; Turksen et al. 1991), a function of critical importance in epidermal wound healing where keratinocytes have to migrate in order to cover the wound surface. Whereas TGFα is produced by keratinocytes KGF is secreted from dermal fibroblasts and this secretion is dramatically increased during wound healing (Werner et al. 1992). Both factors stimulate keratinocyte proliferation but seem to exert different effects on differentiation within the epidermis. TGFα overexpressed in the epidermis of mice leads to a proportional thickening whereas in KGF overexpressing mice the ratio of differentiation stages of epidermal keratinocytes is shifted towards more undifferentiated forms (Vassar and Fuchs 1991; Guo et al. 1993). Lack of KGF in the skin of mice does not impair keratinocyte growth, but leads to disturbed hair follicle formation (Guo et al. 1996).

1.5.2. Keratinocyte differentiation

Keratinocyte differentiation describes the process that enables keratinocytes to form a stratified epithelium and finally leads to the formation of cornified squames on top of the epidermis. It can be divided into two phases: commitment of transit amplifying cells and terminal differentiation.

Commitment occurs within the basal epidermal layer. It is preceded by the transition of an epidermal stem cell to a transit amplifying cell and means the progressive development of a transit amplifying cell from its birth to a postmitotic state.

Until today very little is known about factors that determine proliferative potential of basal keratinocytes. There are reasons to assume that extracellular factors have a function in this regulation. If, due to wounding, parts of the epidermis are lost a wound healing reaction is initiated at the site of injury that dramatically changes the extracellular milieu and promotes proliferation of epidermal cells in order to cover the wound within a short time. After complete wound epithelialisation keratinocytes with characteristics of stem cells can be found in the area of the former wound (Compton et al. 1989) showing that the stem cells lost by wounding have been replaced by new stem cells most likely recruited from the keratinocytes that migrated over the wound. This example shows that the number of stem cells is not static and can be regulated.

Good candidates for regulators of proliferative potential are components of the ECM. Basal keratinocytes with characteristics of stem cells have been found to reside in isolated areas within the epithelium (stem cell niches) for example on top of the dermal papillae and in the bulge region of hair follicles (Jensen et al. 1999; Taylor et al. 2000). It is possible that the basement membrane composition in these places differs from that in the rest of the epidermis and favours stem cell properties (Sengel 1990). A role of ECM in stem cell regulation is also suggested by the fact that a major group of ECM receptors on the keratinocyte surface, β1 integrins, show higher expression and adhesive function in cells that have a high proliferative potential (Jones and Watt 1993; Jones et al. 1995).

In addition to ECM, one extracellular factor exerting an influence on keratinocyte proliferative potential is epidermal growth factor. This soluble factor was first shown to affect keratinocytes more than 20 years ago (Rheinwald and Green 1977). Addition of EGF to the culture medium increases the number of population doublings of keratinocytes. Recently it has been shown that integrins can directly activate the EGF receptor (Moro et al. 1998) and an interdependence between integrin- and growth factor signalling has been demonstrated (Renshaw et al. 1997). It would therefore not be surprising if both receptors would operate a common pathway that can regulate keratinocyte proliferative potential.

An additional regulator of keratinocyte proliferative potential has been described more recently. β catenin is an adapter protein linking E-cadherin mediated cell-cell contacts via its binding partner α catenin to the actin cytoskeleton (McCrea and Gumbiner 1991) (Ozawa and Kemler 1992; Aberle et al. 1994; Jou et al. 1995). In addition, β catenin is a second messenger operating in the Wnt signalling pathway that regulates activity of transcription factors of the Lef-1/TCF family (Hinck et al. 1994; Behrens et al. 1996; Huber et al. 1996; Riese et al. 1997). It is also involved in signalling from E-cadherin mediated cell- cell contacts which have been shown to regulate keratinocyte proliferation and differentiation (Zhu and Watt 1996). Activation of Lef-1 requires nuclear translocation of β catenin (Huber et al. 1996). In cultured keratinocytes nuclear exogenous β catenin has been found in actively proliferating cells and expression of a constitutively active mutant of β catenin increases keratinocyte proliferative potential in vitro (Zhu and Watt 1999). These results are especially interesting in the light of in vivo findings showing that activated Lef-1 expressed in the epidermis of transgenic mice

leads to formation of new hair follicles and development of tumours in these mice which resemble human pilomatrixomas. Beta-catenin-stabilising mutations and Lef-1 activation have been found in spontaneously occurring pilomatrixomas in men (Chan et al. 1999; DasGupta and Fuchs 1999).

In recent years there is accumulating evidence that the decision of a keratinocyte to leave the basal layer is not a passive, random event initiated, for example by a lack of space within the basal layer that forces individual cells out of it. Rather the decision to execute the terminal differentiation program seems to be an active one determined by intrinsic and extrinsic factors (Watt and Hogan 2000). This means that a basal keratinocyte stops cell division at a certain point due to unknown stimuli and enters the terminal differentiation program „voluntarily", following its preprogrammed fate. After withdrawal from the cell cycle, keratinocytes detach from the basement membrane and move into the suprabasal layers of the epidermis. Subsequently they undergo the typical changes that have been described previously (see Figure 1.1). A number of substances and signalling molecules have been described that can regulate expression of markers of terminal differentiation or irreversible cell cycle withdrawal.

The calcium switch (abrupt change of the calcium concentration in the cell culture medium from 50 μM to around 2 mM) has been used widely to study early events in terminal differentiation. A high calcium concentration is, however, not necessary for a keratinocyte in order to withdraw from the cell cycle and express involucrin (Watt and Green 1982; Watt 1984) and a switch from low to a high calcium does not necessarily induce intracellular changes typical of the onset of differentiation in vivo. Instead, calcium is essential for the assembly of cell- cell contacts and epidermal stratification (Watt et al. 1984). This seems to be of importance for the stability and homeostasis of the whole epidermis since disturbances in calcium metabolism as caused by mutations in calcium transport proteins result in the hereditary skin diseases of Darier (Sakuntabhai et al. 1999) and Hailey-Hailey (Hu et al. 2000; Sudbrak et al. 2000).

Retinoids can potently modulate epidermal differentiation via mechanisms involving gene transcription see, for example (Fuchs and Green 1981; Kopan et al. 1987) and receptors of the RAR and RXR types are expressed in human epidermis (Elder et al. 1992). In most cases retinoids seem to inhibit features of terminal differentiation in keratinocytes. Another naturally occurring modulator of keratinocyte differentiation is

Vitamin D which has been observed to stimulate terminal differentiation (Pillai et al. 1988; Itin et al. 1994; Cho et al. 1996).

There are several cytokines that have been shown to be involved in regulation of keratinocyte terminal differentiation. The most established one is transforming growth factor beta (TGFβ) which is expressed within the epidermis by suprabasal, differentiating keratinocytes (Akhurst et al. 1988). Growth factors of the TGFβ family can induce cell cycle arrest in keratinocytes at picomolar concentrations (Bascom et al. 1989) and this seems to be required for maintainance of the epidermal homeostasis (Wang et al. 1997). This growth arrest is reversible initially but is then followed by commitment to terminal differentiation. At those concentrations TGFβ does not influence expression of differentiation markers, however, so that its primary role in the regulation of differentiation in basal keratinocytes may be to induce growth arrest. At higher concentrations TGFβ has been reported to change the expression of differentiation markers in organotypic keratinocyte cultures into a pattern that is more typical for hyperproliferative epidermis, e.g. in wound healing (Choi and Fuchs 1990). The overall role of TGFβ in wound healing is still not clear despite high interest in this topic during recent years. In one study deficiency of TGFβ 1 delayed healing of skin wounds (Crowe et al. 2000) whereas in an in vitro study it had no effect on speed of wound reepithelialisation (Garlick and Taichman 1994). In contrast, deficiency in a downstream signalling molecule in the TGFβ pathway, Smad 3, results in accelerated wound healing in mice (Ashcroft et al. 1999).

Very recently another cytokine has been suggested to regulate differentiation of cultured human keratinocytes in an organotypic in vitro system: Granulocyte/ Macrophage Colony Stimulating Factor (GM-CSF) (Szabowski et al. 2000). From these investigations there is evidence for a paracrine loop between epidermal keratinocytes and dermal fibroblasts that involves interleukin 1 secreted by keratinocytes stimulating, via the transcription factors c-jun and jun B, release of GM-CSF and KGF from fibroblasts. KGF is known to stimulate keratinocyte proliferation and inhibit differentiation (Guo et al. 1993), whereas GM-CSF seems to stimulate later phases of the terminal differentiation process (Szabowski et al. 2000).

The search for signalling pathways that are able to regulate differentiation has led to results suggesting the involvement of different signalling molecules in differentiation pathways. One of the first intracellular signal transducers with such a function was the

small GTPase $p21^{ras}$ (Ras). Like other GTP (guanosintrisphosphate) binding proteins Ras cycles between an activated GTP- and an inactive guanosindiphosphate (GDP)-bound state. GTP binding by Ras stimulates its intrinsic GTPase activity which then leads to cleavage of GTP to GDP and self- inactivation of ras (Marshall 1999). Ras constitutively activated by point mutation and introduced into murine keratinocytes stimulates proliferation and inhibits their differentiation in vitro and in vivo (Roop et al. 1986; Bailleul et al. 1990). Furthermore, tumorigenesis is stimulated by the activated molecule in the epidermis of transgenic mice (Bailleul et al. 1990) and Ras has therefore been implicated in the development of skin cancer. In experimental models of skin cancer mutations of Ras are induced by treatment with 7,12-dimethylbenzanthracene (DMBA) (Bizub et al. 1986).

Another signalling protein that seems to be involved in the regulation of keratinocyte terminal differentiation is the tyrosine kinase Fyn (Calautti et al. 1995). Though in this work differentiation of primary keratinocytes in vitro was induced by a calcium switch and treatment with TPA, alterations in expression of the differentiation markers keratin 1 and filaggrin were also observed in fyn deficient mice in vivo. It is not clear, however, whether fyn deficiency actually alters the program of terminal differentiation or rather impinges on expression of selected proteins associated with epidermal differentiation, like keratin 1 and filaggrin. Expression of other markers like keratin 5 and involucrin was not affected in fyn -/- mice and increased proliferation of epidermal keratinocytes in these mice is not obvious.

Initially based on pharmacological evidence using activators and inhibitors, the family of protein kinases C (PKC) has been thought to be involved in the regulation of differentiation. This has recently been confirmed by experiments on the molecular level for the PKC isoforms eta and delta (Ohba et al. 1998). Since PKC activity can be stimulated by EGF this may be a pathway through which EGF acts as a differentiation promoting factor (Susa et al. 1989). There is also some evidence that fyn and PKC eta could be components of the same pathway that regulates differentiation (Cabodi et al. 2000).

In a recent in vitro study the cyclin kinase inhibitor p21 (cip1/waf1) has been implicated in control of terminal differentiation. Though this protein in many cells has a function in growth arrest, forced expression of p21 inhibited features of terminal differentiation in cultured murine keratinocytes (Dicunto et al. 1998). This is in agreement with other

work showing that p21 is expressed during terminal differentiation but not sufficient for its induction (Harvat et al. 1998).

The transcription factor NFkB is involved in the regulation of terminal differentiation. In particular, there is evidence that components of the NFkB pathway can control cell cycle arrest that occurs prior to the migration of keratinocytes into the suprabasal layers of the skin (Seitz et al. 1998). In addition, targeted deletion of a kinase with a regulatory function for NFkB activity, IkB kinase 1 (IKK1), results in increased thickness of the epidermis and altered expression of the late differentiation markers filaggrin and loricrin (Li et al. 1999) which could suggest that NFkB may be involved not only in the control of cell cycle arrest but also in the regulation of later stages of differentiation. The trimeric kinase complex that regulates NFkB translocation into the nucleus consists of IKK1, IkB kinase 2 (IKK2) and the kinase NEMO. A mutation in NEMO has been shown to cause a rare human skin disease, Incontinentia pigmenti which is characterised by severe disturbances of epidermal proliferation and differentiation (Makris et al. 2000; Schmidt-Supprian et al. 2000; Smahi et al. 2000). Presently it is not clear whether all effects of altered signalling function of the IKK complex are actually due to impaired NFkB activity or whether other, so far unidentified pathways may be involved (Hu et al. 1999).

1.5.3. Epidermal wound healing and hyperproliferative skin disease

There are many skin diseases which are characterised or accompanied by epidermal hyperproliferation. In general, they can be divided into two categories: those in which hyperproliferation of keratinocytes is initiated by a stimulus in the epidermis itself and those in which it is stimulated by a signal coming from another tissue than the epidermis, e.g. the dermis or a wound granulation tissue.

In the first category fall benign and malignant tumours of the epidermis, e.g. virus warts and squamous cell carcinomas. In warts, epidermal keratinocytes that have been infected with a papilloma virus hyperproliferate due to dysregulation of their cell cycle machinery by the virus proteins (Thomas et al. 1999; Duensing et al. 2000). In the latter case, mutations have occurred in epidermal cells, e.g. due to UV light exposure, that lead to alterations in keratinocyte proliferation and differentiation. Interestingly, squamous cell carcinomas often show altered patterns of integrin expression (Jones et

al. 1993) and forced misexpression of the α6β4 integrin in the skin of transgenic mice increases frequency and malignancy of skin carcinomas in tumorigenesis experiments (D.M. Owens, F.M. Watt, unpublished observations).

Other situations in which massive hyperproliferation of epidermal keratinocytes can be found are wound healing and inflammatory skin diseases, with the prototype of those being Psoriasis. The wound healing reaction is a response of the skin to a disturbance of its barrier function caused by destruction of parts of the epidermis. Classically, it is characterised by formation of a fibrin clot which provides provisional wound coverage, subsequent recruitment of inflammatory cells to the wound site and epidermal reepithelialisation which is driven by an increase in keratinocyte proliferation and migration (Martin 1997). In the regulation of the epidermal wound healing reaction the EGF receptor and its ligands EGF and TGFα are thought to play a crucial role. During development in human embryonic skin the EGF receptor is, in contrast to adult skin, distributed throughout all nucleated layers of the epidermis (Nanney et al. 1990). A similar patter of expression has been observed in hyperplastic epidermis at wound edges (Nanney and King) and in other situations of epidermal hyperproliferation, e.g. Psoriasis (Nanney et al. 1986). Thus, upregulation of the EGF receptor seems to be closely associated with epidermal hyperproliferation. Clinical studies on the influence of treatment of skin wounds with soluble EGF revealed that wounds treated with EGF healed faster than control wounds (Brown et al. 1989). Unexpectedly, however, TGFα deficiency in knock out mice does not significantly impair wound healing (Luetteke et al. 1993; Guo et al. 1996). Because of the vital importance of an efficient wound healing response redundancy in the pathways regulating it is very likely. Interestingly, in healing epidermis expression of various β1 integrins is also upregulated. Like the EGF receptor, in these situations integrins are not only found in the basal epidermal layer but also in suprabasal layers (Hertle et al. 1992). This is particularly interesting in the light of new findings by (Moro et al. 1998) showing that integrins can activate EGF receptor and may suggest a cooperation between suprabasally expressed EGF receptor and β1 integrins.

The association of both aberrant integrin and EGF receptor expression with epidermal hyperproliferation raises the question of which cellular functions these receptors exert in keratinocytes. Both are known to participate in the regulation of keratinocyte proliferation although the exact mechanisms are not known. From in vitro studies there

is evidence that both EGF and β1 integrins are involved in the regulation of keratinocyte proliferative potential (Rheinwald and Green 1977; Jones and Watt 1993). Another function that has been shown to be EGF receptor dependent is keratinocyte migration (Barrandon and Green 1987) though the mechanisms are still unclear. Regulation of adhesion and cell migration are also important functions of integrins (Akiyama et al. 1989; Huttenlocher et al. 1998).

Inflammatory skin diseases accompanied by epidermal hyperproliferation can be of different etiology. The changes that they cause within the epidermis, however, bear many similarities. In most cases the epidermis is thickened, the rete ridges are elongated, the cornified layer is thickened (hyperkeratosis) and in some diseases, e.g. Psoriasis, contains keratinocytes that have not completed their differentiation program and are still nucleated (parakeratosis). The changes observed in epidermal architecture can all be interpreted as a function of epidermal hyperproliferation leading to an expansion of the transit amplifying compartment (Iizuka et al. 1996; Iizuka et al. 1997;1999) and it is therefore not surprising that there are many histological similarities between different diseases.

Psoriasis is a common skin disease that affects more than 2% of the world's population. It is characterised by plaques of thickened, scaly epidermis with an underlying inflammatory infiltrate in the dermis and in the epidermis. Histologically the epidermis is three- to fivefold thicker than normal (acanthosis) with dramatic elongation of the epidermal rete ridges (papillomatosis) and shows increased mitotic activity primarily in basal but also in some suprabasal keratinocytes. Further characteristics are reduction or loss of the granular layer (hypogranulosis), a thickened cornified layer (hyperkeratosis) and areas in which corneocytes still contain nuclei (parakeratosis) (Lever and Schaumburg-Lever 1983). The inflammatory infiltrate mainly consists of T lymphocytes both of the CD4+ and CD8+ type and of granulocytes. Most of these inflammatory cells are situated in the dermis directly beneath the epidermal basement membrane but a certain proportion can also enter the epidermis. Granulocytes that have migrated into the epidermis form characteristic aggregates in the upper epidermis, the microabscesses of Munroe (Lever and Schaumburg-Lever 1983).

Following the observation that cyclosporin A, an immunosuppressant inhibiting T cell activation, is highly efficient in the treatment of Psoriasis (Ellis et al. 1991) and

subsequent findings that depletion of activated T cells by use of an immunotoxin can improve severe Psoriasis (Gottlieb et al. 1995) this disease is today widely believed to have an autoimmune pathogenesis. The fact that new episodes of this chronic-intermittent disease can be triggered by infections, especially when caused by streptococci, has been a long standing clinical observation (Rosenberg and Noah 1988). Until today, however, no specific antigen has been identified that could be causally involved in the pathogenesis of Psoriasis. Furthermore, the role of epidermal keratinocytes in Psoriasis has not been sufficiently explored, probably because of the lack of a suitable model to study the development of the disease. It has been demonstrated, however, that the initiation of a Psoriasis like inflammatory skin disease in transgenic mice can be achieved by specifically expressing β1 integrins in suprabasal keratinocytes using the involucrin promoter (Carroll et al. 1995; Romero et al. 1999). Aberrant expression of integrins as observed in Psoriasis and wound healing (Hertle et al. 1992) may therefore not only be a consequence of epidermal hyperproliferation but also contribute to the development of the disease. Thus, though the involvement of the immune system in its pathogenesis is well documented, it is not clear whether the mechanisms that take effect correspond to pathways that have been established in autoimmunity or represent a new type of autoimmune reaction specific for the skin.

Until today a bulk of evidence for a role of integrins in regulating keratinocyte differentiation has accumulated. For a more comprehensive understanding of this role it is now necessary to characterize the intracellular events that transduce integrin dependent signals. I therefore set out to investigate mechanisms of integrin mediated signalling in keratinocytes. I was especially interested to illuminate the role of MAPK signalling in the epidermis since this pathway is emerging as a powerful regulator of cell behavior. Knowledge about MAPK signalling in keratinocytes is therefore likely to be relevant for the physiology and pathophysiology of the skin.

CHAPTER 2
MATERIALS AND METHODS

2.1. Cell Biology

2.1.1. Cell Culture

2.1.1.1. General Solutions

The Central Cell Services of Imperial Cancer Research Fund provided sterile distilled deionised water (dH_2O) and solutions which are indicated by 'ICRF'. All reagents used were of tissue culture grade and kept sterile.

Phosphate buffered saline (PBS, ICRF)

8g NaCl, 0.25g KCl, 1.43g Na_2HPO_4 and 0.25g KH_2PO_4 were dissolved in 1l dH_2O, the pH was adjusted to 7.2 and the solution was autoclaved. PBS$_{ABC}$ was PBS supplemented with 1mM $CaCl_2$ (B) and 1mM $MgCl_2$ (C).

Tris buffered saline (TBS)

10x stock solution was prepared by dissolving 24.2g Trizma base and 80g NaCl in 1l dH_2O. The pH was adjusted to 7.6 and the solution was autoclaved.

EDTA solution (versene, ICRF)

8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4 and 0.2g ethyldiaminotetraacetic acid, disodium salt (EDTA) and 1.5ml 1% (w/v) phenol red solution were dissolved in 1l dH_2O, the pH was adjusted to 7.2 and the solution was autoclaved.

Trypsin solution (ICRF)

8g NaCl, 0.1g Na_2HPO_4, 1g D-glucose, 3g Trizma Base, 2ml 19% (w/v) KCl solution and 1.5ml of 1% phenol red solution were dissolved in 200ml dH_2O, the pH was adjusted to 7.7 and 0.06g penicillin and 0.1g streptomycin (Gibco BRL) were added. 2.5g pig trypsin (Difco, 1:250) was dissolved in 200ml dH_2O; air was bubbled through the solution until the trypsin dissolved. The trypsin solution was added to the Tris-

buffered saline, made up to 1l with dH_2O, sterilised by filtration through 0.22μm filter (Millipore) and stored at -20°C.

Mitomycin C stock solution

Mitomycin C is an inhibitor of DNA synthesis and nuclear division (Tomasz et al. 1987). It is used to metabolically inactivate J2-3T3 cells for the keratinocyte cultures. 4mg mitomycin C powder (Sigma) was dissolved in 10ml PBS. The stock solution (0.4 mg/ml) was sterilised by filtration through a 0.22μm filter, aliquoted and stored at -20°C. In the treatment of J2-3T3 cells, mitomycin C solution was added to the medium at a final concentration of 4 μg/ml.

Puromycin stock solution

100mg puromycin powder (Sigma) was dissolved in 50ml PBS. The stock solution (2 mg/ml) was sterilised by filtration through a 0.22μm filter, aliquoted and stored at -20°C.

2.1.1.2. Cultured Cell Types

Human epidermal keratinocytes were isolated from neonatal foreskins, grown and serially passaged as described in Section 2.1.1.4. J2-3T3 cells were used as feeder cells for supporting epidermal keratinocyte growth. Clone J2 of 3T3 Swiss mouse embryo fibroblasts is a clone selected for its ability to support keratinocyte growth (Rheinwald and Green 1975). Puromycin resistant J2-3T3 cells (J2-puro) were used as feeder cells for retrovirally infected human keratinocytes. Ecotropic retroviral packaging cells, GP + E (Markowitz et al. 1988a), and amphotropic packaging cells, AM12 (Markowitz et al. 1988b), were used to generate high titer retroviruses for infecting human keratinocytes. HeLa cervical carcinoma cells were used to determine the titers of retroviral producer cells. Lines of mouse keratinocytes isolated from transgenic mice expressing β1 integrin under the control of the involucrin promoter (Romero et al. 1999) were used to investigate integrin signalling. All cell lines were cultured on plastic dishes or flasks of tissue culture grade (Becton-Dickinson or Nunc) in a humidified incubator at 37°C with 5% CO_2. Media or any solutions added to cells were first warmed to 37°C. All cell lines were tested for mycoplasma infection by the ICRF Cell Production Unit. If any mycoplasma contamination was detected the whole cell stock was discarded.

2.1.1.3. J2-3T3 Cells and J2-puro Cells

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

This comprised Dulbecco's modification of Eagles' medium (DMEM) (E4, ICRF) supplemented with 10% (v/v) donor calf serum (DCS, Gibco BRL). For J2-3T3 cells transfected with the puromycin resistance gene (J2-puro, prepared by L. Goodman), puromycin was added to the culture medium at a final concentration of 2.5 μg/ml.

J2-3T3 and J2-puro cell cultures

J2-3T3 cells were routinely cultured to generate stocks of feeder cells required in keratinocyte culture (Watt 1998b). When J2-3T3 cells approached confluence, they were subcultured at 1:5 or 1:10. A 75cm^2 flask of confluent J2-3T3 was harvested: cells were first rinsed twice with 5ml versene solution and then incubated with 1 ml trypsin/versene solution (1 part trypsin and 4 parts versene) for 5 minutes at 37°C. 5ml medium was added to the cell suspension and the cell suspension collected in a tube. The cells were pelleted by centrifugation at 1,000 rpm for 5 minutes. The cell pellet was gently resuspended in 10ml medium and the required volume of cell suspension added to 75 cm^2 flasks containing 14 ml medium. J2-3T3 feeder cells were usually cultured for 2-3 months and were discarded when they started to transform. For the keratinocytes infected with retrovirus J2-puro were used as feeder cells.

Freezing and thawing of J2-3T3 cells

Cells were harvested as described above. The cell pellet from a 75 cm^2 flask was resuspended gently in 3 ml DCS containing 10% (v/v) sterile dimethyl sulphoxide (Gibco BRL). 1 ml cell suspension was frozen in each cryotube (Nunc) in an insulated box at -70°C overnight before transferring to liquid nitrogen for long term storage. Thawing of cells was performed quickly by transferring the cryotube of cells from liquid nitrogen to a 37°C water bath. As soon as the cell suspension was thawed, it was added to 10 ml medium and centrifuged at 1,000 rpm for 4 minutes. The recovered cells were plated onto a 25cm^2 flask.

2.1.1.4. Epidermal Keratinocytes

Keratinocyte culture medium (FAD+ FCS + HICE)

FAD powder (Imperial Labs.) consisting of 1 part Ham's F12 and 3 parts DMEM + 1.8x 10^{-4} M adenine (final concentration) was supplemented with 3.07 g/l $NaHCO_3$, 100 IU/l penicillin and 100 µg/l streptomycin. FAD medium (ICRF) was bubbled with CO_2 until acidic in pH before sterilising by filtration through a 0.22µm filter. Medium was stored at 4°C until use.

Stock solutions of additives were prepared. 10^{-5} M cholera enterotoxin (ICN) was stored at 4°C. Hydrocortisone (Calbiochem) was dissolved in 95% ethanol at 5 mg/ml and stored at -20°C. 100 µg/ml recombinant human epidermal growth factor (Sigma) was prepared by first dissolving in 1/100 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 1,000x 'cocktail' (HCE): 1ml hydrocortisone, 100µl cholera enterotoxin and 1ml epidermal growth factor stock solutions were added to 7.9ml FAD medium with 10% FCS and stored at -20°C. The final concentrations in the medium were 10^{-10} M cholera enterotoxin, 0.5 µg/ml hydrocortisone and 10 ng/ml epidermal growth factor. 1,000x insulin stock solution (5 mg/ml in 5mM 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 culture medium at 1 µg/ml. Complete medium was stored at 4°C for up to 10 days.

Preparation of J2-3T3 cells as feeder cells

The culture of human keratinocytes required co-cultivation with mitotically inactivated 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 2-3h at 37°C. Cells were then harvested and plated onto flasks as described earlier. Typically, cells from a confluent 75cm^2 flask were plated equally between 3x 75cm^2 or 9x 25cm^2 flasks. J2-puro cells were used as feeder cells when keratinocytes were infected with retroviruses.

Isolation of primary human keratinocytes

Neonatal foreskins were kindly provided by Dr Cohen, London. Isolation of primary keratinocytes was carried out as soon as possible after circumcision. 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 $5mm^2$ and transferred into a Wheaton Cellstir (Jencons) containing 5ml trypsin and 5ml versene and stirred over a magnetic stirrer at 37°C. Dissociated cells were collected every 30 minutes and added to 5ml keratinocyte culture medium. The number of cells obtained was determined 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 1-5x 10^7 cells. Feeder cells had been plated onto $25cm^2$ flasks in readiness. Isolated cells were pooled, pelleted and plated at a density of 10^5 cells per $25cm^2$ flask. Cells were cultured until just confluent. One flask of cells was tested for mycoplasma infection by the ICRF Cell Production Unit, while the remaining cells were harvested and frozen at 10^6 cells per ml as for J2-3T3 cells. If mycoplasma infection was detected, the stocks were discarded.

Serial culture of human keratinocytes

Frozen keratinocytes (strains kb, km, kq; passage 2-4) were thawed as described for J2-3T3 cells. The usual number of cells seeded in 1x $75cm^2$ flasks was 2x 10^5 cells; but 5x 10^5 cells from a frozen cryotube were plated in a $75cm^2$ flask to allow for loss of viability resulting from freezing and thawing. Fresh medium was given to keratinocytes every 2- 3 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 2 ml trypsin/versene solution (1 part trypsin and 4 parts versene) at 37°C until all keratinocytes had detached from the flask. This usually required between 10-20 minutes. 5 ml medium were added to the suspension and the number of cells was determined 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 $25cm^2$ flask or 3x 10^5 cells were added to a $75cm^2$ flask.

Culture of murine keratinocyte lines

Keratinocyte lines isolated from transgenic mice expressing β1 integrins under the control of the involucrin promoter were used (Carroll et al. 1995; Romero et al. 1999). These cells were grown and passaged like human keratinocytes on feeder layer in FAD medium, but at a temperature of 32°C. For experiments keratinocytes of passages 20-30 were used.

2.1.1.5. Retroviral Producer Cells

Retroviral producer cell culture

Helper free ecotropic packaging cells, GP + E-86 (abbreviated as GP + E), and amphotropic packaging cells, GP + *env*AM12 (abbreviated as AM12), were used in conjunction with the pBabe puro retroviral vector to minimise the risk of generation of wild type MoMuLV via recombination events (Markowitz et al. 1988a) (Markowitz et al. 1988b). The packaging cells were cultured in E4 medium supplemented with 10% (v/v) FCS (Sigma). For transfected or infected retroviral producer cells the culture medium was supplemented with 2.5 μg/ml puromycin. Procedures for harvesting, freezing and thawing retroviral producer cells were the same as for J2-3T3 cells.

Transfection of GP + E cells by calcium phosphate co-precipitation

Calcium-phosphate co-precipitation was used to generate ecotropic producer cell lines. The ecotropic GP + E cells were seeded onto a 100 mm dish at a density of 2-5x 10^5 the day before transfection. In a round bottom plastic tube (6ml, 12 x 75mm), 20μg DNA was made up to 160μl with 1mM Tris-HCl, pH7.6, 0.1mM EDTA, mixed thoroughly but gently on a whirling mixer and incubated for 5 minutes at 37°C. 160μl of 0.5M $CaCl_2$ and 0.1M HEPES, pH6.74 solution pre-warmed to 37°C was added, mixed thoroughly and incubated for 10 minutes at 37°C. 320μl of 0.28M NaCl, 0.75mM Na_2HPO_4, 0.75mM NaH_2PO_4, 0.05M HEPES, pH6.74 solution was prewarmed to 37°C and added drop-wise while gently vortexing the contents of the tube. This was incubated for 15 minutes at 37°C and then added to the cells whilst gently swirling the medium. The cells were left in the incubator at 37°C for about 16h and then inspected under a microscope for presence of a suspension of fine, black precipitate in the medium. The medium was discarded and a sterile solution of 25mM Tris-HCl, pH7.4, 137mM NaCl, 5mM KCl, 0.7mM $CaCl_2$ and 0.6mM Na_2HPO_4 was added to the cells and incubated for 10 minutes at 37°C. This was repeated twice and then the medium was replaced. Puromycin (2.5 μg/ml) was added to the medium 48h later. The

selection medium was changed every 2 days until cells reached confluence. At this point, the selection medium was replaced with half the volume of normal culture medium (E4 + FCS), and active retroviral particles were enriched in the supernatant for 2 days. Floating cells were removed from the conditioned medium by filtration through a 0.22μm filter. The filtered supernatant was used directly to infect amphotropic AM12 cells.

Infection of AM12 cells

Producer lines that are generated by retroviral infection have higher viral titers than those generated by transfection, hence virus released into the culture medium by confluent GP + E cells was used to infect the amphotropic packaging line AM12 (Markowitz et al. 1988b; Levy et al. 1998). AM12 cells were seeded on 100 mm dishes at a density of 1-2x 10^5 the day before infection. 2.5ml infection medium (conditioned medium from the GP + E cells supplemented with 8 μg/ml polybrene; Sigma) was added to the AM12 cells. After 3h 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.

Ring cloning of AM12 cells

The viral titer of the polyclonal AM12 population was only 10^3-10^4 cfu/ml. In order to obtain maximum infection efficiency it was therefore necessary to clone the AM12 cells (Levy et al. 1998). The polyclonal population of infected AM12 cells were seeded on 100mm dishes at 100- 2000 cells per dish and fed every third day until drug resistant colonies were visible without the aid of a microscope (normally 7-10 days after plating). At least 24 sparsely located colonies were chosen and marked at the bottom of the dishes. The culture medium was then removed and glass cloning rings (5mm diameter, 8mm height, Howe Instrumentation) dipped into autoclaved silicon grease were placed over marked colonies. 50μl trypsin solution was added into each ring to release cells from the dish. Cloned cells were transferred to 24 well plates and eventually expanded onto 100 mm dishes. Expression of MAPK constructs in cloned AM12 cells was examined by Western blotting with an antibody against phosphorylated MAPK (MAPK-P183/185). For analysis of MAPKK1 activity AM12 clones were starved overnight in DMEM without serum. This medium was changed against fresh DMEM without serum on the next morning 1 hour prior to cell lysis. For analysis of MANA activity AM12 clones were starved overnight and on the next morning stimulated with DMEM containing 10% DCS for 10 minutes.

Cultivation of HeLa cells and titration of retroviral producer cells

HeLa cells are human epithelial carcinoma cells and were chosen for the determination of retroviral titers because of their similarity to human keratinocytes. HeLa cells were obtained from the ICRF Cell Production Unit. The culture medium comprised DMEM (E4, ICRF) supplemented with 10% (v/v) FCS (Imperial Labs.).
HeLa cells were seeded on 100mm dishes at 4x 10^5 density the day before titration. 50µl filtered supernatant of AM12 producer cells was added to 9.95ml fresh culture medium containing 8 µg/ml polybrene. The resulting infection solution was used to infect HeLa cells at 37°C. The infection medium was discarded 4h later and fresh culture medium was added to the cells and incubated overnight at 37°C. Infected HeLa cells were then split with a series of dilutions (1:5, 1:10, 1:50, 1:100 and 1:500) and grown in the selection medium containing 0.4 µg/ml puromycin for a week. Surviving colonies were fixed in 3.7% formaldehyde (BDH) and stained with 1% methylene blue (Sigma). Blue colonies were counted and the number of counted colonies was converted to the retroviral titer using the following formula: retroviral titer (colony forming unit/ml) = 200 x number of the colonies x dilution factor.

Expanding retroviral producer clones

Amphotropic producer clones were selected based on the activity of the MAPK constructs, as examined by Western blotting, and of their retroviral titers. In every clone examined high expression levels correlated with high retroviral titers. Clones with highest titers and expression levels were selected, expanded and used to infect primary human epidermal keratinocytes.

Infection of human keratinocytes

In order to achieve maximum efficiency retroviral infection was carried out by plating human keratinocytes (10^5 per 100mm dish) onto 70% confluent AM12 producer cells that had been pre-treated with 4 µg/ml mitomycin C. After 2 days 1 µg/ml puromycin was added to the medium. The producer cells were usually removed after 3-4 days and replaced with puromycin resistant J2-3T3 cells, J2-puro. Double infections were performed in the same way. Infected keratinocytes were used for experiments immediately or following passage on J2-puro in medium supplemented with 1 µg/ml puromycin. The titers of AM12 producer cells used for infections were as follows: MAPKK1: 1.9x10^6 cfu/ml; MANA: 2x10^5 cfu/ml. Polyclonal producer cells expressing the empty retroviral vector were also used (3.2x10^6 cfu/ml).

2.1.2. Analysis of Keratinocyte Proliferation and Differentiation

2.1.2.1. Counting Viable Cells Using Trypan Blue

The trypan blue solution was made by dissolving 20mg trypan blue powder (Gibco BRL) in 50ml PBS, filtered with 0.22μm filter to remove crystals. Cells were harvested with the versene/trypsin solution. 100μl cell suspension was mixed with 100μl trypan blue solution. Mixed solution was pipetted thrice very gently. Cells were scored in a haemocytometer; only small golden cells were scored as viable cells; giant golden cells and blue or dark brown cells were regarded as nonviable ones.

2.1.2.2. Setting up Growth Curves and measuring BrdU Incorporation

Equal numbers of viable cells were seeded onto 35mm dishes in the presence of mitomycin C-treated feeder cells. Growth curves were obtained by harvesting triplicate dishes at days 7, 9, 11, 13, 15, 21 and counting cells in a Neubauer chamber.
For measuring BrdU incorporation preconfluent keratinocytes were grown on culture dishes or trypsinised and kept in FAD+FCS+HICE supplemented with methylcellulose for 24 hours. Cells were pulse labelled with 10 μg/ml BrdU for 1 hour, harvested and fixed in 70% ice cold ethanol. Immunodetection of incorporated BrdU using a monoclonal antibody and FACS analysis was performed by the FACS laboratory of ICRF as described previously (Gandarillas and Watt 1997).

2.1.2.3. Clonogenicity Assays

Equal numbers of viable keratinocytes were plated per 35mm dish in triplicate on mitomycin C-treated J2-3T3 feeder cells. After 2 weeks the cultures were washed with PBS and the cells were fixed in 3.7% formaldehyde for 5 minutes at room temperature. After further washing in PBS, the cultures were stained for 30 minutes at room temperature with rhodanile blue (Rheinwald and Green, 1975; Jones and Watt, 1993). All the visible colonies (i.e. >1 cell) were scored on each dish and colony forming efficiency was calculated as % of plated cells that formed colonies. Abortive colonies were defined as colonies that were less than 0.4mm in diameter and contained fewer than 40 cells; the majority of which were large and terminally differentiated.

2.1.2.4. De-epidermised Dermis (DED) Culture

Preparation of DEDs

The method for growing keratinocytes on DEDs was based on the Pruniéras model (Pruniéras et al. 1983) to achieve histological differentiation as close as possible to the epidermis *in vivo*. Human breast skin was cut into 10cm^2 pieces and the subcutaneous fat was scraped off. The skin was heated in PBS to 56°C for 20 minutes and the epidermis was peeled off. The remaining dermis was cut into 1cm^2 pieces, placed into cryovials and snap frozen in liquid nitrogen. The vials were allowed to thaw at room temperature for 20 minutes and then frozen again in liquid nitrogen. This cycle of freezing and thawing was repeated for 10 times. After the last cycle the vials were stored at -70°C until use.

Growing keratinocytes on DEDs

The vials were thawed and the DEDs were placed on tissue culture inserts (Becton Dickinson) in six well plates, one DED per well, with the denuded epithelial surface uppermost. 10^5 cells were prepared by the versene/trypsin treatment, washed twice in the culture medium and spun down. The cell pellet was resuspended in 20µl culture medium and then plated onto the denuded epithelial surface of the DEDs (Rikimaru et al. 1997). Cultures were fed every 2 days and after 10- 14 days they were mounted in O.C.T. compound (BDH), snap-frozen in isopentane pre-cooled in liquid nitrogen, and stored at -70°C until use.

2.1.2.5. Induction of Terminal Differentiation

Medium for keratinocyte suspension culture

Keratinocytes were induced to undergo terminal differentiation by placing them as a single cell suspension in methyl cellulose supplemented medium. 3.5g methyl cellulose (Mecel, viscosity 4000 centipoises, Aldrich Chemical Co.) was autoclaved in a 400ml centrifuge tube together with a magnetic stirrer bar. 180ml FAD medium was heated to 60°C and added to the tube. The contents were stirred at room temperature for 30 minutes and then at 4°C overnight (Green 1977) (Adams and Watt 1989). After addition of 20ml FCS the medium was centrifuged at 9,500rpm in a Beckman J2-21 at 4°C for 30 minutes. Methyl cellulose (1.75%, w/v) medium was aliquoted and stored at

-20°C until use. Immediately before use 'cocktail' and insulin solutions were added as for keratinocyte medium and mixed thoroughly.

Preparation of polyHEMA-coated dishes

To minimize keratinocyte attachment to the substrate during suspension culture, bacteriological grade plastic petri dishes were used. Petri dishes were coated with polyHEMA (type NCC, cell culture grade, Hydro Medical). A 10% polyHEMA (w/v) stock 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/acetone (50%/50%, v/v). 5ml diluted solution was added to a 100mm dish and swirled quickly to coat the base of the dish and then removed immediately. This was repeated once. Coated dishes were left under sterile conditions to dry completely in a tissue culture hood before use.

Suspension culture

Newly confluent cultures of keratinocytes were incubated briefly with the versene solution to remove any remaining feeder cells, harvested with the versene/trypsin solution and resuspended at 10^6 cells per ml in complete FAD medium. The cells were then added slowly to the methyl cellulose medium stirring continuously to ensure that keratinocytes were homogeneously distributed in the suspension. Keratinocytes were at a final cell density of 2x 10^5 cells per ml. 50ml cell suspension was poured into 100mm polyHEMA-treated dishes and cultured for up to 24h in a humidified 37°C incubator. The starting population of cells (i.e. before suspension) was recovered from the methyl cellulose medium immediately. Keratinocytes were recovered from suspension by diluting methyl cellulose medium 10 fold with ice cold versene solution and centrifuging in 250 ml conical bottom Beckman bottles at 2,000rpm for 10 minutes at 4°C. The cell pellet obtained was resuspended gently in 15ml ice cold PBS.

To assess the extent of induction of terminal differentiation, 5x 10^5 cells were permeabilised with 0.3% saponin (Sigma), stained for involucrin, cornifin and transglutaminase I, markers of keratinocyte terminal differentiation, and analyzed by flow cytometry (see Section 2.1.6.2).

2.1.3. Adhesion Assays

2.1.3.1. Extracellular Matrix (ECM) Proteins

Collagen IV

Human placental type IV collagen (Sigma) was dissolved in sterile filtered 0.25% acetic acid at 2 mg/ml by mixing end over end at 4°C for 2-4h. The solution was aliquoted, stored at -20°C and thawed at room temperature when needed.

Fibronectin

Human plasma derived fibronectin (Becton Dickinson) was dissolved in sterile PBS at 5 mg/ml. The solution was aliquoted, stored at -20°C and thawed at 37°C for 2h before use.

Laminin 5

The surfaces to be coated with keratinocyte matrix enriched with laminin 5 were seeded with feeder cells treated with mitomycin C. Normal human keratinocytes were plated onto the feeders at a density that ensured that the keratinocytes would reach confluence in 5-8 days. The cells were fed every 2 days and grown until a confluent sheet of keratinocytes was present in the dishes. The medium was removed and the cells were washed thrice with cold PBSABC. Lysis buffer (1% triton X-100, 10mM EDTA and 25mM Tris-HCl, pH 7.5) (Rousselle et al. 1991) was added to the keratinocytes and left at 4°C for 15-20 minutes. The cells and lysis buffer were removed. The matrix proteins remaining on the dishes were washed thrice with cold PBSABC and then used immediately.

2.1.3.2. Preparation of Assay Plates

Petri dishes or bacteriological 96 well plates (Flow Laboratories) were coated overnight at 4°C with ECM proteins in PBSABC at appropriate concentrations. 100µl dilution solution was applied to each well of a 96 well plate, or 1ml to a 60mm dish. Uncoated areas were blocked by 0.5 mg/ml heat denatured BSA in PBSABC at 37°C for 1h and washed thrice in PBSABC. The coated plates or dishes were then used immediately.

2.1.3.3. Adhesion Assays

Subconfluent keratinocytes were harvested using the versene/trypsin solution, washed twice in complete FAD medium to inactivate trypsin activity. The cell pellet was resuspended at a density of 10^5 cells per ml of serum-free FAD medium supplemented with 25μM cycloheximide (Sigma) to inhibit protein synthesis. 10^4 or 2x 10^4 viable cells in a volume of 100μl were applied to each well in a 96 well plate and incubated for 3h in a 37°C incubator. After incubation the plate was washed thrice in PBS, dried and the number of cells attached was quantified by measuring the amount of lactate dehydrogenase (LDH) released from adherent cells upon cell lysis, using a CytoTox 96™ colorimetric kit (Promega) according to manufacturer's instructions. Adhesion assays with doubly infected cells were carried out by Alan Zhu.

2.1.4. Cell Motility and Spreading Assays

Sub-confluent human keratinocytes were harvested with the versene/trypsin solution and washed twice in complete FAD medium to inactivate trypsin. 2x 10^4 keratinocytes were plated onto bacteriological dishes coated with 10 μg/ml fibronectin and kept in 10% CO_2 at 37°C and monitored for 24h. Frames were taken every 2 minutes using Olympus IMT1 or IMT2 inverted microscopes fitted with monochrome CCD cameras driven by Broadcast Animation Controllers (BAC900) and video recorders (Sony SCC M370CE and PVW-2800P, respectively. Recordings were digitized and the sequence of all frames was run on a PC. 20-23 individual cells per sample were tracked manually and speed was calculated using an algorithm of the computer program Mathematica (kindly provided by Dr. Daniel Zicha). Analysis of Variance was carried out using Mathematica.

For assessment of cell spreading cells were plated onto bacteriological dishes coated with 10 μg/ml fibronectin and allowed to attach for 2 hours. EGF (10ng/ml) and PD098059 (25 μM) or DMSO (0.1%; solvent control) were then added. After 1 hour of incubation cells were fixed in 4% formaldehyde and stained with 1% methylene blue.

2.1.5. Immunological Methods

2.1.5.1. General Solutions

Paraformaldehyde solution

The stock solution of 10% paraformaldehyde was prepared by adding paraformaldehyde powder (BDH) to PBS pre-warmed to 60°C and stirring in a fume hood until the

solution appeared almost clear. Complete clearance was achieved by adding 1 drop of 1M NaOH. Aliquots were stored at -20°C. 4% paraformaldehyde solution was used to fix tissues and cells. The frozen solution was thawed completely at 37°C and then filtered through a 0.22μm filter before diluting to 4% with PBSABC.

Blocking solution

This comprised 0.2% cod fish skin gelatin (FSG, Sigma) solution in PBS. The stock solution of 1% (w/v) was prepared, filtered through 0.22μm filter and stored sterile at room temperature.

Gelvatol mounting solution

The Gelvatol mounting solution was prepared as described by (Harlow and Lane 1988). 2.4g Gelvatol (Monsanto Chemicals) was mixed with 6g glycerol (Sigma) and vortexed. 6ml dH_2O was added, mixed and left to stand for 90 minutes at room temperature. 12.5ml of 200mM Tris-HCl, pH8.5 was added and the solution was vortexed, heated to 50°C and vortexed again. Heating and vortexing were repeated thrice and the solution placed on an end over end mixer overnight at room temperature. The solution was then centrifuged at 2,000rpm for 10 minutes at room temperature and stored in aliquots at 4°C.

2.1.5.2. Antibodies

List of antibodies used

All antibodies used are listed in Table 2.1.-2.5.

Table 2.1. Antibodies to integrins

Antibody name	Antigen specificity	Species	Reference
P5D2	human β1 integrin	mouse monoclonal	purchased from DSHB; (Dittel et al. 1993)
CD29 conjugated to FITC	human β1 integrin	mouse monoclonal	purchased from DAKO; (Koenigsmann et al. 1992)
mAb13	human β1 integrin	rat monoclonal	a gift from Dr K. Yamada, (Akiyama et al. 1989)
JG 22	chicken β1 integrin	mouse monoclonal	(Greve and Gottlieb 1982)

Table 2.2. Antibodies to chimeric constructs

Antibody name	Antigen specificity	Species	Reference
UCHT4 conjugated to FITC	human CD8α	mouse monoclonal	purchased from Sigma
UCHT4	human CD8α	mouse monoclonal	a gift from Dr P. C. Beverley; (Beverley et al. 1980)
OKT8	human CD8α	mouse monoclonal	purchased from ECACC; (Thomas et al. 1980)
K9-18	mouse H-2K^d	mouse monoclonal	a gift from Dr B. Arnold; (Arnold et al. 1985)

Table 2.3. Antibodies to epidermal differentiation markers

LL001	human keratin 14	mouse monoclonal	Skin Tumour Laboratory, ICRF; (Purkis et al. 1990)
SY5	human involucrin	mouse monoclonal	Keratinocyte Laboratory, ICRF; (Hudson et al. 1992)
DH1	human involucrin	rabbit polyclonal	Keratinocyte Laboratory, ICRF; (Dover and Watt 1987)
B.C1	epidermal transglutaminase	mouse monoclonal	gift of Dr. R. Rice; (Thacher and Rice 1985)
SQ37C	α cornifin	rabbit polyclonal	gift of Dr. A. Jetten; (Fujimoto et al. 1997)

Table 2.4. Miscellaneous antibodies

Antibody name	Antigen specificity	Species	Reference
SC-1647	MAPK	mouse monoclonal	purchased from Santa Cruz
SC-153	MAPK	rabbit polyclonal	purchased from Santa Cruz
MAPK-YT	phosphorylated p42 and p44 MAPK	mouse monoclonal	purchased from Sigma
MAPK-P183/185	phosphorylated p42 and p44 MAPK	rabbit polyclonal	purchased from NEB
C-20	FAK	rabbit polyclonal	purchased from Santa Cruz
4G10	phospho-tyrosine	mouse monoclonal	purchased from Upstate
anti laminin	human laminin	mouse monoclonal	provided by the ICRF Histopathology Unit
MIB 1	Ki67 antigen	mouse monoclonal	purchased from Novacastra

Table 2.5. Secondary antibodies

Antigen specificity	Conjugate	Species	Source
mouse IgG, whole molecule	FITC	Goat	Jackson ImmunoResearch
mouse IgG, whole molecule	Texas Red	Donkey	Jackson ImmunoResearch
mouse IgG, whole molecule	HRP	Sheep	Amersham
rabbit IgG, whole molecule	FITC	Goat	Jackson ImmunoResearch
rabbit IgG, whole molecule	Texas Red	Donkey	Jackson ImmunoResearch
rabbit IgG, whole molecule	HRP	Donkey	Amersham
rat IgG, whole molecule	Alexa 488	Goat	Molecular Probes
rat IgG, whole molecule	Texas Red	Donkey	Jackson ImmunoResearch
mouse IgG, whole molecule	Alexa 488	Goat	Molecular Probes
rabbit IgG, whole molecule	Alexa 488	Goat	Molecular Probes
mouse IgG, whole molecule	Alexa 594	Goat	Molecular Probes
rabbit IgG, whole molecule	Alexa 594	Goat	Molecular Probes

2.1.5.3. Preparation of Cells for Immunofluorescence Staining

Cells were cultured on chamber slides (Nunc). Culture medium was aspirated from cells before fixing in 4% paraformaldehyde solution for 20 minutes at room temperature. The specimens were rinsed thrice in PBS, and then blocked in 0.2% FSG or 10% goat serum solution to reduce binding to non-specific proteins, for at least 30 minutes at room temperature. Cells for staining with anti- involucrin, anti- cornifin and anti- transglutaminase antibodies were fixed in 4% formaldehyde for 10 minutes at room temperature.

If the epitope of the primary antibody was intracellular, after fixing, cells were permeabilised using 0.4% Triton X-100 in PBS for 4 minutes or 0.3% saponin (see Section 2.1.6.2.) at room temperature before rinsing thrice in PBS and then blocked in 0.2% FSG solution.

2.1.5.4. Preparation of paraffin- and cryosections of DEDs and skin

Samples of psoriatic skin were kindly provided by Dr. Karin Hartmann, University of Cologne. Small pieces of DED, human neonatal foreskin, human abdominal skin, psoriatic skin and wounded and unwounded mouse skin were fixed in 4% paraformaldehyde at 4°C overnight or placed in a plastic mould containing O.C.T. compound and frozen for 1-2 minutes on isopentane pre-cooled in liquid nitrogen.

Fixed tissues were given to the ICRF Histopathology Unit where they were embedded in paraffin blocks from which 5 μM sections were cut using a microtome. Paraffin sections were mounted onto glass slides and dried overnight at 37°C. Prior to immunostaining sections were dewaxed by washing twice in Xylol followed by four washes in isopropanol solutions of decreasing concentration (100%, 96%, 75%, 50%). Sections were then stained with hematoxylin/eosin or prepared for immunostaining by blocking with 10% goat serum in PBS. For staining of laminin dewaxed sections were incubated in trypsin solution for 20 minutes at room temperature and then washed in PBS prior to blocking. For staining of the Ki 67 antigen sections were boiled in citrate buffer for 20 minutes prior to blocking using a microwave oven.
The tissue embedded in O.C.T. compound was stored at -70°C. Using a cryomicrotome (Reichart-Jung), 6μm sections were cut from the tissue block in an orientation perpendicular to the surface of the tissue. The sections were mounted onto slides (Superfrost Plus, BDH) and stored at -70°C. All sectioning was performed by the ICRF Histopathology Unit. To examine the morphology of the tissues frozen sections were stained with haematoxylin and eosin (H&E). For immunofluorescence staining, frozen sections were thawed at room temperature, fixed in 4% paraformaldehyde for 20 minutes at room temperature or dried unfixed, blocked with 10% FCS in PBS for 1h before incubating with the primary antibodies.

2.1.5.5. Immunofluorescence and Immunohistochemistry Staining Protocol

This was the general method used for staining tissue sections or cultured cells. Primary antibodies were diluted in the blocking solution, dilutions ranging from 1:100 to 1:500. The hybridoma supernatant of LL001 was used neat. In most cases sections were incubated with primary antibodies for 1h at room temperature. For staining of phosphorylated MAPK using the antibody MAPK-YT cells or sections were incubated with a 1:50 dilution for 2 hours at 37°C. The cells and sections were rinsed twice in the blocking solution and then incubated with the secondary antibody conjugated to a fluorophore diluted 1:500 in the blocking solution for 1h at room temperature. For staining of Ki 67 positive nuclei in DEDs sections were incubated in 0,1% H_2O_2 after dewaxing. The secondary antibody used was coupled to HRP. Sections were developed in DAB substrate solution and counterstained with Azan. After three washes in PBS_{ABC} and an additional washing step in dH_2O slides were mounted in the 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. In some experiments DAPI (Sigma) or ToProIII

(Molecular Probes) was used to stain the nucleus. Stained samples were viewed with a Zeiss Axiophot microscope (Carl Zeiss) or with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss) using an argon laser at 488 and 568 nm and a helium-neon laser at 633 nm.

2.1.6. Flow Cytometry

2.1.6.1. Cell Surface Epitopes

Cells were harvested with the versene/trypsin solution. The cells were resuspended in ice cold complete medium and transferred to a 1.6ml Eppendorf tube. The cells were pelleted again by pulse spinning in a bench top centrifuge (14,000rpm for approximately 8 seconds), resuspended in 100μl primary antibody diluted 1:100 in medium and incubated for 20 minutes at 4°C. The cells were agitated occasionally. 1ml medium was added and cells were pelleted again. Cells were washed twice in medium and then incubated with secondary antibody conjugated to FITC diluted 1:100 in medium for 30 minutes at 4°C. Cells were washed twice in medium and once in PBS. Finally cells were filtered through 70μm nylon mesh (Becton-Dickinson) and resuspended in 200μl PBS and transferred to round bottom plastic FACS tubes (6ml, 12 x 75mm). Immediately before analysis on the Becton-Dickinson FACScan, a drop of propidium iodide (5 mg/ml) was added to the sample for viability gating. 10,000 events were acquired in list mode for each sample. If not analyzed immediately samples were fixed in 4% paraformaldehyde solution for 5 minutes at 4°C, washed thrice and stored at 4°C for up to 3 days. In some experiments the differentiating cells were gated out as previously described (Jones and Watt 1993)

2.1.6.2. Intracellular Epitopes

FACS staining of intracellular epitopes was carried out with the help of Robin Hobbs. Harvested keratinocytes were permeabilised by resuspending in 10% FCS and 0.3% saponin in PBS (FSP) for 20 minutes at room temperature. The stock saponin solution was 3% (w/v) solution in PBS, filtered through a 0.22μm filter and stored at 4°C. 5x 10^5 cells were stained for each sample. The method was as described for staining cell surface epitopes except cells were resuspended in FSG instead of medium and after the final antibody incubation, keratinocytes were fixed in 1% paraformaldehyde for 5 minutes at room temperature. Keratinocytes were resuspended in 200μl PBS and analyzed on a Becton-Dickinson FACScan machine (Gandarillas and Watt 1997).

2.2. Biochemistry

2.2.1. Protein Lysis

2.2.1.1. Extraction of Total Proteins

SDS extraction buffer was used. It comprised 1% SDS, 2mM $CaCl_2$ and 15mM Tris-HCl, pH7.5. Cells were washed twice in PBS and lysed for 10 minutes on ice. The cells were scraped from the Petri dishes, boiled for 5 minutes and centrifuged at 14,000 rpm for 5 minutes. The protein lysates were assayed for protein content (Section 2.2.1.4.). 1mM PMSF and 1 μg/ml leupeptin were then added and the lysates were aliquoted and stored at -70°C until use.

2.2.1.2. Extraction of Proteins for MAPK and FAK Assays

Newly confluent human keratinocytes were serum and growth factor starved for 1h and harvested using versene/trypsin solution. Cell pellets were washed 3 times in serum-free medium before any further manipulations. The cells were either suspended in serum-free medium in an end over end mixer for 30 minutes or plated onto dishes pre-coated with fibronectin (25 μg/ml) and incubated at 37°C for 30 minutes. After incubation cells were washed twice with ice cold PBS and lysed in a modified RIPA buffer containing 5mM EDTA, 1% Triton X-100, 20μM leupeptin, 0.5 mg/ml soybean trypsin inhibitor, 0.5mM $NaVO_3$, 10 mg/ml p-nitrophenylphosphate and 1mM PMSF. The cell lysates were sonicated at 4°C for 30 seconds and the cell debris was pelleted by centrifugation at 14,000rpm for 10 minutes at 4°C. In some experiments cells were harvested and the cell pellet was extracted directly. Protein content of the lysates was determined. Cell lysates were then aliquoted and stored at -70°C until use.

2.2.1.3. Bradford Assay for Measuring Protein Concentration

This assay is based on binding of the dye Coomassie Brilliant Blue G-250 to protein which causes a shift in the absorption maximum of the dye from 465nm to 595nm. Reagents for the assay were obtained from Bio-Rad. A standard curve was prepared by making triplicate dilutions of a standard protein solution (BSA, 2 mg/ml, Pierce). Lysis buffer was used as the "blank" and to dilute samples. Samples were applied neat, 1:2 dilution and 1:5 dilution. 5μl protein standard or sample were added into triplicate wells in a 96-well plate (Immulon Dynatech). The assay was carried according to

manufacturer's instructions. Since detergent (Triton X-100 or SDS) was present in the lysis buffer, 20µl reagent S was added to 1ml reagent A. 25µl working reagent A was added to each well. 200µl reagent B was then added to each well. The plate was gently agitated and incubated for 15 minutes at room temperature. Any bubbles present in the wells were dispersed. The $O.D._{690}$ was measured for the contents of each well on a Titertek Multiscan MCC/340 MKII spectrophotometer. The protein concentration of sample was determined against the standard curve.

2.2.2. SDS-PAGE and Immunoblotting

2.2.2.1. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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 buffer had additional 10% (v/v) β-mercaptoethanol.

SDS-PAGE

Vertical gel electrophoresis apparatus systems (Model SE400, Hoefer Scientific Instruments) were used. 1.5mm thick gels were prepared between glass plates using the method of Laemmli (Laemmli 1970). The composition of the gels are presented in Table 2.6. After pouring the resolving gel solution, 1ml of water saturated butanol was applied immediately to ensure a level surface as well as to eliminate an air-acrylamide interface. Gels were allowed to polymerize at room temperature for a minimum of 1h. The overlaying butanol was discarded, and the stacking gel solution was poured. A 1.5mm thick comb was inserted to create wells and then the gel was left to polymerize. After the gel had set, the comb was removed and the wells were flushed with SDS-PAGE running buffer which comprised 50mM Trizma base, 384mM glycine and 0.1% SDS. Samples were applied to the wells using capillary pipette tips. 10µl pre-stained rainbow molecular weight markers (Amersham) were added to 10µl Laemmli sample buffer, boiled for 5 minutes and then loaded into one of the wells. Samples were separated by electrophoresis overnight at 50- 60V. When the dye front reached the end of the gel, the gel was removed and prepared for Western blot.

Table 2.6. Preparation of SDS-PAGE gels

Stock solutions

Solution A	30% acrylamide / 0.8% bisacrylamide (Millipore), 4°C
Solution B	3M Tris-HCl, pH8.8 (BDH)
Solution C	10% SDS
Solution D	2M Tris-HCl, pH6.8 (BDH)
AP	10% ammonium persulphate (Bio-Rad) in dH_2O, freshly prepared
TEMED	N,N,N',N'-tetramethylethylenediamine (Bio-Rad)

Resolving gels (30ml)

stock solutions

	A	B	C	dH_2O	TEMED	AP
5%	5ml	3.75ml	0.3ml	20.95ml	30µl	300µl
7.5%	7.5ml	3.75ml	0.3ml	18.45ml	30µl	300µl
10%	10ml	3.75ml	0.3ml	15.95ml	30µl	300µl
12.5%	12.5	3.75ml	0.3ml	13.45ml	30µl	300µl

Stacking gel (10 ml)

stock solutions

	A	C	D	dH_2O	TEMED	AP
	1.5ml	100µl	0.625ml	7.775ml	10µl	100µl

2.2.2.2. Western Blotting

Semi-dry transfer buffer

10x stock solution comprised 0.2M Tris-HCl, pH7.5 and 1.5M Glycine. The solution was filtered through 0.22µm filter and stored at room temperature. The semi-dry transfer buffer was made by diluting the stock solution 10 fold in 0.1% SDS and 20% (v/v) absolute methanol.

Semi-dry transfer protocol

20- 50µg protein lysate were loaded per lane on an SDS-PAGE gel and separated. After electrophoresis, proteins were transferred onto a Millipore Immobilon PVDF (polyvinyldifluoride) membrane (pre-wet in absolute methanol) using a semi-dry

transfer unit (Millipore MilliBlot graphite electroblotter I system, Hoefer Scientific Instruments) at 200mA for up to 1h (Figure 2.1). After transfer, the membrane was stained using a 1:1000 dilution of Indian ink in PBS containing 0.1% Tween-20 (PBST) for 15 minutes to check whether the transfer had worked. The membrane was rinsed briefly in PBST and then was incubated in 5% milk powder (99% fat free, Premier Brands UK Ltd.) solution in PBST for 1h at room temperature or overnight at 4°C to block non-specific binding of antibodies.

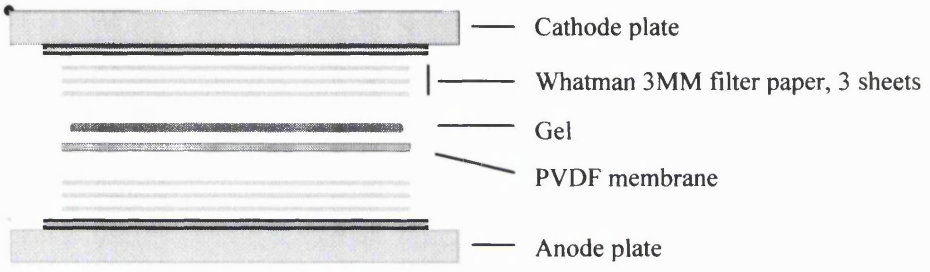


Figure 2.1. Assembly of the transfer stack for semi-dry transfer

Probing with antibodies and detection with ECL

Membranes were incubated with antibody diluted in 2.5% milk powder solution in TBS containing 0.1% Tween-20 (TBST) or PBST. Incubation with primary antibody was for 1h at room temperature or overnight at 4°C with agitation. This was followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham). After each antibody incubation, membranes were washed thoroughly with at least 3 changes of TBST or PBST. Detection of HRP-bound antibody was by chemiluminescence using a kit (ECL, Amersham) according to manufacturer's instructions. X ray films were exposed in autoradiography cassettes lined with reflective screens. If blots were to be reprobed they were stripped for 15-30 minutes at 50°C in a buffer containing 2% SDS, 62.5mM Tris-HCl, pH6.7 and 100mM β-mercaptoethanol and than incubated in 5% milk powder prior to reprobing with antibody.

2.3. Molecular Biology

2.3.1. Bacterial Culture

2.3.1.1. Bacterial Media and Selection Antibiotics Stocks

Brain Heart Infusion (BHI) broth (ICRF)

This comprised 3.7% Brain Heart Infusion (BHI) broth powder (Difco) in dH_2O and the solution was autoclaved.

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 100mm 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 dH_2O) was used as a selection antibiotic and was added to BHI or L-agar to a final concentration of 100 μg/ml.

2.3.1.2. Bacterial Transformation

Buffers I and II for preparing competent bacteria

Buffer I comprised 30mM sodium acetate, pH6.0, 50mM $MnCl_2$, 100mM RbCl, 10mM $CaCl_2$ and 15% (v/v) glycerol. The solution was adjusted to pH5.8, filtered through 0.22μm filter and stored sterile at 4°C. Buffer II comprised 10mM 3-(N-Morpholino)propane-sulfonic acid (MOPS, Sigma), pH7.0, 10mM RbCl, 75mM $CaCl_2$ and 15% (v/v) glycerol.

Preparation of competent bacteria

A single colony of bacteria strain *Escherichia coli* DH5α was inoculated overnight in BHI medium and grown in a 37°C agitator (250rpm). A 2ml overnight culture was used to inoculate 200ml BHI, this was cultured in a 37°C agitator until $O.D._{600}$ was about 0.6 (for 3-4h). The bacteria were pelleted by centrifugation at 3,000rpm for 10 minutes at room temperature. Bacteria were resuspended in ice cold buffer I and incubated on ice for 10 minutes. Bacteria were pelleted again by centrifugation at 3,000rpm for 10 minutes at 4°C. The pellet was resuspended in 4ml ice cold buffer II and aliquoted in eppendorf tubes which were pre-cooled in dry ice. Aliquots were stored at -70°C. Competence was tested by transformation with a known plasmid.

Transformation of bacteria

50μl of competent bacteria were thawed at room temperature and then incubated on ice for 10 minutes. DNA (0.1μg for ligations or 1 ng for plasmids) was added, mixed and left on ice for 20 minutes. The bacteria were then heat-shocked at 42°C for 45 seconds and transferred on ice for 2 minutes. 4 volumes of BHI medium was added and the tube was agitated gently in a 37°C water bath for 40 minutes. 100μl culture was spread out onto plates containing L-agar and 100 μg /ml ampicillin and incubated overnight at 37°C. Single colonies were picked with a sterile loop to inoculate LB medium.

2.3.2. DNA Techniques

All reagents used were of molecular biology grade. Methods and solutions prepared were according to (Sambrook et al. 1989). When possible, solutions were autoclaved after preparation to destroy DNases.

2.3.2.1. General Solutions

Tris/EDTA buffer (TE)

TE was used as a general storage buffer for DNA and comprised 10mM Tris-HCl and 1mM EDTA, pH8.0.

Tris-acetate-EDTA buffer (TAE)

A 50x stock solution was prepared by dissolving 242g Trizma base and 57.1ml glacial acetic acid (BDH) in dH_2O. 100ml 0.5M EDTA, pH8.0 were added and the final volume was made up to 1l.

Plasmid preparation solutions I, II, and III

Solution I comprised 50mM glucose, 25mM Tris-HCl, pH8.0 and 10mM EDTA pH8.0 and 5 mg/ml lysozyme (Sigma). Solution II comprised 0.2M NaOH and 1% (w/v) SDS and was prepared freshly. Solution III comprised 3M potassium acetate and 11.5% (v/v) glacial acetic acid to give pH4.8.

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 80-100V in 1x TAE buffer.

DNA loading buffer

6x DNA gel loading buffer comprised of 0.25% bromophenol blue (Sigma), 0.25% xylene cyano (Sigma) and 30% (v/v) glycerol in dH_2O. The loading buffer was stored at 4°C.

2.3.2.2. General DNA Techniques

Phenol/chloroform extraction

This was used to remove protein from preparations of DNA. 1 volume of phenol (pH7.8-8)/chloroform/isoamyl alcohol (25:24:1 by volume, Amresco) was mixed with 1 volume of DNA solution, vortexed thoroughly and centrifuged at 14,000rpm for 5 minutes at room temperature. As much as possible of the upper aqueous phase (DNA) was removed without disturbing the interface. Recovered DNA solution was then further mixed with equal volume of chloroform and extracted as for phenol/chloroform/isoamyl alcohol.

Ethanol precipitation of nucleic acids

To DNA solution, sodium acetate (pH5.2) was added to a final concentration of 0.3M. The solution was mixed thoroughly before 2 volumes of ice cold ethanol was added, mixed thoroughly and left at -20°C to precipitate for at least 30 minutes. DNA was pelleted by centrifugation at 14,000rpm for 10 minutes at room temperature, washed with 70% ethanol, air dried and dissolved in TE, pH8.0 or dH_2O.

Quantitation of nucleic acids

Nucleic acids solution was diluted and placed in a clean quartz cuvette with a path length of 1cm. The absorbance at wavelengths 260nm and 280nm was read in a spectrophotometer (Pharmacia Biotech, Ultrospec 2000). An $O.D._{260}$ reading of 1 corresponded to 50 μg/ml double stranded DNA, 40 μg/ml RNA and 33 μg/ml oligonucleotides.

Preparation of plasmid DNA

To screen colonies after transformation, single colonies were picked and inoculated in LB-media containing a suitable antibiotic, grown overnight in a 37°C agitator. Plasmid DNA was isolated from the pelleted bacteria by resuspending in 100μl ice cold solution I, followed by addition of 200μl solution II and mixing, then 150μl solution III and mixing, centrifugation at 14,000rpm for 10 minutes at room temperature, and finally precipitation with 0.7 volumes of isopropanol. Precipitated DNA was washed in 70% ethanol, air dried and finally dissolved in 50μl TE, pH8.0 containing 100 μg/ml RNase A (Sigma).

For maxi preparations, 0.5ml of the 10ml overnight culture were added to 100ml LB medium containing suitable antibiotic and grown overnight in a 37°C agitator. Bacteria were pelleted by centrifugation at 6,000rpm for 15 minutes at 4°C in a Beckman J2-21 centrifuge. The plasmid DNA was purified using a Qiagen plasmid maxi kit (Qiagen Ltd.) according to the manufacturer's instructions.

List of cDNA constructs

All the cDNA constructs used for generation of recombinant retroviral vectors are listed in Table 2.7.

Table 2.7. List of cDNAs used

Plasmid	Encoded protein	Reference
pBabepuro-CD8	full length CD8α (CD8)	(Zhu et al. 1999)
pBabepuro-CD8β1	CD8α/β1A integrin (CD8β1)	(Zhu et al. 1999)
pBabepuro-MAPKK1	mutant MEK1 with S? E substitutions at positions 217 and 221 (MAPKK1)	(Cowley et al. 1994)
pBabepuro-MANA	mutant MEK1 with S? A substitution at position 221 (MANA)	(Cowley et al. 1994)
pBabepuro-β1 integrin	wild type chicken β1 integrin chain	(Levy et al. 1998)

2.4. List of Suppliers and Distributors

Advanced Protein Products Ltd. Brockmoor, West Midlands, UK.
American Tissue Culture Collection (ACTT), Rockville, Maryland, USA.
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 Products Ltd., Elstree, UK.
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.
Costar Europe Ltd. Badhoevedorp, Holland.
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.
European Collection of Animal Cell Cultures (ECACC), Salisbury, UK.
Flow Laboratories Ltd., Aryshire, Scotland, UK.

Gibco BRL/Life Technologies Ltd. Paisley, Renfrewshire, UK
Hoefer Scientific Instruments is distributed by Biotech Instruments Ltd., Beds, UK.
ICN Pharmaceuticals Ltd. Thame, Oxon, UK.
Imperial Laboratories (Europe) Ltd. Andover, Hampshire, UK.
Immunlon Dynatech, Chantilly, Virginia, USA.
Integrated Separation Systems, Natick, Maryland, USA.
Jackson Immunoresearch Laboratories, Luton, Bedfordshire, UK
Monsanto Chemicals. Springfield, Massachusetts, USA.
Millipore, Harrow, Middlesex, UK
New England Biolabs (NEB) UK Ltd. New York, USA.
Nunc A/S, Roskilde, Denmark.
Perkin-Elmer Co. Foster City, Carlifornia, USA.
Pharmacia Biotech. Uppsala, Sweden.
Pharmingen, San Diego, Carlifornia, USA.
Pierce, Rockford, Illinois, USA.
Qiagen Ltd. Crawley, UK.
Santa Cruz Biotech. Inc. Santa Cruz, Carlifornia, USA.
Serotec Ltd. Kidlington, Oxford, UK.
Sigma Chemical Co. Poole, Dorset, UK.
Transduction Laboratories, Lexington, Kentucky, USA.
Whatman International Ltd. Maidstone, Kent, UK
Dr K. Aktories, Institute of Biochemistry, University Freiburg, Germany
Dr B. Arnold, German Cancer Research Center, Heidelberg, Germany.
Dr P. C. Beverley, The Jenner Institute, Compton, Berkshire, UK.
Dr K. Hartmann, University of Cologne, Germany
Dr A. Jetten, NIEHS, Research Triangle Park, USA
Dr I. Leigh, London Hospital, UK
Dr C. J. Marshall, Institute of Cancer Research, London, UK.
Dr. R. Rice, University of California, Davis, USA
Dr K. Yamada, National Institute of Dental Research, Bethesda, Maryland, USA.

CHAPTER 3

SIGNALLING OF β1 INTEGRINS TO MAP KINASE REGULATES KERATINOCYTE PROLIFERATIVE POTENTIAL

Human epidermis contains two types of proliferating keratinocyte: epidermal stem cells and transit amplifying cells. These two populations can be distinguished on the basis of their expression level of β1 integrins. It is not clear, however, whether the surface level of integrins is only a marker for stem cells or whether there is a causal relationship between integrin expression and function, on the one hand, and keratinocyte proliferative potential, on the other. Should integrin function be critical for determination of keratinocyte proliferative potential then this should be reflected by the activity of integrin dependent signalling pathways within the cell. I therefore set out to investigate integrin mediated signalling in keratinocytes.

3.1. Integrin mediated activation of MAPK in primary human keratinocytes

A good candidate for an integrin derived signal that could be able to regulate proliferative potential was the classical MAP kinase cascade since it had been shown to be activated upon integrin engagement in NIH3T3 fibroblasts (Chen et al. 1994) and also to be a key pathway involved in the regulation of cell proliferation and differentiation (Cowley et al. 1994). I therefore carried out experiments investigating the role of integrins in the regulation of MAP kinase activity in primary human keratinocytes. I trypsinised cultured keratinocytes and kept them in suspension or plated them onto dishes coated with different ECM molecules: collagens I and IV, fibronectin, keratinocyte ECM enriched for laminin 5, or with an antibody to β1 integrins, mAb 13 or with non specific substrate to which cells adhere without integrin engagement, poly-L- lysine (PLL). As a readout for MAP kinase activity I determined phosphorylation of the enzyme by Western Blot using an antibody that only recognises the phosphorylated, not the unphosphorylated, form (MAPK-P183/185, New England Biolabs; upper panel in Fig. 3.1). In order to control for equal loading of the gels membranes were stripped and incubated a second time with an antibody that recognises both phosphorylated and

unphosphorylated forms of MAP kinase (lower panel). Phosphorylation of MAP kinase at threonine183 and tyrosine185 always correlates with activation of the enzyme (C. Marshall, personal communication).

I found that in suspended keratinocytes and in keratinocytes plated onto PLL MAPK phosphorylation was very low and in some experiments undetectable. In contrast, plating of keratinocytes onto dishes coated with collagens I or IV, fibronectin, keratinocyte laminin (laminin 5) or the monoclonal antibody mAb 13 resulted in strong activation of MAP kinase (Fig. 3.1). These results indicate that MAPK is activated in keratinocytes upon engagement of β1 integrins. Since keratinocytes can adhere to laminin 5 via the α6β4 and α3β1 integrins I blocked β1 integrin function by addition of the antibody P5D2 at 100 μg/ml allowing adhesion only via α6β4 (Levy et al. 2000). In this situation MAPK was still activated, indicating that keratinocytes can support activation of MAPK by adhesion to both β1 integrin and α6β4 integrin ligands. The experiments shown in Fig. 3.1 were carried out in the presence of 10 ng/ml EGF in the medium. Similar results were obtained in the absence of EGF though in those experiments the extent of MAP kinase phosphorylation was generally lower (data not shown).

I also studied the kinetics of MAPK activation through β1 integrin engagement by plating primary keratinocytes onto fibronectin coated dishes and analysing MAPK phosphorylation at different time points after plating. Phosphorylation was strongest 30 minutes after plating, the earliest time point examined, and still found to be increased after two hours (Fig. 3.1 b). Four hours and eight hours after plating MAPK phosphorylation was as low as in suspended keratinocytes. These results show that MAPK activation is dependent on integrin engagement in keratinocytes and that integrin engagement causes a transient increase in MAPK activation.

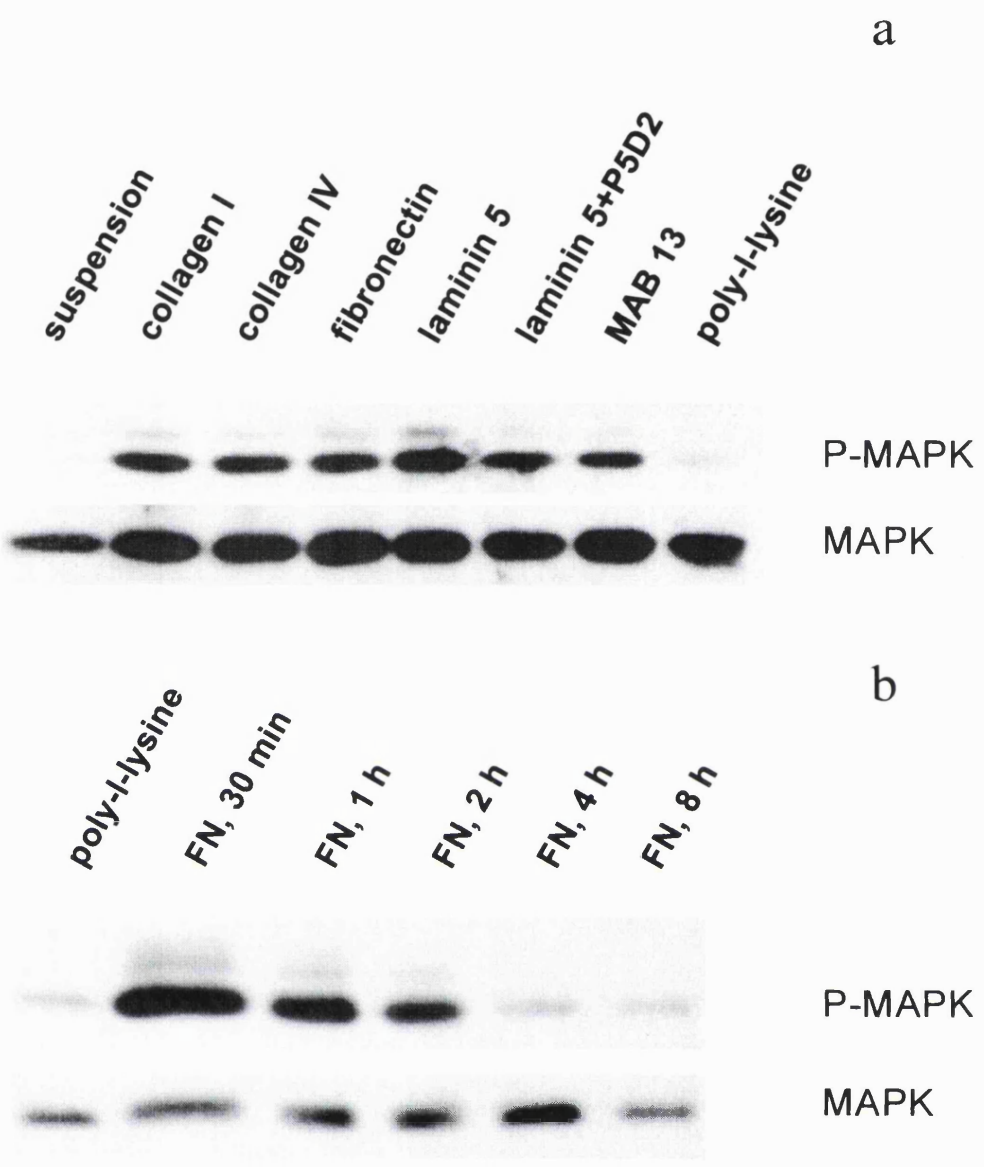


Figure 3.1: Adhesion dependent phosphorylation of MAPK.

Western blot analysis of MAPK phosphorylation: upper panels show threonine/tyrosine phosphorylation of ERK 1/2 visualised with a phosphorylation-specific antibody; lower panels show the same blots incubated with an antibody against total ERK2 as a loading control. **a**: human keratinocytes in FAD medium containing 10 ng/ml EGF were held in suspension or plated for 30 minutes onto dishes coated with poly-L-lysine, the ECM proteins shown or the antibody mAb13. Keratinocytes were also plated onto a laminin coated dish in the presence of 100 µg/ml of the adhesion blocking antibody to the β1 integrin chain, P5D2. **b**: Keratinocytes were plated onto dishes coated with poly-L-lysine or fibronectin (FN) and MAPK phosphorylation was analysed at different time points after plating.

3.2. Inhibition of β1 integrin mediated signalling to MAPK by CD8β1

Investigation of integrin functions in keratinocyte proliferation and early differentiation had been hampered by the fact that detachment is a very potent stimulus for keratinocytes to undergo terminal differentiation. In order to answer the question whether in adherent keratinocytes β1 integrin function was necessary to maintain proliferative potential Alan Zhu, a PhD student in the laboratory, generated retrovirally infected lines of primary human keratinocytes expressing a chimeric receptor that consisted of the cytoplasmic domain of the β1A integrin subunit and the extracellular domain of the CD8 molecule (Bishop et al. 1998; Zhu et al. 1999). Such proteins have been shown earlier to trans- dominantly inhibit integrin functions partially (LaFlamme et al. 1992; Chen et al. 1994; LaFlamme et al. 1994; Lukashev et al. 1994; Smilenov et al. 1994).
Using this approach Alan was able to demonstrate that CD8β1, but not full length CD8 expressed as a control, could:

1. inhibit the adhesive function of the integrins α2β1 and α5β1, but not α6β4,
2. reduce the β1 integrin expression level, a marker for epidermal stem cells,
3. dramatically reduce proliferative potential of the expressing keratinocytes,

and that adhesiveness and proliferative potential were reversed by overexpressing a functionally active wild type chicken integrin.
These results demonstrate that β1 integrins are not only a marker for keratinocytes with high proliferative potential but that their function is required in order to maintain this potential (Zhu et al. 1999).

It is thought that chimeric proteins consisting of the intracellular domain of an integrin and an irrelevant extracellular domain act through competitive binding of limited quantities of intracellular effector molecules (Chen et al. 1994; Lukashev et al. 1994). I was interested in what effect inhibition of integrin function by CD8β1 would have on MAPK activation. I therefore determined whether activation of MAPK or, in comparison FAK, which also acts downstream of β1 integrins (Zachary and Rozengurt 1992) was affected by expression of the CD8β1 chimera.

Primary human keratinocytes were infected with retroviruses encoding CD8β1 or CD8 as a control (Zhu et al. 1999). Retroviral infection of human keratinocytes is highly efficient and, when used in combination with a selectable marker, leads to stable transduction of the entire cell population (Levy et al. 1998). To analyse the effect of the CD8β1 chimera on activation of FAK or MAPK, keratinocytes expressing puro, CD8 or CD8β1 were starved in serum- and growth factor-free medium and then either left in suspension or plated on fibronectin for 30 minutes after which phosphorylation of each kinase was measured by immuno-blotting with phosphorylation-specific antibodies. Blots were also probed with antibodies to FAK or $p42^{mapk}$ (Erk2) as loading controls. There was no activation of FAK or MAPK in suspended cells of all three populations (Figure 3.2 c and data now shown). FAK tyrosine phosphorylation on fibronectin was the same between keratinocytes expressing puro, CD8 or CD8β1 (Figure 3.2 a). The level of MAPK tyrosine/threonine phosphorylation was the same in puro- and CD8-expressing cells, but was reduced approximately two fold, as estimated by densitometry, in CD8β1-expressing cells (Figure 3.2 b, left panel). When the level of functional β1 integrins on the surface of CD8β1 expressing keratinocytes was elevated by expression of a wild type chicken integrin MAPK activation was again increased (Figure 3.2 b, right panel, CD8β1+wt).

In order to determine whether the reduction in MAPK activation in CD8β1 cells was specifically associated with β1 integrin signalling I examined the MAPK response of CD8β1-expressing keratinocytes to α6β4-mediated adhesion (Mainiero et al. 1997) and to stimulation with mitogens (Marshall 1995). When keratinocytes were plated on ECM enriched for laminin 5 for 30 minutes the level of MAPK phosphorylation was the same in CD8- and CD8β1-expressing cells (Figure 3.2 c). MAPK activation on fibronectin declines to a basal level within 4 hours (Figure 3.1 b, 3.2 c). When keratinocytes were plated on fibronectin for 4 hours in serum-free medium and then treated with complete medium containing serum and growth factors (FCS/HICE) for 15 minutes the level of MAPK activation was the same in CD8- and CD8β1-expressing keratinocytes (Figure 3.2 c).

These results demonstrate that MAPK activation upon adhesion of keratinocytes via α6β4 is not reduced in CD8β1 expressing cells. They also show that the CD8β1 construct does not inhibit MAPK in adherent cells when these are stimulated with growth factors contained in complete keratinocyte culture medium (FAD+FCS+HICE).

Thus CD8β1 specifically inhibits MAPK activation in response to β1 integrin mediated adhesion.

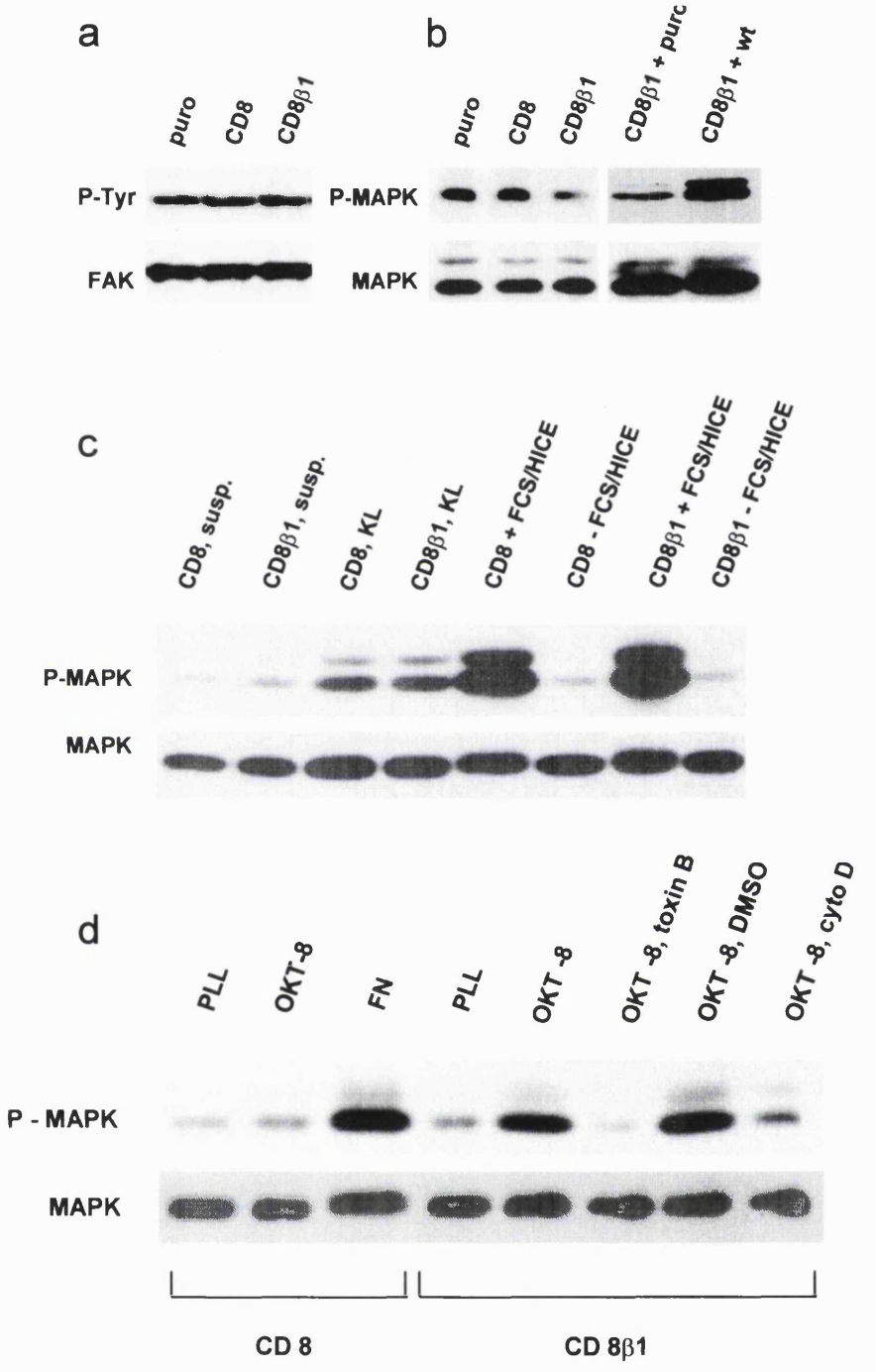


Fig. 3.2 FAK and MAPK phosphorylation in CD8-, CD8β1- and CD8β1+wt-expressing cells.

Western blots showing tyrosine phosphorylation of FAK (P-Tyr) (**a**) and threonine/tyrosine phosphorylation of MAPK (P-MAPK) (**b-d**). The lower panels are loading controls for FAK (**a**) and MAPK (in b a doublet of ERK1 and ERK2 is detected). **a**, **b**. Cells were plated onto fibronectin coated dishes for 30 minutes in serum-free medium. **c**. Cells were held in suspension for 30 minutes (susp.); plated on dishes coated with keratinocyte ECM for 30 minutes in serum-free medium (KL); or

plated on dishes coated with 25 μg/ml fibronectin in serum-free medium for 4 hours and then treated with fresh serum-free medium (-FCS/HICE) or complete medium, containing serum and growth factors (+FCS/HICE), for 15 minutes. **d.** Keratinocytes infected with CD8β1 or CD8 were plated onto dishes coated with poly-L-lysine (PLL), fibronectin (FN) or an antibody against the CD8 extracellular domain (OKT-8) in medium containing 10 μg/ml EGF without further additives or in the presence of 4 μM cytochalasin D or 0.1% DMSO as a solvent control for 30 minutes. Toxin B: CD8β1 expressing keratinocytes were pre-treated with 5ng/ml toxin B from Clostridium difficile, washed and plated on an OKT-8 coated dish in the presence of 10 μg/ml EGF.

The finding that CD8β1 was able to interfere with integrin signalling to MAPK suggested that the intracellular part of the construct could mimic the cytoplasmic tail of an integrin and probably bind to intracellular integrin effectors. This led me to investigate whether ligating the extracellular domain of CD8β1 using an antibody would elicit a similar signalling response as integrin binding to ECM. I therefore compared MAPK phosphorylation in cells expressing CD8β1 or CD8 when plated onto dishes coated with OKT-8 antibody, fibronectin or poly-L-lysine (Figure 3.2 d). I also pre-treated cells with cytochalasin D, an inhibitor of actin polymerisation, and toxin B from Clostridium difficile which inhibits small GTPases of the rho family by glucosylation (Aktories et al. 2000). Keratinocytes expressing CD8 showed a low level of MAPK phosphorylation when plated onto PLL or OKT-8 antibody. This was not due to a general disability of these cells to activate MAPK since plating on fibronectin coated dishes led to a strong phosphorylation of MAPK (Figure 3.2 d). CD8β1 expressing cells also showed a low level of MAPK activation when plated on PLL, but in contrast to CD8 expressing cells activated MAPK upon plating to OKT-8 antibody. This CD8β1 stimulated MAPK phosphorylation was diminished when keratinocytes were preincubated with cytochalasin D or toxin B. This result demonstrates that CD8β1 can directly connect to signalling pathways that regulate MAPK activity and that actin polymerisation and activity of Rho GTPases are involved in this regulation.

3.3. Modulation of MAPK activity in keratinocytes using retroviral vectors

Since expression of CD8β1 reduced the level of MAPK activation, but not FAK phosphorylation, it was logical to ask whether reduced MAPK activity was responsible for the effects on proliferative potential observed upon expression of CD8β1. I

therefore sought a way to activate or inhibit MAPK independently of integrin engagement.

The only mechanism known so far to activate MAPK physiologically is through phosphorylation of threonine 183 and tyrosine 185 by its upstream regulating kinase, MAP kinase kinase (MEK), which is a dual specific kinase (Canagarajah et al. 1997). MEK itself is regulated by an upstream serine kinase, Raf-1, that catalyses phosphorylation of Ser 217 and Ser 221 of MEK1 (Alessi et al. 1994). The two serine residues are critical for MEK1 activation and mutation of these sites partially uncouples MEK1 from upstream regulatory events. Mutation of Ser 217 and 221 to glutamic acid leads to constitutive activation of MEK1 and substitution of Ser 221 by alanine to dominant inhibition (Cowley et al. 1994). The cDNAs of these mutants, cloned into the retroviral vector pBabepuro, were kindly provided by Prof. C.J. Marshall, Institute of Cancer Research, London. I named the corresponding protein products MAPKK1 (for the constitutively active mutant) and MANA (for the dominant inhibitory mutant). Using these retroviral constructs I established ecotropic and amphotropic retroviral producer lines and infected primary human keratinocytes.

Infection of primary keratinocytes with retroviruses encoding MAPKK1 led to a constitutive submaximal phosphorylation of MAPK, lower than that observed upon stimulation of keratinocytes expressing the empty vector puro with complete FAD medium (FAD + FCS/HICE) for 15 minutes (Fig. 3.3 a). The increased phosphorylation was not transient and not dependent on cell adhesion: in freshly trypsinised keratinocytes (start) or keratinocytes kept in suspension culture for 24 hours (susp) MAPK phosphorylation was still elevated compared to cells expressing empty vector puro (Fig. 3.3 b). Conversely, keratinocytes expressing MANA showed reduced phosphorylation of MAPK as compared to control cells upon adhesion to fibronectin (Fig. 3.3 d) or after stimulation with complete FAD medium (Fig. 3.3 a).

Using the constitutively active mutant of MEK I had established an experimental system that allowed activation of the MAPK pathway independently of cell adhesion. Since I had observed decreased activation of MAPK in cells expressing the CD8β1 chimera I tried to „repair" this signalling defect by introducing MAPKK1 into keratinocytes expressing CD8β1 or the CD8 control. This was achieved by double infection of keratinocytes with CD8β1 or CD8 and subsequently with MAPKK1 or empty vector puro. Analysis of lysates prepared from doubly infected, trypsinised cells showed that

indeed introduction of MAPKK1, but not the empty vector puro, led to an elevated level of MAPK phosphorylation in those cells (Fig. 3.3 c). MAPK activation in CD8β1 cells and CD8 cells expressing the MAPKK1 construct was the same and was increased relative to cells doubly infected with the empty vector, puro.

These doubly infected keratinocytes were used to investigate whether expression of activated MAPKK1 could overcome the effects of the CD8β1 chimera on keratinocyte adhesion, integrin expression and proliferative potential that Alan had observed.

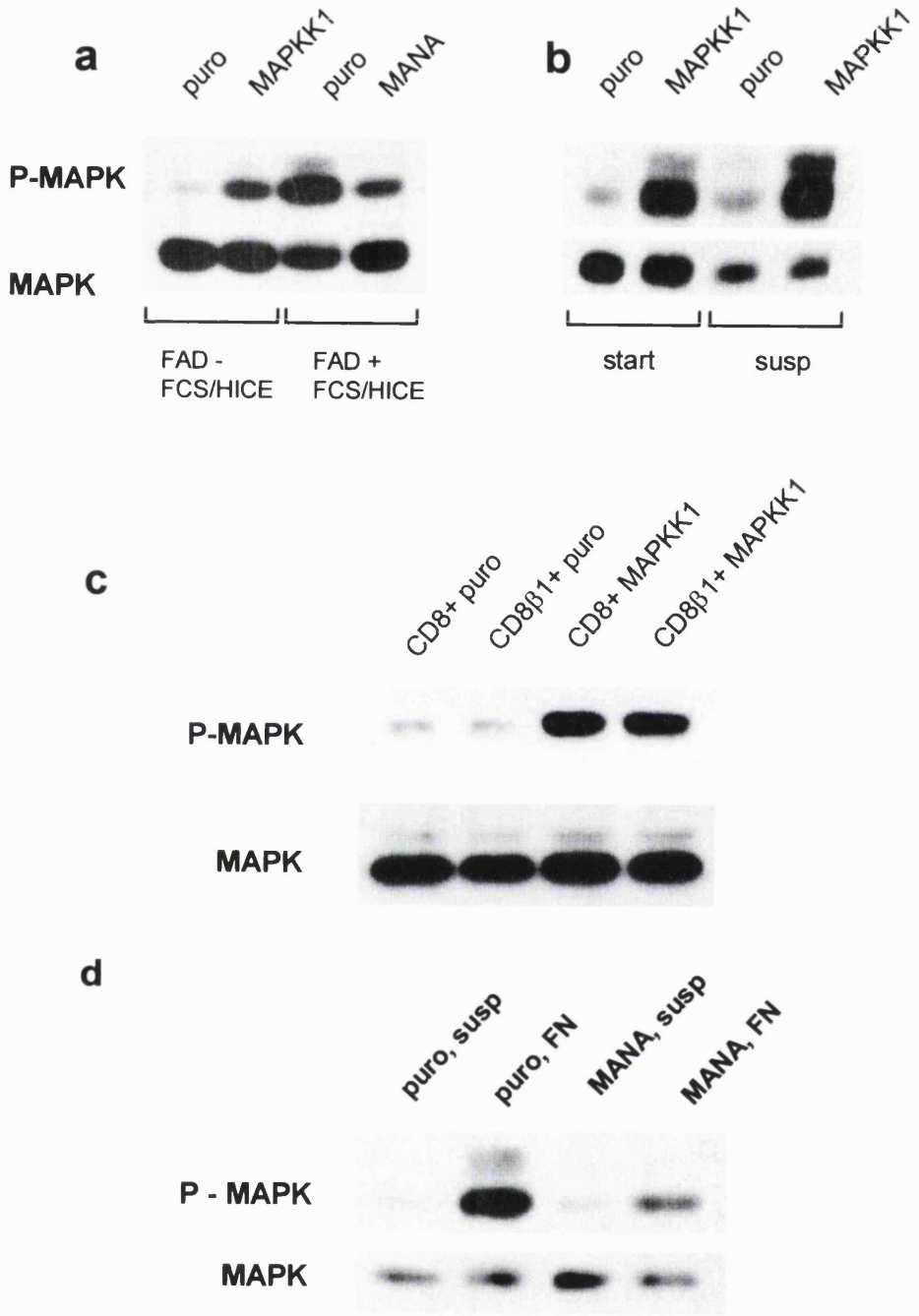


Figure 3.3: Modulation of MAPK phosphorylation by MAPKK1 and MANA.

Western blot detection of MAPK phosphorylation as described in Figure 3.1 legend. **a**: Adherent keratinocytes infected with empty vector puro, MAPKK1 or MANA were starved overnight and lysed 15 minutes after addition of fresh medium with or without FCS/HICE. **b**: Keratinocytes infected with puro or MAPKK1 were trypsinised, held in suspension for 15 minutes (start) or 24 hours (susp) and then lysed. **c**: MAPK activity in pellets of keratinocytes doubly infected with CD8β1/CD8 and MAPKK1 or empty vector puro. **d**: Keratinocytes expressing puro or MANA were held in suspension or plated onto fibronectin coated dishes and then lysed.

3.3.1. Effects of modulating MAPK activity on keratinocyte adhesion

I first tested whether the activated MAPKK1 mutant could restore the adhesiveness of CD8β1 expressing cells to extracellular matrix proteins. For the adhesion assay equal numbers of doubly infected cells were plated onto heat denatured bovine serum albumin, type IV collagen or fibronectin for 3h in the presence of cycloheximide and the number of adherent cells was determined (Figure 3.4 a). Activated MAPKK1 restored adhesiveness of CD8β1-expressing cells to control levels when assayed on type IV collagen and also increased adhesion to fibronectin. In contrast, introduction of activated MAPKK1 into keratinocytes expressing CD8 had no effect on the proportion of cells that attached to fibronectin or type IV collagen. There was no difference in the number of cells attached to bovine serum albumin.

The next parameter affected by introduction of CD8β1 was the level of β1 integrin expression. Surface levels of doubly infected keratinocytes were examined by flow cytometry (Figure 3.4 b,c). There was no effect on surface β1 integrin levels upon introduction of MAPKK1 into CD8-expressing keratinocytes (Figure 3.4 b). In contrast, constitutively activated MAPKK1 increased surface β1 levels in CD8β1-expressing cells to 90% of the control levels (Figure 3.4 c). Cell surface CD8 and α6β4 levels were unaffected by the introduction of MAPKK1 into CD8- or CD8β1-expressing keratinocytes (data not shown).

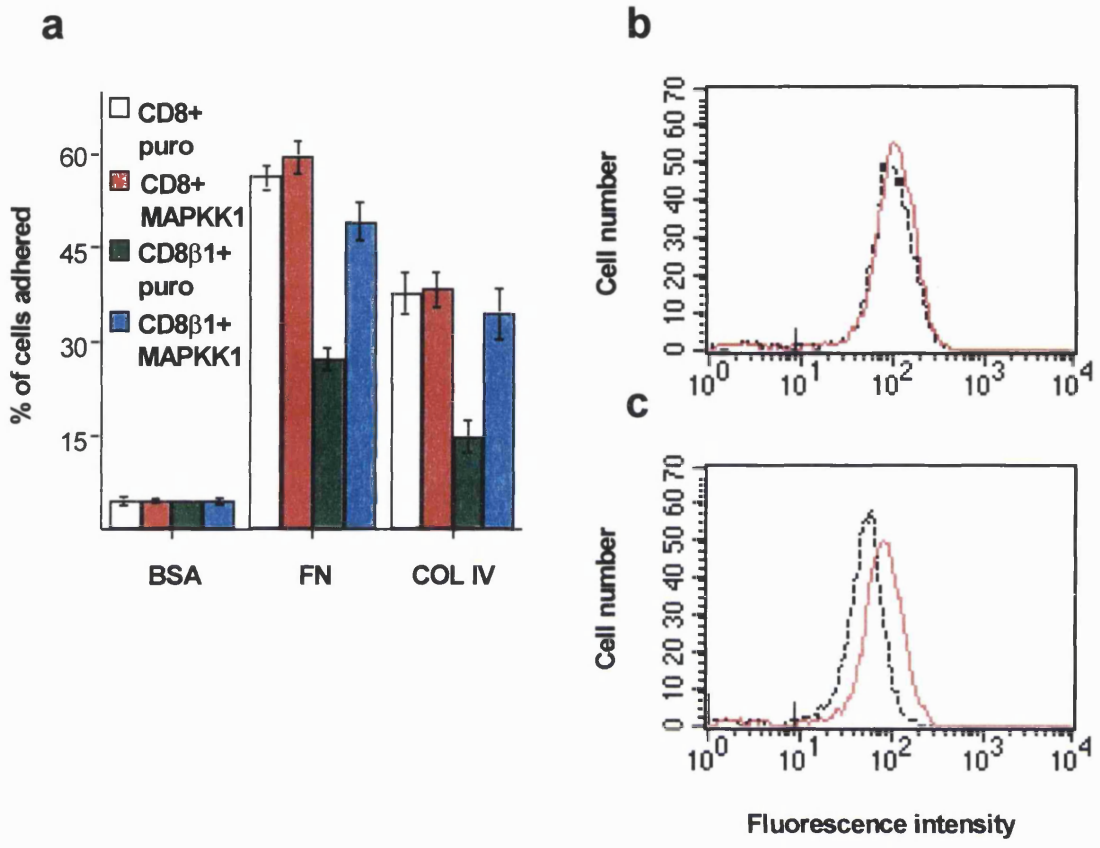


Figure 3.4: Effect of MAPKK1 on adhesiveness and integrin expression.

a: 10^4 doubly infected cells were plated for 3 hours in the presence of 25 μM cycloheximide on 96-well plates coated with heat denatured bovine serum albumin (BSA, 0.5 mg/ml), fibronectin (FN, 25 μg/ml) or type IV collagen (COL IV, 50 μg/ml) and the number of adherent keratinocytes was determined. Error bars represent standard deviation of the mean of triplicate samples within a single experiment. **b, c**: Flow cytometry analysis of surface β1 integrin levels on doubly infected cultured keratinocytes. Black lines: CD8- (b) or CD8β1- (c) expressing cells infected with puro. Red lines: CD8- (b) or CD8β1- (c) expressing cells infected with MAPKK1. Marks on x axes show maximum fluorescence of cells labelled with a control K9-18-FITC antibody.

Expression of activated MAPKK1 increased the adhesiveness and integrin surface levels of keratinocytes expressing CD8β1 but not of control keratinocytes. In order to test a causal role for MAPK signalling in the effects observed upon expression of CD8β1 I wanted to investigate whether reducing MAPK activity in keratinocytes that had not been transduced with CD8β1 was sufficient to reduce adhesiveness and β1

integrin surface levels. For this purpose I used primary human keratinocytes that I transduced with the dominant negative MEK mutant, MANA.

Expression of MANA using retroviruses from a highly expressing AM12 clone reduced MAPK phosphorylation by approximately 50% when keratinocytes where plated onto fibronectin coated dishes (Fig. 3.3 d). This reduction is comparable with the efficiency of MANA in inhibiting MAPK activity that has been reported previously (Cowley et al. 1994).

I carried out adhesion assays in which I compared adhesiveness of MANA infected keratinocytes with that of cells infected with the empty vector puro. MANA reduced the adhesiveness of keratinocytes plated on fibronectin or type IV collagen by more than 50% (Figure 3.5 a). In addition, expression of MANA led to a decrease in cell surface β1 integrin levels (Figure 3.5 b) of the same order of magnitude as observed with CD8β1 (Figure 3.4 b).

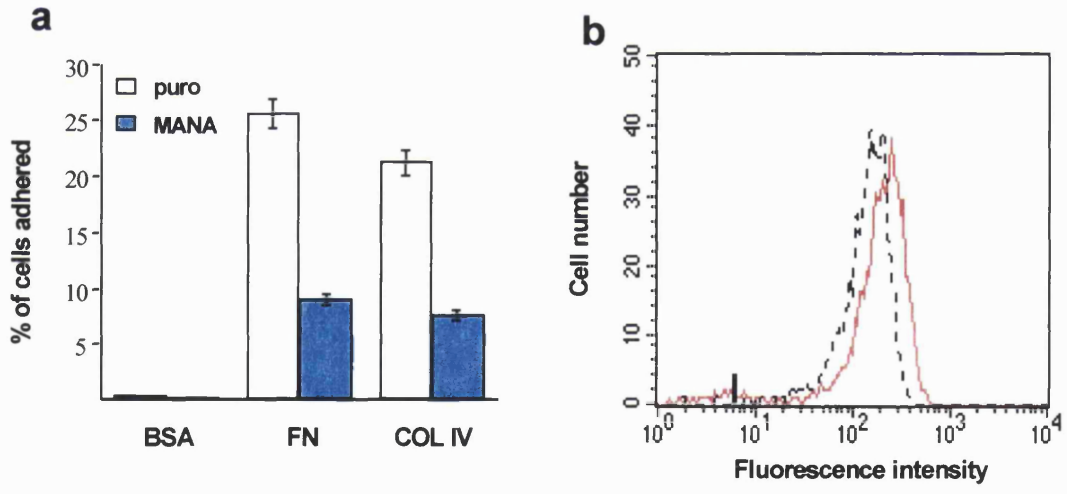


Fig. 3.5 Effects of MANA on adhesion and integrin expression.

a: 10^4 cells expressing puro or MANA were plated for 3 hours on heat denatured bovine serum albumin (BSA, 0.5 mg/ml), fibronectin (FN, 25 μg/ml) or type IV collagen (COL IV, 50 μg/ml) and the number of adherent keratinocytes was determined. Error bars represent standard deviation of the mean of triplicate samples within a single experiment. **b**: Flow cytometry analysis of surface β1 integrin levels on basal keratinocytes. Red line: puro-expressing cells; black line: MANA-expressing cells. Mark on x axis shows maximum fluorescence of cells labelled with UCHT4-FITC antibody as a control.

3.3.2. Effects of MAPK activity on keratinocyte proliferative potential at clonal density

Colony forming efficiency (CFE, clonogenicity) of keratinocytes is a functional parameter for their proliferative potential. CD8β1 severely compromises this function by stimulating commitment to terminal differentiation. This results in the formation of a high proportion of small, abortive colonies in cultured keratinocytes expressing CD8β1 (Zhu et al. 1999). I carried out clonogenicity assays in order to determine whether constitutive activation of MAPK could overcome the effects of CD8β1 on proliferative potential. Table 3.1 shows results from doubly infected cells either expressing CD8β1 + MAPKK1 or empty vector puro, or expressing CD8 + MAPKK1 or puro. The first two rows show the results for CD8 expressing cells. Infection with MAPKK1 had no effect on the total number of colonies formed (middle column) and no effect on the proportion of abortive, transit amplifying, colonies (right column). Rows 3 and 4 show the results for CD8β1 expressing keratinocytes with or without MAPKK1. In these cells activated MAPKK1 increased the total number of colonies founded by plated cells (middle column) and reduced the proportion of abortive colonies to the same levels as in cells expressing the full length CD8 (right column).

	% colony forming efficiency	% abortive colonies
CD8 + puro	10.9 ± 0.5	27.4 ± 2.4
CD8 + MAPKK1	11.6 ± 1.0	28.1 ± 3.6
CD8β1 + puro	4.1 ± 0.3	53.9 ± 3.9
CD8β1 + MAPKK1	10.4 ± 1.2	25.3 ± 2.9

Table 3.1 Effect of the constitutively activated MAPKK1 mutant on colony formation.
Clonogenicity assays were carried out with equal numbers of doubly infected keratinocytes (CD8 + puro, CD8 + MAPKK1, CD8β1 + puro, CD8β1+ MAPKK1) were plated on 35mm dishes and were cultured for 2 weeks. The cultures were fixed and stained with rhodanile blue. All visible colonies (i.e. >1 cell) were scored and colony forming efficiency was calculated as % of plated cells that formed colonies. Colonies that were less than 0.4mm in diameter and contained fewer than 40 cells, the majority of which were large and terminally differentiated, were classified as abortive

colonies. Data are means of triplicate dishes from a typical experiment. Experiment was carried out 3 times.

The results shown in Table 3.1 raised the question whether MAPK activity was required in order to maintain a high proliferative potential in keratinocytes. To answer this question I used primary keratinocytes infected with MANA and analysed their colony forming abilities. The results of these clonogenicity assays are shown in Table 3.2. MANA reduced total colony forming efficiency (middle column) and increased the proportion of abortive, transit amplifying clones (right column) to the same extent as introduction of CD8β1 (Table 3.1). Inhibition of MAPK activity by introduction of MANA thus had similar consequences on proliferative potential of keratinocytes as compared to the CD8β1 construct.

	% colony forming efficiency	% abortive colonies
puro	28.3 ± 1.4	27.5 ± 2.4
MANA	14.5 ± 0.2	52.7 ± 6.1

Table 3.2 Effect of the dominant negative MEK mutant MANA on colony formation.
Clonogenicity assays were carried out with equal numbers of keratinocytes expressing MANA or empty vector puro. Cells were plated on 35mm dishes and cultured for 2 weeks. Colonies were scored as in table 3.1. Data are means of triplicate dishes from a typical experiment. Experiment was carried out 3 times.

3.4. Discussion

Upon engagement of integrins by extracellular matrix molecules signals are generated and sent into the cell. I have investigated one signalling pathway that is known to be stimulated by integrin engagement in other cell types, the MAPK cascade. Under the experimental conditions that I used MAPK was almost undetectable in suspended keratinocytes that were not in contact with extracellular matrix molecules, even in the presence of EGF, and dramatically increased when cells were plated onto dishes coated with ECM ligands for α6β4 or the β1 integrins α2β1, α3β1 and α5β1, or with a monoclonal antibody against the β1 integrin subunit, mAB13. The monoclonal antibody 13 (mAb13) recognises an epitope that is present only in the unoccupied integrin conformation and can inhibit ligand binding by an allosteric mechanism (Mould et al. 1996). The effect of mAb13 on MAPK activation can therefore not be explained by a mechanism in which mAb13 would mimic ligand binding. This is in agreement with the results shown in Figure 3.2 d: a chimeric molecule that lacks the ligand binding site can still promote activation of MAPK signalling. These results are inconsistent with a model in which integrin receptor occupancy by its ligand is required to stimulate MAPK signalling (Miyamoto et al. 1996). They suggest that other consequences of integrin ligation, e.g. receptor aggregation, might be sufficient for transduction of the signal.

Since the standard culture system for primary keratinocytes depends on the addition of EGF to the culture medium and keratinocytes are known to produce growth factors that bind to the EGF receptor in an autocrine manner (Cook et al. 1991; Shirakata et al. 2000) I added 10 ng/ml EGF to the plating medium in order to achieve maximal MAPK stimulation by integrins. The results presented in Fig. 3.1. do therefore not represent a pure integrin signal but rather a signal generated jointly by extracellular matrix molecules and EGF. A synergistic role of integrins and EGF in signalling has been proposed recently (Miyamoto et al. 1996; Renshaw et al. 1997; Moro et al. 1998). In agreement with those results my data show that efficient activation of MAPK signalling in keratinocytes in the presence of EGF requires contact to ECM molecules. Activation of MAPK upon integrin engagement has previously been shown in different cell types including keratinocytes (Chen et al. 1994; Morino et al. 1995; Mainiero et al. 1997). In primary human keratinocytes, Mainiero et al found that MAPK was activated after plating onto laminin 5, which binds both to α6β4 and α3β1 integrins, or an

antibody specific for the α6β4 integrin, but not after plating to collagen I, a ligand for the α2β1 integrin, or an antibody against the α3 integrin subunit. The authors concluded that ligation of α6β4 but not the integrins α2β1 and α3β1 induces MAPK signalling in keratinocytes. This seems to be inconsistent with my results demonstrating MAPK activation after plating onto collagen I. There are, however, major differences between my experimental conditions and those used by Mainiero et al. First, primary human keratinocytes used in the experiments were cultured in different media. Whereas Mainiero et al used a commercial serum- free culture medium with a low calcium concentration in the absence of feeder cells I cultured primary keratinocytes in FAD medium containing 10% FCS at a calcium concentration of 1.8 mM using NIH-3T3 feeders. All feeders were completely removed prior to the plating experiments. Second, plating and ligation experiments carried out by Mainiero et al were always performed in the absence of growth factors with cells that had been kept in medium deprived of growth factors for 36 hours prior to the experiment. In my experiments I usually starved keratinocytes for 2- 4 hours but never for longer than for 12 hours. In my experiments I added 10 ng/ml EGF to the plating medium.

Although primary cells isolated from human tissues, in contrast to established cell lines, are thought to represent the status of the biological system they are derived from more closely, they are exposed to a completely unnatural environment. It seems logical that cellular signalling pathways that transduce signals of the environment into the cell are altered under these conditions. Whereas individual cell culture systems bear specific advantages or disadvantages they are mostly tailored to provide optimum conditions for survival and growth. In this context it seems unusual to deprive primary cells from growth factors for 36 hours and it is perceivable that this could lead to the temporary or permanent downregulation of signalling pathways. On the other hand, starvation of primary keratinocytes in serum- and growth factor free medium for a few hours is probably not sufficient to eliminate all soluble factors that could potentially activate MAPK and activation of MAPK that I observed in my experiments is most likely the consequence of co-operative signalling by integrins and growth factors. The results of Mainiero et al and my results are therefore not directly comparable. However, my data provide clear evidence that in keratinocytes both α6β4 and β1 integrins are capable of activating MAPK in the presence of growth factors.

Specific inhibition of β1 integrin function by introduction of CD8β1 led to reduced phosphorylation of MAPK but not FAK. Following introduction of more wild type β1

integrin molecules into cells expressing CD8β1 MAPK activation was again increased. This clearly shows that the number of functional β1 integrin receptors available is a determinant for the strength of the MAPK signal in keratinocytes. My results shown in Figures 3.1 a and 3.2 d suggest that these receptors do not necessarily have to be occupied by ligand in order to signal to MAPK.

The MAPK activation observed in my plating experiments was of a transient nature: 4 and 8 hours after plating MAPK phosphorylation was not elevated in comparison to suspended cells. This is in agreement with previous results showing transient MAPK activation after integrin engagement (Howe and Juliano 1998). Recent work suggests that fibronectin mediated stimulation of MAPK activity comprises two phases: A first phase lasting 20- 40 minutes during which strong MAPK activation is triggered by Raf and that requires Ras- Raf interaction, and a second phase of more moderate MAPK activity that is also Raf dependent and lasts for additional 80- 100 minutes. This second phase is not dependent on an interaction between Ras and Raf but on activity of protein kinase C (PKC) (Howe and Juliano 1998). These results were obtained in NIH-3T3 cells, a fibroblast line, and it would therefore be interesting to test whether this holds also true for primary keratinocytes.

Integrins are believed to be able to activate MAPK in different ways (Howe et al. 1998). MAPK can be activated via Ras, either through FAK (Schlaepfer et al. 1994) or Shc (Wary et al. 1996; Wary et al. 1998). These two models propose linear signalling cascades that are very similar to and partly include elements of the EGF receptor signalling pathway. In keratinocytes FAK phosphorylation was not inhibited by CD8β1, as observed when similar dominant negative β1 constructs are expressed in other cell types (Akiyama et al. 1994; Lukashev et al. 1994). The Shc-mediated pathway is believed to involve the interaction of the transmembrane and juxtamembrane extracellular domains of integrin α subunits with caveolin (Wary et al. 1996) and it is difficult to envisage how this process would be affected by overexpression of the β1 cytoplasmic domain. Furthermore, these two mechanisms of MAPK activation do not take into account that MAPK could be activated in cooperation between integrins and growth factors.

A third mechanism of adhesion dependent MAPK activation that has been described (Lin et al. 1997; Renshaw et al. 1997) that seems to be more applicable to my results. In this model the crucial function of integrins is the spatial organisation of signalling components of the EGF receptor signalling pathway, Ras, Raf and MEK, which

increases the likelihood of their interaction. This is an attractive hypothesis since integrins are known to be cardinal organisers of the spatial distribution of many molecules within a cell (Hynes 1992) and compartmentalisation is in many cases mediated by elements of the cytoskeleton to which integrins are connected via their cytoplasmic domains. In favour of this hypothesis is the finding that activated MAPK has been found to be concentrated in focal adhesion (Fincham et al. 2000). Interestingly, integrin mediated MAPK activation can be blocked by preincubation of cells with cytochalasin D, an inhibitor of actin polymerisation, indicating that the actin cytoskeleton is an important element in this signalling process (Zhu and Assoian 1995), see Figure 3.2 d. Using this model to explain the mechanism of action of CD8β1 on MAPK activation in keratinocytes one arrives at a scenario in which CD8β1 connects to elements of the cytoskeleton (Bishop et al. 1998) or their regulators, but not FAK, and decreases their availability for endogenous integrins. This then leads to an altered or less efficient spatial organisation of upstream signalling molecules involved in MAPK activation, such as Ras, Raf or MEK, or MAPK itself, possibly due to an impairment of normal actin polymerisation. This causes a diminished interaction of these molecules leading to reduced activation of MAPK. Such a view would be supported by the fact that in cells adherent for 4 hours on fibronectin there was no difference in the level of MAPK phosphorylation after stimulation with cell culture medium containing serum and supplements. This could indicate that cellular events occurring immediately after plating, like the assembly of the actin cytoskeleton, could be involved in the inhibitory effect of CD8β1 on MAPK activation. Phosphorylation of MAPK in CD8β1 expressing keratinocytes, but not CD8 expressing cells demonstrates that CD8β1 can connect to the downstream machinery that regulates MAPK activity. As has been shown previously for integrin mediated stimulation of MAPK this machinery requires actin polymerisation and the activity of rho family GTPases (Zhu and Assoian 1995; Renshaw et al. 1996).

Proliferative potential is the ability of a keratinocyte to permanently populate a certain surface area of the skin, or, in vitro, of the culture dish. This potential is highest in keratinocytes with high surface levels and function of β1 integrins which are therefore thought to be epidermal stem cells (Jones and Watt 1993). My data show that signalling activity of the MAPK cascade depends on the number of functionally active β1 integrin molecules on the surface of keratinocytes and it was therefore interesting to investigate whether MAPK activity would correlate with stem cell characteristics. A technique that

allowed manipulation of MAPK activity independently of upstream regulatory mechanisms provided a tool to bypass the signalling defect caused by CD8β1.

Increasing MAPK function in CD8β1 expressing keratinocytes restored adhesiveness, β1 integrin expression and proliferative potential, and inhibition of MAPK signalling led to a loss of these stem cell characteristics. The underlying mechanisms for this remain unclear: there are no established mechanisms through which MAPK could increase adhesiveness of keratinocytes. On the contrary, signals derived from Ras and MAPK have been shown to downregulate ligand binding affinity of exogenously expressed integrins in CHO cells (Hughes et al. 1997). Since constitutive activation of MAPK in CD8β1 expressing cells also led to an increase of β1 integrin surface levels it is possible that increased adhesion is caused by the higher number of available adhesion receptors. In agreement with that, activation of MAPK in CD8 expressing cells caused neither higher integrin surface levels nor increased adhesion.

Taking into consideration that keratinocyte culture conditions have been optimised over the years in order to maximise proliferative potential it is not surprising that the submaximal activation of MAPK achieved by expression of MAPKK1 did not cause a further increase of proliferative potential of cells with normal integrin function (cells expressing CD8), as determined by the proportion of abortive colonies formed. Activation of MAPK only led to a detectable increase when integrin function was inhibited indicating that the integrin function that supports MAPK activation is indeed critical for a high proliferative potential. The fact that MAPK activation could prevent and MAPK inhibition induce the loss of stem cell properties in keratinocytes identifies MAPK activity as an anti commitment signal during the first phase of keratinocyte differentiation. A similar function for MAPK has been reported in smooth muscle cells where MAPK activation inhibited early differentiation related events (Bennett and Tonks 1997).

CHAPTER 4
THE ROLE OF MAP KINASE IN KERATINOCYTE PROLIFERATION, DIFFERENTIATION AND MIGRATION

The unique structure of the epidermis is maintained by a tightly regulated balance between keratinocyte proliferation and differentiation. Proliferation is largely confined to the basal cell layer, where keratinocytes are in contact with the underlying basement membrane. During terminal differentiation keratinocytes withdraw from the cell cycle, detach from the basement membrane and move into the suprabasal layers of the epidermis.

When cultured keratinocytes are deprived of contact with the extracellular matrix both stem and transit amplifying cells enter the second differentiation phase without further rounds of division. Suspension-induced terminal differentiation can be inhibited with extracellular matrix proteins or antibodies to β1 integrins, suggesting that integrin ligation is a negative regulator of terminal differentiation (Adams and Watt 1989; Watt et al. 1993; Levy et al. 2000).

In view of the role of integrins in regulating keratinocyte adhesion and differentiation it is not surprising that integrin expression is altered in both benign and neoplastic keratinocyte disorders (reviewed by Watt and Hertle 1994). In particular, although integrin expression is normally confined to the basal layer of the epidermis, suprabasal integrin expression is a feature of hyperproliferative epidermis, as found, for example, following wound closure or in lesions of the benign human skin disorder, psoriasis (Hertle et al. 1992). That suprabasal integrin expression can play a causal role in the onset of psoriasis has been demonstrated by creating transgenic mice in which various integrin subunits are expressed under the control of the involucrin promoter (Carroll et al. 1995; Romero et al. 1999). In these mice sporadic epidermal hyperproliferation with accompanying histological features of psoriasis, including a lymphocytic infiltrate, is observed.

Given the role of β1 integrin signalling via MAPK in the stem to transit amplifying cell transition I decided to investigate whether it also plays a role in the second phase of

keratinocyte differentiation and, specifically, whether suprabasal integrin expression might exert its effects via the MAPK cascade.

4.1. The Effect of MAPK activity on proliferation and terminal differentiation of keratinocytes in vitro

4.1.1. Effect of MAPK activation on proliferation of adherent and suspended keratinocytes

There is evidence from a number of in vitro systems that MAPK is able to regulate proliferation and differentiation (Cowley et al. 1994; Bennett and Tonks 1997; Schramek et al. 1997). In Chapter 3 I have shown that MAPKK1 was able to increase the proliferative potential of CD8β1 expressing keratinocytes, but had no influence on the ratio of clones derived from stem cells and transit amplifying cells in CD8 expressing cultures. This does not rule out an effect of MAPK activity on the overall growth rate of keratinocytes or on the proliferative potential of transit amplifying cells. Using primary keratinocytes transduced with MAPKK1 and MANA I therefore first analysed the influence of MAPK on keratinocyte proliferation by establishing growth curves. Since I had found before that inhibition of MAPK activity in keratinocytes by expression of MANA reduces the proportion of adherent keratinocytes by about 50% (see Chapter 3) in preparing growth curves I plated twice as many cells infected with MANA as cells expressing puro or MAPKK1. Expression of MAPKK1 always led to an increased growth rate compared to the empty vector control, puro, whereas introduction of MANA caused a reduction of cell proliferation. The results of one experiment out of three are shown in Figure 4.1. This is consistent with results I obtained in adherent keratinocytes pulse labelled with BrdU, showing a higher proportion of S phase cells in cultures expressing MAPKK1 (Table 4.1). The extent of growth stimulation or inhibition varied between individual experiments, but the effects of MAPKK1 and MANA relative to puro were the same in every experiment.

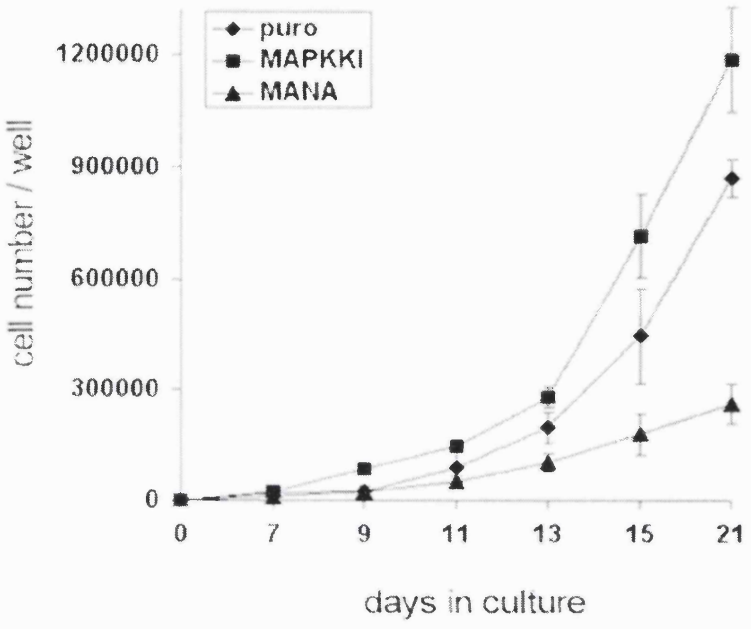


Figure 4.1: Effect of MAPK activity on keratinocyte proliferation.
500 (puro, MAPKK1) or 1000 (MANA) keratinocytes were plated per 35 mm dish and cultured for 21 days. Triplicate dishes were harvested on the days shown. Error bars show SD. Representative result from one experiment out of three.

Activation of MAPK in keratinocytes upon infection with MAPKK1 led to an adhesion independent stimulation of MAPK activity (Figure 3.3). Since constitutive MAPK activity is sufficient to transform cultured mammalian cell lines (Brunet et al. 1994; Cowley et al. 1994; Mansour et al. 1994) which then show anchorage independent growth I asked whether expression of MAPKKI in primary keratinocytes would lead to proliferation of suspended cells. I measured the proportion of keratinocytes expressing puro or MAPKK1 that incorporated BrdU during a 1 hr incubation after 24 hours in suspension culture. Suspension led to a reduction in the proportion of BrdU-positive cells in both puro- and MAPKK1-expressing populations, indicating that modulation of MAPK activity alone in primary keratinocytes did not result in anchorage independent growth (Table 4.1).

	PURO		MAPKK I	
	adherent	suspended	adherent	suspended
% BrdU positive cells	21.5 ± 3.5	1.8 ± 0.9	27.7 ± 1.2	1.0 ± 0.14

Table 4.1: BrdU incorporation by adherent and suspended keratinocytes.
Cells were grown on culture dishes (adherent) or trypsinised and held in FAD medium supplemented with FCS/ HICE and methylcellulose for 24 hours (suspended). Adherent and suspended cells were then pulse labelled with 10μg/ml BrdU for 1 hour and incorporation was measured by flow cytometry. Data are mean values obtained from triplicate samples ± SD. Representative result of one experiment out of three, performed in triplicate.

4.1.2. Effect of MAPK activation on suspension-induced terminal differentiation

MAPK has been shown to be a potent regulator of cell differentiation in several cell types (Cowley et al. 1994; Mamajiwalla and Burgess 1995; Crompton et al. 1996; Bennett and Tonks 1997). In keratinocytes loss of integrin engagement is a strong trigger for the onset of terminal differentiation and simultaneously results in disabled signal transduction via MAPK (Figure 3.1). I was therefore interested to know whether forced activation of MAPK signalling in the absence of integrin engagement would alter the onset of the differentiation program that is normally observed after keratinocyte detachment (Adams and Watt 1989). Primary human keratinocytes infected with puro or MAPKK1 were placed in suspension medium supplemented with methylcellulose, harvested at different time points and analysed for expression of several differentiation markers using flow cytometry. In cells infected with puro, expression of involucrin, cornifin and transglutaminase 1 was induced in suspension, the proportion of cells expressing each protein increasing from 8 to 24 hours in suspension; this is revealed as an increase in the size of the cell peak with high fluorescence and a corresponding decrease in the cell peak with low fluorescence (Figure 4.2). Keratinocytes infected with MAPKK1 also showed an increase in expression of these differentiation markers; however the number of cells expressing each protein (cell peak with high fluorescence) was always much lower in the MAPKK1 infected as compared to the puro infected populations (Figure 4.2). This shows that forced activation of MAPK signalling was sufficient to delay the onset of suspension induced keratinocyte differentiation.

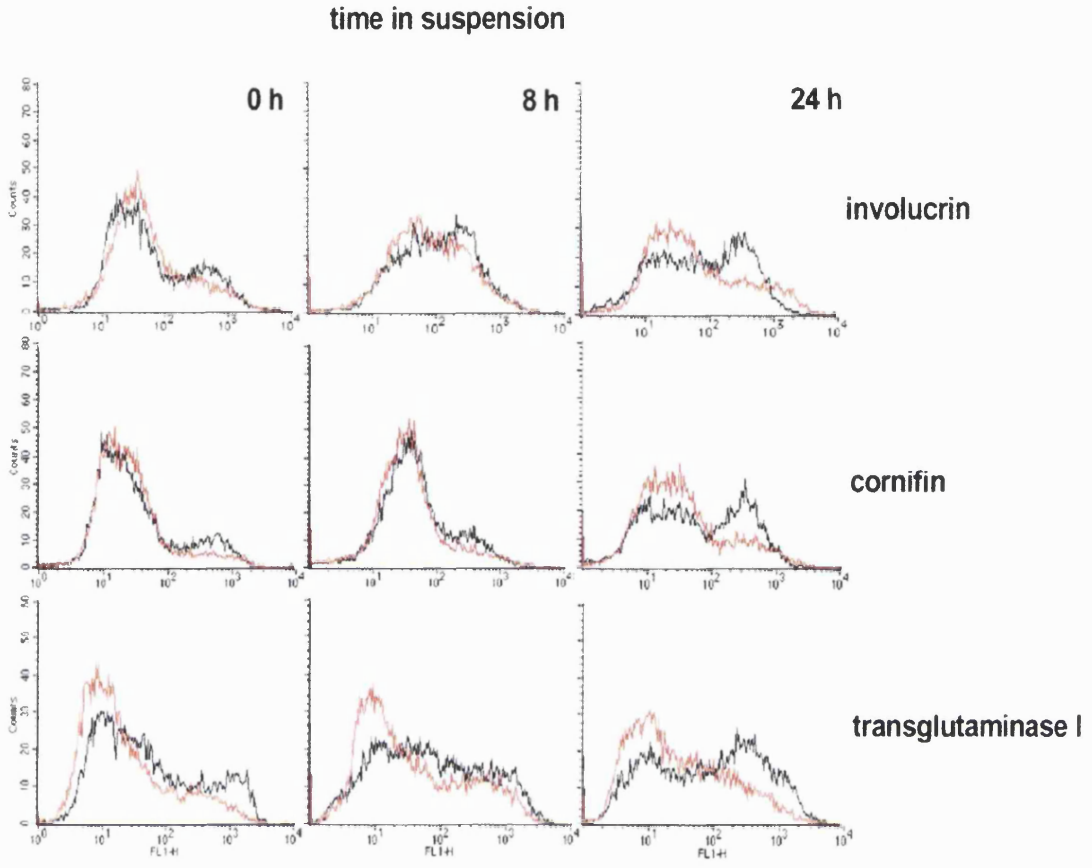


Figure 4.2: Effect of constitutive MAPK activation on suspension-induced terminal differentiation.

Flow cytometry profiles of keratinocytes fixed immediately after trypsinisation (0 h) or after 8 or 24 hours in suspension. Cells were labelled with antibodies to the differentiation markers indicated. Black lines: cells expressing empty vector puro, red lines: cells expressing MAPKK1. x axes show fluorescence intensity, y axes show cell counts.

4.2. The Effect of MAPK activation on proliferation and differentiation of reconstituted epidermis

When keratinocytes are seeded on dead, de-epidermised dermis and cultured at the air-liquid interface they are able to reconstitute an epidermis with many of the characteristics of the normal tissue (Rikimaru et al. 1997 and references cited therein). I compared epidermis reconstituted by keratinocytes infected with puro, MAPKK1 and MANA. At day 11 of culture keratinocytes infected with puro had formed a stratified

epithelium with a single basal cell layer and several suprabasal layers, the uppermost of which started to form the dead, cornified layers characteristic of the final stage of keratinocyte terminal differentiation (Figure 4.3 a). By day 14 the epidermis formed by puro infected cells had slightly increased in thickness and a largely anucleate cornified layer had developed with an underlying granular layer, thus bearing a close resemblance to normal epidermis (Figure 4.3 d).

Keratinocytes expressing MAPKK1 formed an epidermis which was already slightly thicker than the puro controls at day 11 and by day 14 showed pronounced thickening and elongation of the rete ridges (Figure 4.3 b,e). Immunostaining with an antibody to laminin demonstrated that the basement membrane was intact and so the down growths of keratinocytes were true rete ridges and not invasive fronts (Figure 4.4). In contrast to cells expressing puro there was no granular layer and a thick cornified layer had formed by day 14 in which many cell nuclei were still present (Figure 4.3 e). Keratinocytes expressing MANA gave rise to a thinner epidermis than the puro controls in which the basal cell layer was not clearly distinguishable and which by day 14 had only a thin, anucleate cornified layer (Figure 4.3 c,f).

To analyse proliferation in the reconstituted epidermis I used immunostaining with an antibody against the Ki 67 proliferation-associated antigen. Immunohistochemistry using a peroxidase labelled secondary antibody and DAB as substrate yielded a dark brown staining for Ki 67 positive nuclei (Figure 4.5). In the microscopical analysis a higher number of Ki 67 positive nuclei in MAPKK1 infected cultures was apparent (Figure 4.5 b, compare to puro expressing keratinocytes shown in 4.5 a). Conversely, epidermis reconstituted by keratinocytes expressing MANA showed fewer Ki 67 positive nuclei (Figure 4.5 c). In all cultures the majority of Ki 67 positive cells were localised in the basal cell layer. Whereas in puro and MANA expressing cultures no suprabasal Ki 67 staining was observed, in the MAPKK1 expressing cultures few suprabasal Ki 67 positive cells were present.

Table 4.2 shows a quantitation of the number of Ki 67 positive cells per high power field in cultures of keratinocytes expressing puro, MAPKK1 or MANA after 11 days of culture on dead, de-epidermised dermis. Even allowing for differences in the cellularity

of the basal layer, introduction of MAPKK1 led to an almost twofold increase in the number of Ki 67 positive cells, whereas MANA caused a reduction in the frequency of Ki 67 expression.

	PURO	MAPKK 1	MANA
number of Ki 67 positive cells	16.4 ± 1.65	29.6 ± 4.6	9.9 ± 2.4

Table 4.2: Quantitation of Ki 67 expression in reconstituted epidermis.
Sections of paraffin embedded day 11 cultures were stained with a monoclonal antibody against the Ki 67 antigen and the number of positive cells in 12 high power fields per section was counted. The average number of basal cells per field was 115 for puro-, 99 for MANA- and 134 for MAPKK1-expressing keratinocytes. Values represent the average number of Ki 67 positive cells per field ± SD.

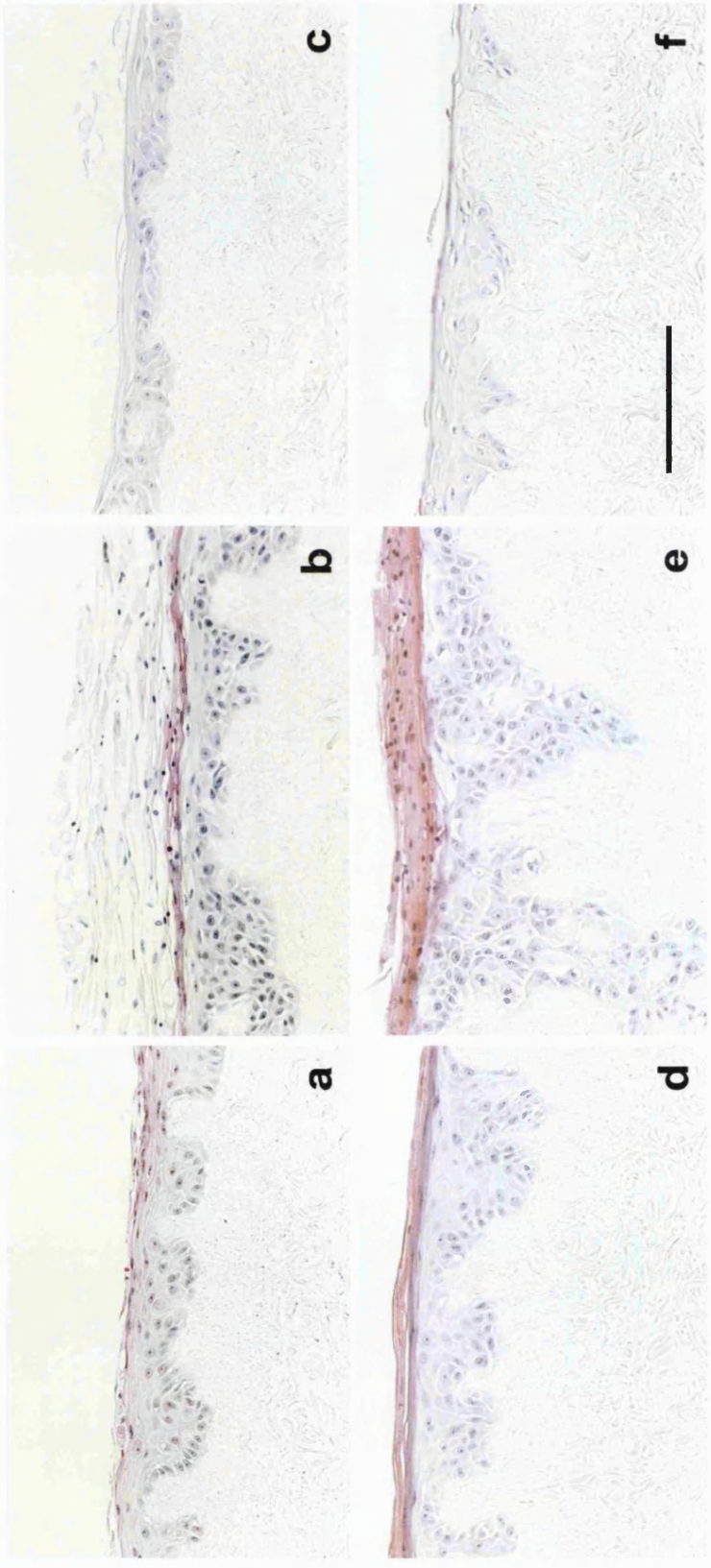


Figure 4.3: Effects of constitutive activation or inhibition of MAPK on reconstituted epidermis.
Human keratinocytes infected with puro (a,d), MAPKK1 (b,e) or MANA (c,f) were cultured for 11 (a,b,c) or 14 (d,e,f) days on dead, de-epidermised dermis. H/E staining of 5 μm sections of paraffin embeded tissue. Scale bar: 100µm.

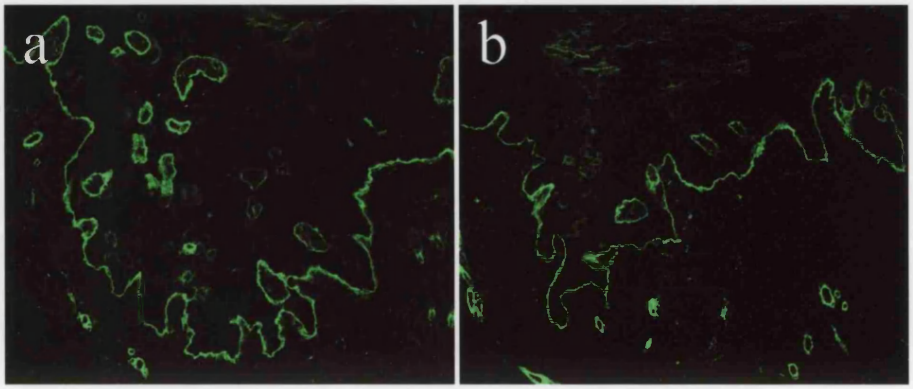


Figure 4.4: Basement membrane in epidermis reconstituted by MAPKK1.
a,b: Confocal images of sections from different samples of epidermis reconstituted in vitro by keratinocytes expressing MAPKK1 as in Figure 4.3 e, stained with an antibody against laminin. The secondary antibody was coupled to Alexa 488 producing a green fluoresence.

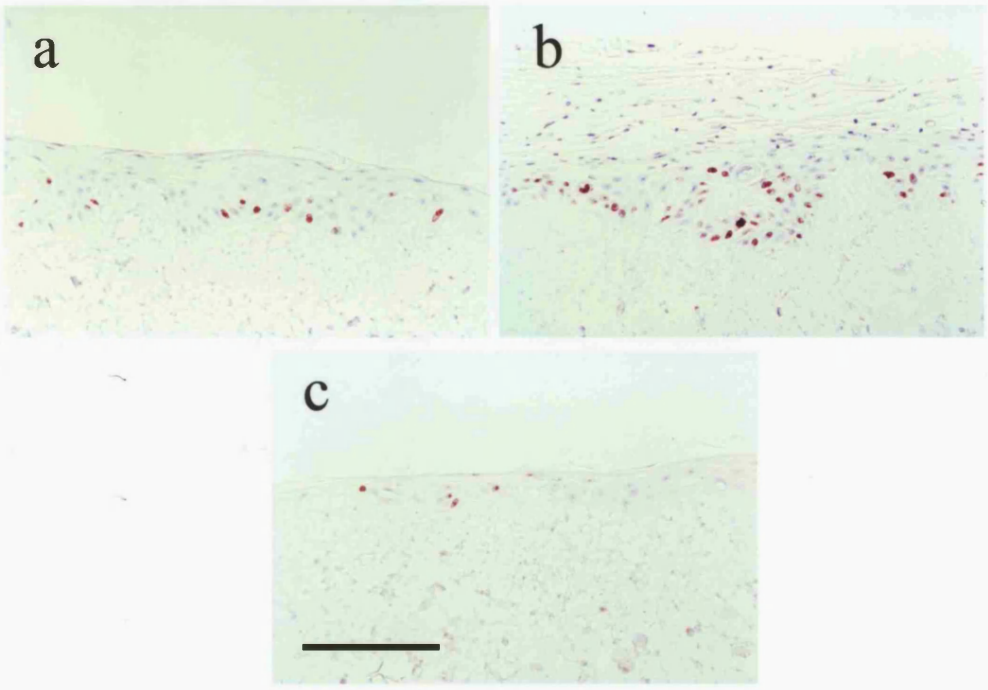


Figure 4.5: Expression of the Ki 67 antigen in reconstituted epidermis.
Sections of paraffin embedded samples of epidermis reconstituted in vitro by primary human keratinocytes expressing empty vector puro (**a**), MAPKK1 (**b**) and MANA (**c**) at day 11 were stained with a monoclonal antibody against Ki67 (MIB-1). Sections were incubated with a HRP-coupled secondary antibody and Ki67 expression was visualised using DAB substrate. Positive nuclei appear brown. Scale bar: 100 μm.

In order to determine the degree of terminal differentiation in organotypic cultures the reconstituted epidermis was stained with antibodies against keratin 14 and involucrin (Figure 4.6). All keratinocytes were keratin 14 positive. In cultures of keratinocytes expressing puro basal cells were involucrin negative (red) and all suprabasal layers were involucrin positive (green or yellow in Figure 4.6 a). In contrast, cultures infected with MAPKK1 showed a delay in the onset of involucrin expression and contained several suprabasal involucrin negative cell layers (red in Figure 4.6 b); involucrin expression was not completely suppressed but confined to a small compartment of cells directly beneath the cornified layer (green or yellow in Figure 4.6 b). In epidermis reconstituted by MANA expressing cells the expression pattern of involucrin was similar to the puro control, in that all the suprabasal layers were positive; however, there were more involucrin positive basal cells in the MANA cultures (Figure 4.6 c).

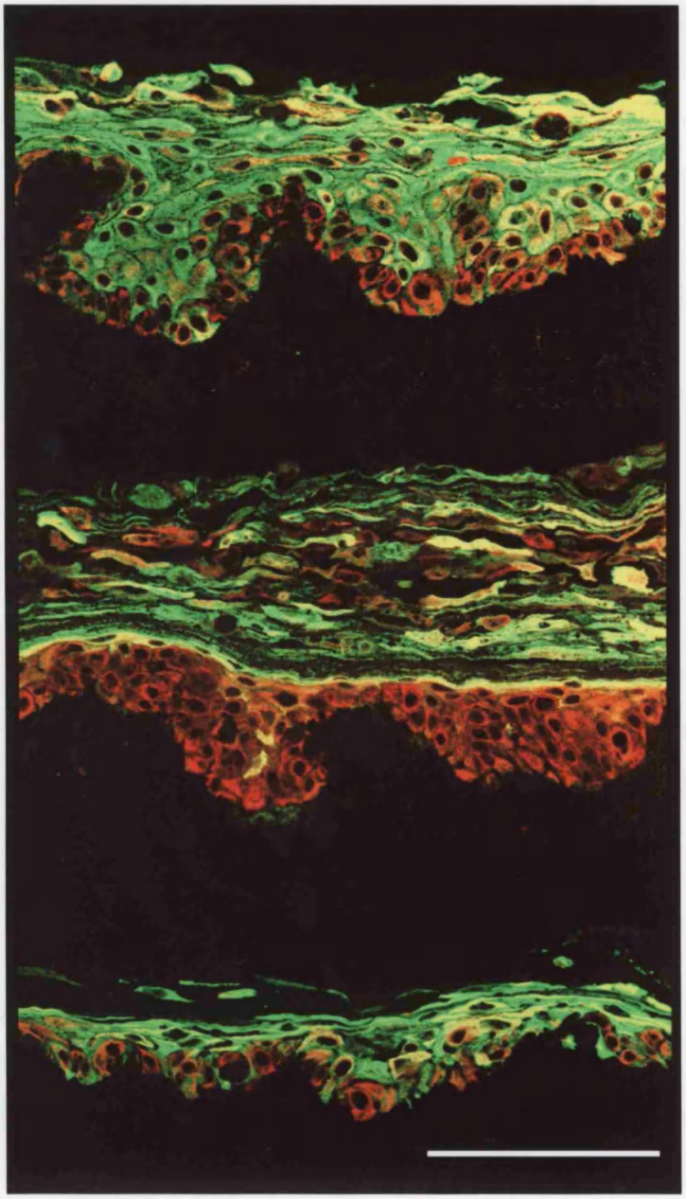


Figure 4.6: Effects of constitutive activation or inhibition of MAPK on involucrin expression in reconstituted epidermis. Keratinocytes infected with empty vector puro (**a**), MAPKK1 (**b**) or MANA (**c**) were cultured on dead, de-epidermised dermis for 11 days and stained with antibodies against keratin 14 (red) and involucrin (green). Scale bar: 100μm.

4.3. The Effects of MAPK activation on keratinocyte motility and spreading

The finding that MAPK activation provides an anti- commitment signal for keratinocytes together with the data on regulation of proliferation and inhibition of terminal differentiation suggests that MAPK activity could be important for the regeneration response of the epidermis after wounding. Apart from increased proliferation and suppressed differentiation epidermal regeneration requires an additional function of epidermal keratinocytes: migration. I therefore used cells infected with MAPKK1 or MANA to investigate this function.

Keratinocyte motility was monitored by time lapse video microscopy over 24 hours. Constitutive activation of MAPK by expression of MAPKK1 led to an increase in the total distance moved by single cells (Figure 4.7 a, b) and a two-fold increase in average cell speed (Figure 4.7 c). Figure 4.7 c shows the results of four separate experiments in which the speed of cells expressing the empty vector puro, or MAPKK1 was compared. The number of cells moving at the same speed within each experiment is represented by the width of the bars. Analysis of variance revealed that the difference between puro expressing (16.9 ± 1.8 μm/h) and MAPKK1 expressing (32.8 ± 1.2 μm/h) keratinocytes was statistically significant ($p<0.01$).

Consistent with previous findings (Barrandon and Green 1987) incubation of keratinocytes with 10ng/ml EGF led to a three fold increase in cell speed ($p<0.001$; compare puro expressing cells in Figure 4.7 c,d). Since EGF is also a strong stimulus for MAPK activation I was interested in whether EGF stimulated cell motility was mediated by MAPK. Inhibition of MAPK activity with the specific MAPK kinase inhibitor PD098059 or by introduction of MANA reduced keratinocyte motility in the presence of 10 ng/ml EGF (Figure 4.7 d). The histogram in Figure 4.7 d shows the speed of all cells analysed in five independent experiments in which MAPK activity was inhibited either by incubation with PD98059 (25 μM) or by expression of MANA. Analysis of variance revealed a significant difference in speed between the puro/DMSO and the MANA/PD98059 groups ($p<0.001$). The direction of all cell movement observed was always completely random (Figure 4.7 a, b) and neither activation nor inhibition of MAPK signalling had any influence on the directionality of cell movement.

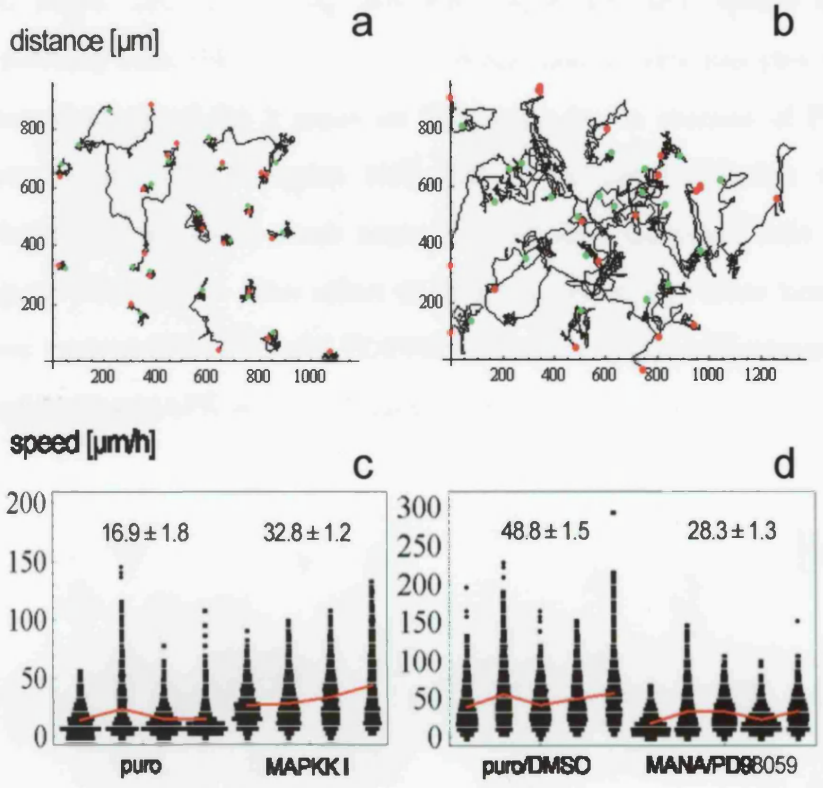


Figure 4.7: Effects of MAPK on keratinocyte motility. a,b: Trajectories of cells expressing puro (**a**) or MAPKK1 (**b**) on 10 μg/ml fibronectin for 24 hours. 20 cells per sample are shown. Origin and end point for each cell are indicated by coloured dots. **c,d**: Histograms showing speed of cell movement. Each bar represents a separate experiment; 20 cells were analysed per experiment. The number of cells moving at the same speed within an experiment is represented by the width of the bar. Red lines connect the means of individual experiments. The means and standard errors of the mean for all replicate experiments are given above the bars. **c**: Comparison between cells expressing puro and MAPKK1. **d**: Comparison between puro expressing- or uninfected keratinocytes treated with 0.1% DMSO and MANA expressing- or uninfected keratinocytes treated with 25 μM PD098059 in the presence of 10 ng/ml EGF.

While studying keratinocyte motility I noticed an effect of MAPK activity on cell shape: cells expressing MAPKK1 were less well spread than puro expressing cells (Figure 4.8 a, b). When normal keratinocytes that were allowed to spread for 2 hours on fibronectin in the absence of FCS/HICE were treated with 10 ng/ml EGF they underwent contraction within 30 minutes and appeared much more rounded than untreated cells (compare Figure 4.8 a and c). This effect of EGF was attenuated when keratinocytes were pretreated with 25 μM PD098059, indicating that cell contraction was mediated by MAPK activity (Figure 4.8 d).

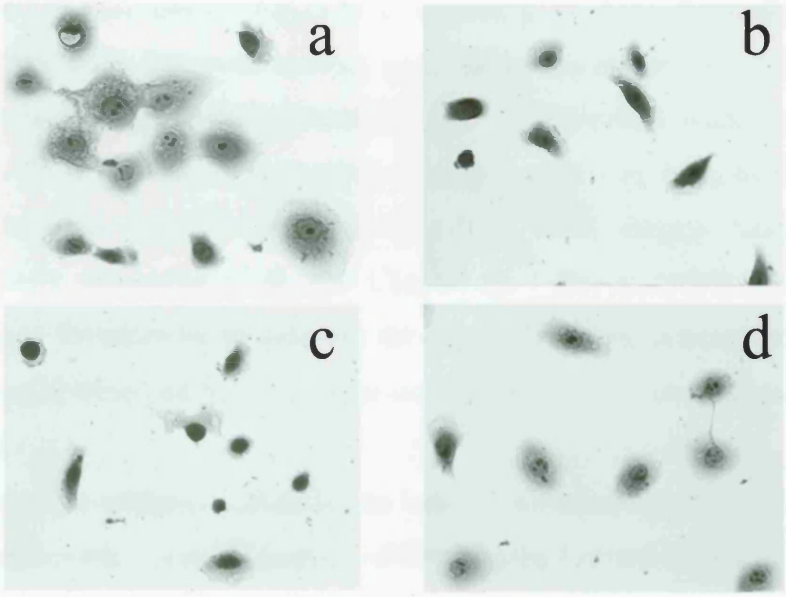


Figure 4.8: Effect of MAPK activation and inhibition on cell spreading. Microscopical images of primary keratinocytes attached to fibronectin and spreading for 2 hours. Cells expressing puro (**a**) or MAPKK1 (**b**) were plated in the absence of EGF or uninfected keratinocytes were pretreated with 0.1% DMSO (**c**) or 25 μM PD098059 (**d**) and stimulated with 10 ng/ml EGF.

4.4. Discussion

The functions of the classical MAPK cascade in cell biology are diverse and it seems that the specificity of certain tasks of MAPK signalling are determined by the respective cell type. In keratinocytes there is evidence that this signalling pathway is activated concomitantly with signals promoting cell cycle progression (Mainiero et al. 1997) without an exact definition of the function that MAPK exerts in this process. There is also evidence that MAPK acts as a survival factor for keratinocytes protecting them from UV induced apoptosis (Peus et al. 1999). In my experiments expression of MAPKK1 resulted in an increased growth rate of keratinocytes both under conventional culture conditions (Figure 4.1) and in reconstituted epidermis (Figure 4.5, Table 4.2); conversely, inhibition of MAPK signalling by expression of MANA reduced cell proliferation. On the first glance these results seem to confirm a role for MAPK in the regulation of cell cycle progression in keratinocytes. In the light of my results described in Chapter 3 there is, however, another possible mechanism by which MAPK could stimulate proliferation of cultured keratinocytes. My previous work has shown that although MANA increases the proportion of transit amplifying cells, MAPKK1 has no effect on the proportion of stem cells in culture when integrin function was not perturbed (cells expressing CD8, see Chapter 3). The stimulation of growth by MAPKK1 may therefore be by delaying the onset of terminal differentiation of transit amplifying cells, observed both in suspension and in reconstituted epidermis (Figures 4.2 and 4.6).

In support of the hypothesis postulating an indirect influence of MAPK on keratinocyte proliferation through a delay of terminal differentiation I observed that in keratinocytes cultured in suspension, activated MAPK could delay, but not inhibit, the onset of expression of proteins related to keratinocyte differentiation Figure 4.2). A delay in expression of the differentiation marker involucrin was also observed in the organotypic cultures (Figure 4.6).

Inhibition of cell differentiation upon activation of MAPK has also been observed in other epithelial cells. In MDCK cells cytokeratin expression, a feature of differentiation of these cells, was suppressed (Schramek et al. 1997). In skeletal muscle cells MAPK activity inhibited early events in cell differentiation; at later stages, however, MAPK stimulated differentiation (Bennett and Tonks 1997). Stimulation of neuronal differentiation by MAPK has also been reported from PC12 cells where MAPK

activation induced neurite outgrowth (Cowley et al. 1994). Thus MAPK seems to be able to both inhibit and stimulate cell differentiation depending on the cell type and the respective stage of the differentiation process. In primary human keratinocytes cultured on dishes, in suspension or in an organotypic culture system constitutive activation of MAPK clearly inhibited features of differentiation.

In a recent paper by (Schmidt et al. 2000) the authors propose a role for MAPK activation in the induction of early steps in keratinocyte differentiation. As a model they used an immortalised keratinocyte line, HaCaT, which was grown at a calcium concentration of 50 μM and then switched to 1 mM calcium ions (high calcium). Upon treatment with high calcium the authors observed a transient increase in MAPK activity and they speculate that this could be an event causally related to the increased expression of differentiation markers observed after calcium treatment (Boyce and Ham 1983). Evidence for this is based on an experiment in which calcium induced expression of involucrin could be inhibited by treatment of HaCaT cells with pharmacological inhibitors of MAPK for 48 hours. Though MAPK is known to induce features of differentiation in other systems (Cowley et al. 1994; Crompton et al. 1996; Bennett and Tonks 1997) these data do not allow a conclusion on a role of MAPK in keratinocyte differentiation. First, HaCaT cells are immortalised, aneuploid cells with a mutation in the p53 oncogene and are therefore an imperfect model for keratinocyte differentiation (Boukamp et al. 1988). Second, prolonged treatment of cultured cells with high concentrations of MAPK inhibitors may affect the viability of these cells either specifically, since MAPK activity has been reported to be a survival signal in keratinocytes (Peus et al. 1999) or unspecifically due to toxicity. Assays of cell viability that would be required in this experimental setup were not performed. Third, stimulation of primary keratinocytes with high concentrations of calcium ions does not only lead to an increase in the number of involucrin positive cells but also to stimulation of DNA synthesis (Watt et al. 1991). In this context it is interesting that (Efimova et al. 1998) have investigated the influence of MAPK activity on the activity of transcription from the involucrin promoter. They found that expression of wild type Raf-1, ERK1 and ERK2 had no influence on promoter activity.

Motility of keratinocytes is known to be stimulated by EGF and its related growth factors TGFα and HB-EGF (Martin 1997). Since I found MAPK activity to be increased after treatment with EGF and MAPK has recently been shown to phosphorylate a key

enzyme involved in cell motility, myosin light chain kinase (Klemke et al. 1997) I was interested in whether the motility stimulating function of EGF was exerted by MAPK. MAPK activation achieved by expression of MAPKK1 increased random migration of keratinocytes about two-fold in the absence of EGF, while inhibition of MAPK reduced EGF-dependent motility. This demonstrates that activation of MAPK is sufficient to stimulate keratinocyte motility and that EGF induced migration is partly dependent on MAPK activity.

Though MAPK is apparently important for the stimulation of keratinocyte motility in wound healing there are probably other pathways involved in the regulation of reepithelialization. Members of the rho family of small GTPases are known to mediate growth factor induced cytoskeletal rearrangements (Nobes and Hall 1995) and are therefore thought to be of crucial importance for migration of keratinocytes in healing skin wounds (Nobes and Hall 1995; Martin 1997). The small GTPase Rac stimulates cortical actin filament assembly, ruffling and membrane protrusion, which leads to an enlargement of the contact area of the cell with its underlying substrate (Nobes and Hall 1995). In my experiments keratinocytes expressing MAPKK1 or stimulated with EGF were less well spread than cells expressing puro or those in which MAPK activity was inhibited. This suggests that MAPK counteracts such spreading signals by regulating the actin-myosin motor that generates forces for cell contraction (Klemke et al. 1997; Cheresh et al. 1999). MAPK dependent cell contraction, mediated by phosphorylation of myosin light chain kinase could be an important part of the epidermal migration process in wound healing.

A certain physiological level of MAPK activity seems to be required in order to form a normal stratified epithelium, because epidermis reconstituted by MANA expressing cells was hypocellular, with decreased proliferation and incomplete terminal differentiation. Constitutive activation in reconstituted epidermis, on the other hand, gave rise to histological changes that mimicked hyperproliferative epidermis, as found in psoriatic lesions (Lever and Schaumburg-Lever 1983): the epidermis was thickened, with an increased number of proliferating cells, a reduction in the granular layer and a highly thickened, nucleated cornified layer. Although these changes are typical for psoriatic epidermis they are not absolutely specific, and similar changes can be found in epidermis that is hyperproliferative for other reasons, in squamous cell carcinomas and

during wound healing. The changes that I observed in the organotypic cultures reflect a state of the epidermis that can be found in several skin diseases.

Pathological activation of the MAPK pathway has been observed in various tumours (Towatari et al. 1997; Salh et al. 1999; Sebolt-Leopold et al. 1999). So far no mutations in MAPK itself have been found and it is therefore likely that dysregulation of MAPK activity in these situations is due to mutational changes in upstream regulating molecules or possibly disturbed growth factor synthesis and -function (Donnelly et al. 1993; Stoll et al. 1997). Since there is experimental evidence that the ras oncogene can be involved in the development of skin papillomas (Bailleul et al. 1990) and MAPK is a downstream target of Ras the possibility exists that constitutive activation of MAPK could cause the development of a malignant phenotype in the transduced keratinocytes (Brunet et al. 1994; Cowley et al. 1994; Mansour et al. 1994). Whereas increased MAPK activity may be a feature of skin malignancies, in my experiments malignant transformation was not induced by chronic MAPK activation: first, the cells transduced were primary keratinocytes and in the light of recent findings (Hahn et al. 1999) it seems unlikely that a single alteration like MAPK activation would be sufficient to transform primary cells. Second, though MAPK activation delayed differentiation in suspended keratinocytes the transduced cells did not show a key feature of transformation: anchorage independent growth (Table 4.2). Third, in organotypic cultures malignantly transformed keratinocytes would be likely to grow invasively and destroy the underlying basement membrane. By immunostaining of a component of the epidermal basement membrane, laminin, I was able to show that this did not occur (Figure 4.4).

CHAPTER 5
A POTENTIAL ROLE OF MAP KINASE IN EPIDERMAL HYPEPROLIFERATION AND WOUND HEALING

The MAP kinases ERK1 and ERK2 act by phosphorylation of nuclear and cytoplasmic target proteins and have been implicated in the regulation of a large variety of transcription-dependent and -independent cellular functions. Nuclear translocation, which is strictly dependent on enzyme activation (Lenormand et al. 1998; Brunet et al. 1999), enables the enzymes to phosphorylate transcription factors, such as c-jun and elk-1 (Seger and Krebs 1995) and this is likely to be the mechanism by which MAP kinases exert their crucial functions in the regulation of cell proliferation and differentiation (Brunet et al. 1999). Immunodetection of nuclear translocation of MAPK protein in cells or tissue can therefore be used as a reporter of MAPK activation (Arendt et al. 1995; Lenormand et al. 1998; Tarnawski et al. 1998).

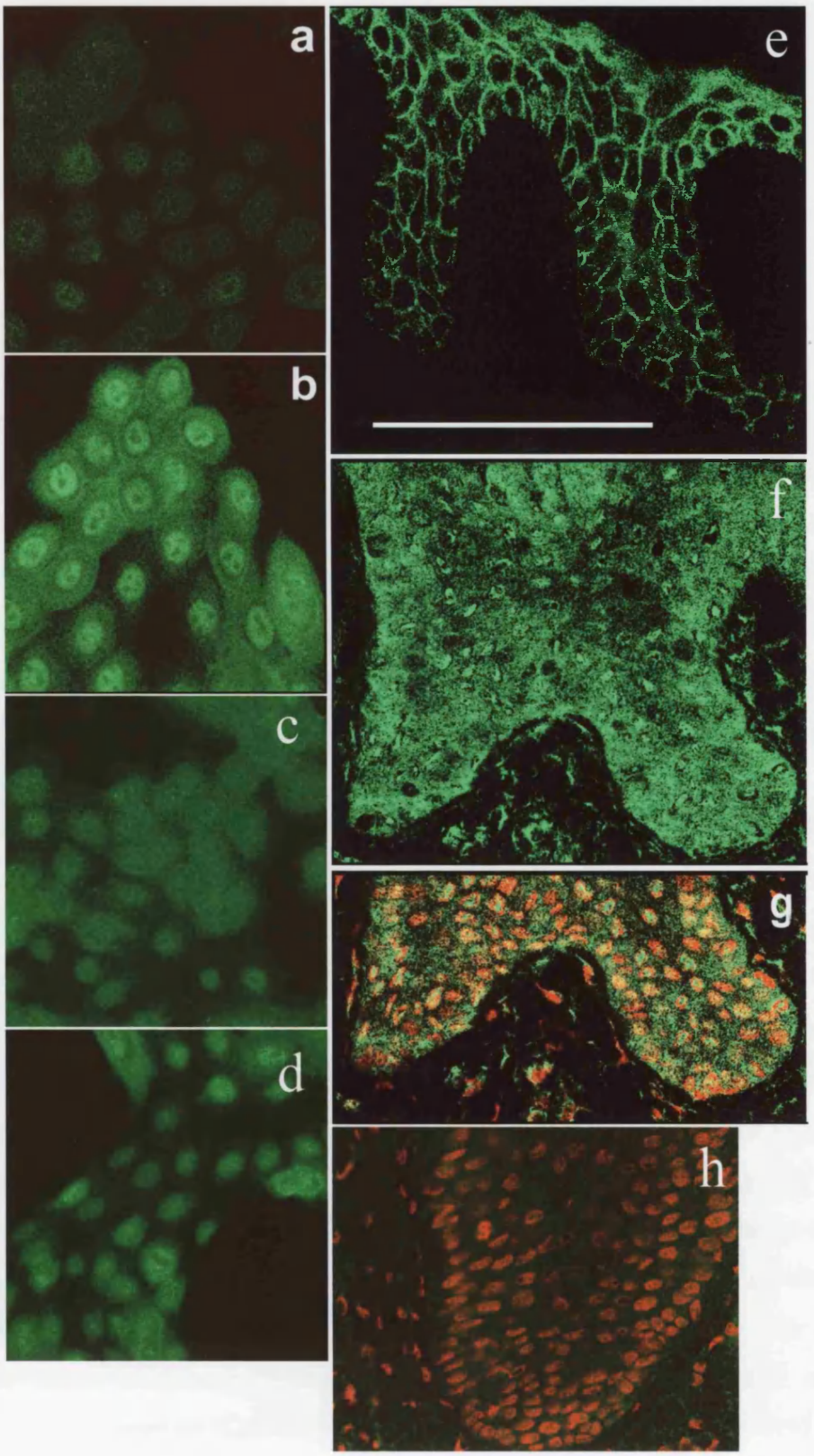
5.1. Activation of MAPK in psoriatic epidermis

To confirm that nuclear localisation was a reporter of MAPK activation in keratinocytes I starved normal human epidermal keratinocytes overnight in FAD medium without serum or growth factors (HICE cocktail) and then treated the cultures for 15 min with FAD alone or FAD supplemented with 10% FCS and HICE prior to fixation. Western blotting had previously shown that MAPK was activated in the presence, but not in the absence, of FCS and HICE (see Chapter 3). In serum starved keratinocytes there was no staining above background with an antibody specific for phosphorylated MAPK (Figure 5.1 a) and a diffuse cytoplasmic and nuclear staining with a monoclonal antibody against total MAPK (Figure 5.1 c). In contrast, cells treated with serum and growth factors exhibited strong nuclear staining and increased cytoplasmic staining for phosphorylated MAPK (Figure 5.1 b) and pronounced nuclear staining, but decreased cytoplasmic staining with the antibody against total MAPK (Figure 5.1 d). This indicates that in human epidermal keratinocytes MAPK becomes phosphorylated and translocates to the cell nucleus upon stimulation with serum and growth factors. A similar nuclear translocation of MAPK was observed with the monoclonal anti-ERK2

antibody sc-1647, which detects both phosphorylated and unphosphorylated ERK2 ($p42^{mapk}$).

I next used immunofluorescence staining to examine MAPK localisation in normal and hyperproliferative human epidermis. I obtained samples of normal skin from three different donors and samples from psoriatic lesions of two patients and stained frozen sections with a monoclonal antibody against ERK2 ($p42^{mapk}$) (sc-1642). This antibody is highly specific for ERK2 and shows only slight crossreactivity with $p44^{mapk}$ (ERK1) on Western blots of keratinocyte lysates (see, for example, loading controls in Figure 3.2 c). In healthy skin the antibody predominantly stained the membrane region of epidermal keratinocytes with little cytoplasmic and no nuclear staining (Figure 5.1 e). In contrast, staining for MAPK in psoriatic epidermis showed a more uniform expression pattern with staining of the cell membrane and the cytoplasm. In addition, the majority of cells in the basal and lower suprabasal layers of the rete ridges showed a positive nuclear signal for MAPK (Figure 5.1 f, g). Staining with the secondary antibody alone (data now shown) or with MAPK antibody preincubated with its corresponding blocking peptide gave no signal for MAPK (Figure 5.1 h).

Figure 5.1: Distribution of $p42^{mapk}$ in cultured keratinocytes and in normal and psoriatic epidermis.
Confocal immunofluorescence microscopy of growth factor-starved cultured keratinocytes (**a**,**c**) or keratinocytes treated with medium containing FCS+HICE for 15 min (**b**,**d**); and of normal human epidermis (**e**) or psoriatic epidermis (**f**,**g**,**h**). Green staining in **a-g**, is for MAPK with an antibody specific for activated (MAPK-YT, a,b) or total MAPK (sc-1647, c-h); red staining in **g** and **h** is with a nuclear dye, Topro III. The field shown in **g** is part of the field shown in **f**. In **h** the antibody sc-1647 against total MAPK was preincubated for 30 minutes with the corresponding blocking peptide and then used for staining.


a
b
c
d
e
f
g
h

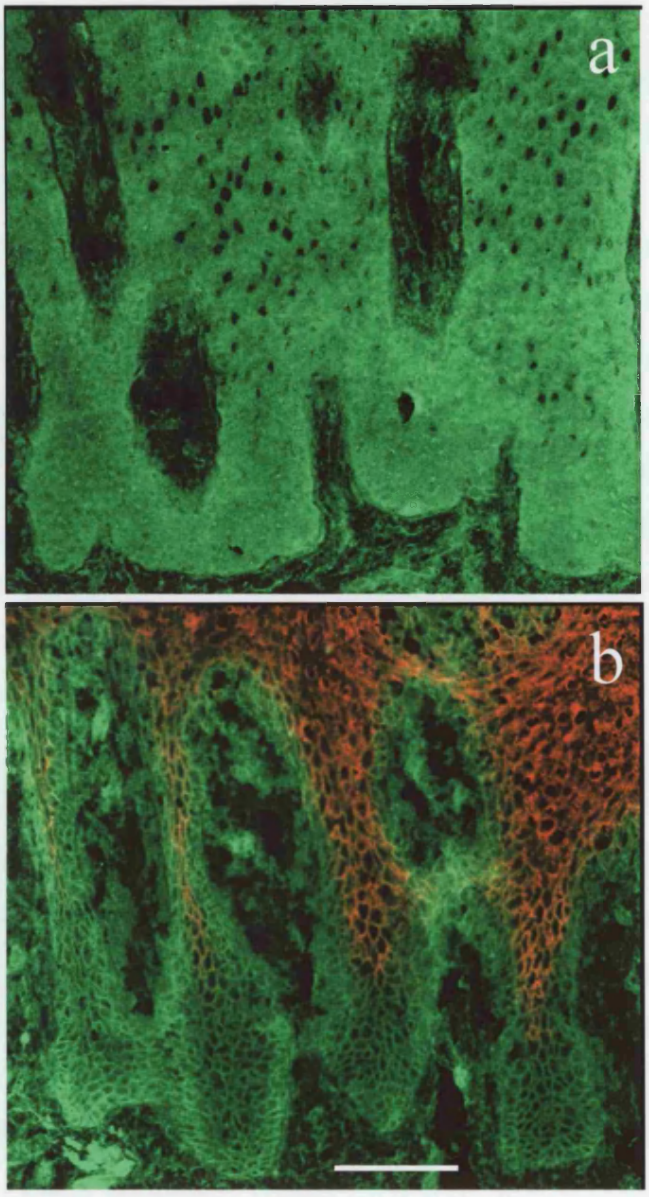
5.2. Correlation of MAPK activation with integrin expression

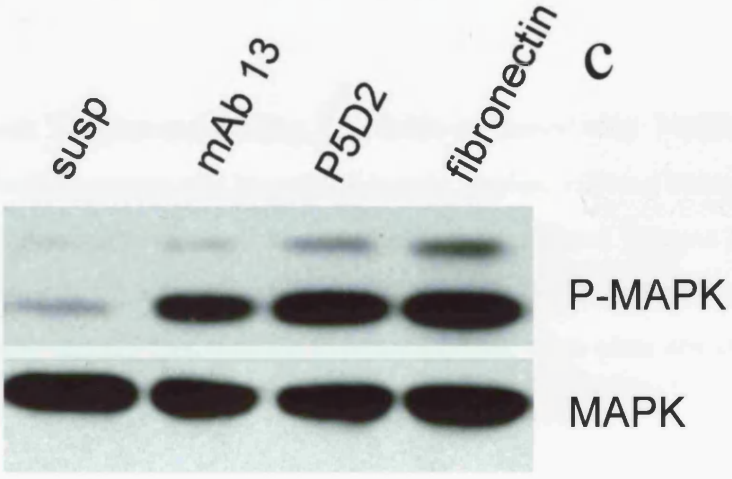
MAPK activity in keratinocytes is dependent on integrin function and β1 integrins are expressed in the suprabasal layers of psoriatic epidermis (Hertle et al. 1992). It has also been shown that β1 integrins can trigger the onset of hyperproliferative skin disease when expressed in the suprabasal layers of mouse epidermis (Carroll et al. 1995). I therefore asked whether aberrant integrin expression correlated with nuclear localisation of MAPK in psoriatic skin. Since staining of tissue sections for MAPK requires fixation with paraformaldehyde and integrin staining works best without fixation I obtained serial sections of psoriatic skin samples and stained them either with an antibody against MAPK (sc-1642) or with antibodies against β1 integrin (mAb 13) and involurin (DH-1). Results are shown in Figure 5.2. Nuclear MAPK staining was only present in basal and suprabasal keratinocytes in the most proximal third of the rete ridges (Figure 5.2 a). Keratinocytes in the same region also stained with an antibody against β1 integrin (green in Figure 5.2 b) but not with an antibody against involucrin (red in Figure 5.2 b). I conclude that nuclear localisation of MAPK is present in keratinocytes that express β1 integrin, but not involucrin.

The results described in Chapter 3 show that MAPK activation in keratinocytes by EGF is dependent on integrin engagement. This raised the question whether β1 integrin receptors aberrantly expressed in the suprabasal layers of psoriatic epidermis are functional with regards to MAPK activation. When normal human keratinocytes become committed to terminal differentiation β1 integrin transcription and intracellular traffic are inhibited and the receptors on the cell surface are no longer able to bind ligand (Adams and Watt 1990; Hotchin and Watt 1992; Hotchin et al. 1995); in addition the suprabasal integrins in hyperproliferative human epidermis are in a non-ligand binding conformation (Bishop et al. 1998; Penas et al. 1998). In order to address the question of whether integrins expressed by suprabasal keratinocytes are able to signal to MAPK I made use of a line of keratinocytes isolated from the skin of mice with suprabasal integrin expression in the epidermis under the control of the human involucrin promoter (Carroll et al. 1995; Romero et al. 1999). When held in suspension these cells, like normal human keratinocytes, are induced to undergo terminal differentiation: expression of the endogenous integrins is downregulated while expression of involucrin and the β1 transgene is induced (Romero et al. 1999). The

transgenic integrin is human and can therefore be distinguished from the endogeous mouse integrins using species-specific antibodies (Romero et al. 1999). I cultivated the cells in suspension for 16 hours in methylcellulose-supplemented medium before plating them onto dishes coated with fibronectin or the human β1-specific antibodies mAb13 and P5D2 (Figure 5.2 c). As observed in primary human keratinocytes MAPK activity was low in transgenic keratinocytes in suspension (or plated onto PLL, data not shown) but increased when the transgenic integrin was ligated by extracellular matrix or anti β1 antibody (Figure 5.2 c). Thus β1 integrins expressed on the surface of terminally differentiating keratinocytes can signal through MAPK.

Figure 5.2: MAPK activation in β1 integrin expressing differentiating keratinocytes.
a,b: Frozen sections of psoriatic epidermis were stained with antibodies against MAPK (**a**, sc-1647, green) or β1 integrin (**b**, mAb 13, green) and involucrin (**b**, DH-1, red). **c**: Western blot showing MAPK phosphorylation in β1 integrin transgenic murine keratinocytes held in suspension or plated onto dishes coated with antibodies against human β1 integrin (mAb 13, P5D2) or fibronectin for 30 minutes in the presence of 10 μg/ml EGF. Scale bar: 100 μm.


a
b


susp
mAb 13
P5D2
fibronectin
c
P-MAPK
MAPK

5.3. Activation of MAPK in wounded mouse skin

A function of the epidermis that requires temporary hyperproliferation of epidermal keratinocytes is the regeneration response after wounding. For ethical reasons it is difficult to obtain samples of wounded human epidermis and I therefore stained unwounded and wounded epidermis from transgenic mice that expressed β1 integrins suprabasally under the control of the involucrin promoter and their littermate controls (Carroll et al. 1995). In phenotypically normal epidermis from transgenic mice and transgene-negative littermate controls there was weak cytoplasmic and plasma membrane staining with the anti-ERK2 antibody, but no nuclear staining (Figure 5.3 a). The tissue used for staining of wounded skin was from epidermis of β1 transgenic mice and littermate controls 6 or 7 days after creating full thickness wounds. At this time the epidermis had migrated over the wound and was hyperproliferative by several criteria, including increased number of viable cell layers (compare Figures 5.3 a and b,c), induction of keratin 16 and suprabasal expression of the endogenous β1 integrins (data not shown). Immunostaining showed localisation of ERK2 in the nuclei of many basal and suprabasal keratinocytes in the hyperproliferative area covering the wound (Figure 5.3 b,c), but not in the adjacent non-affected skin. The nuclear localisation of MAPK was confirmed by double staining with a fluorescent DNA dye, Topro III (Molecular Probes, red in Figure 5.3). Positive staining could be abolished by preincubation of the antibody with its corresponding peptide antigen (sc-153P) (Figure 5.3 d). In the skin samples of the transgenic animals nuclear staining appeared to be more intense and present in a higher proportion of cells when compared with the transgene-negative mice (data not shown).

On the basis of immunostaining I provide evidence that MAPK is activated in hyperproliferative mouse and human epidermis, nuclear staining being observed in basal and suprabasal integrin-positive cells. However, suprabasal integrin expression did not result in constitutive activation of MAPK since no nuclear staining was observed in phenotypically normal epidermis from the transgenic mice (data not shown).

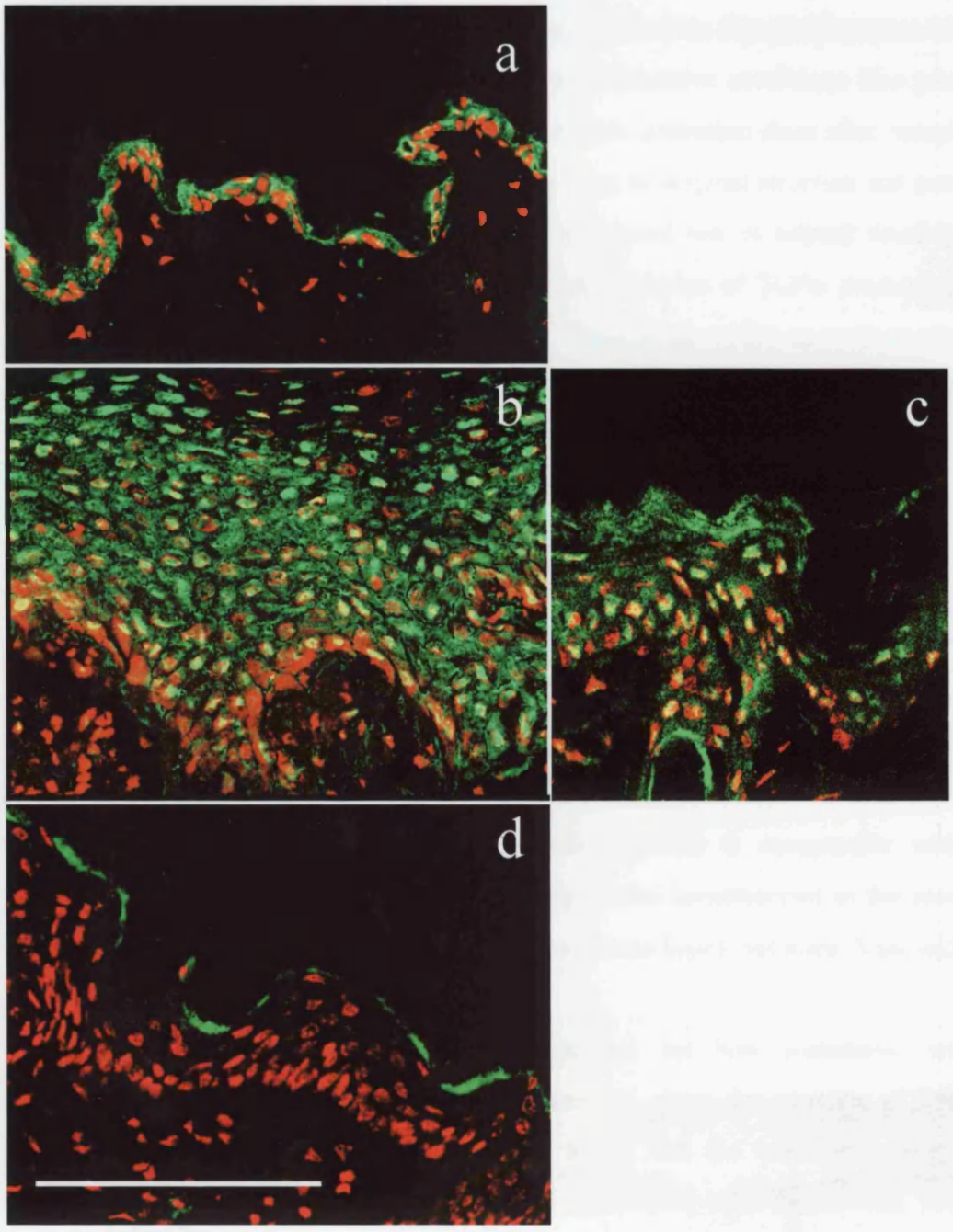


Figure 5.3: Distribution of ERK 2 ($p42^{mapk}$) in normal and wounded mouse epidermis.
Confocal images of sections of normal mouse skin (**a**) and skin of β1 integrin transgenic mice (**b**) or normal littermates (**c**,**d**) seven days after creating full thickness wonds (**b**,**c**,**d**). Green staining is for MAPK with a rabbit polyclonal antibody, sc-153. Red staining is with a nuclar dye, ToProIII. In **d** the antibody was preincubated with its corresponding blocking peptide. Scale bar: 100 μm.

5.4. Discussion

The role of MAPK in regulating keratinocyte proliferation and differentiation raises the possibility that this pathway could not only be involved in the development of skin malignancies but also of benign, transient hyperproliferative conditions like psoriasis and wound healing. This would have to be a reversible activation since after completion of wound healing the epidermis is remodeled to form its original structure and psoriatic lesions are fully reversible and do not bear an increased risk of tumour development (Krueger and Duvic 1994). For psoriasis, both upregulation of TGFα production and EGF receptor expression (Nanney et al. 1986; Elder et al. 1989) as well as aberrant suprabasal epidermal integrin expression (Hertle et al. 1992) have been described. Furthermore overexpression of either a member of the EGF family, amphiregulin, or expression of a transgenic integrin in the suprabasal layers of the epidermis were able to induce a psoriasis like phenotype in mice (Carroll et al. 1995; Cook et al. 1999), demonstrating a causal role for growth factors and integrins in the development of psoriasis- like inflammatory skin disease. This being established, the downstream mechanisms that finally cause the changes in cell behaviour are elusive.

Nuclear localisation of MAPK in psoriatic skin was observed in a population of basal and suprabasal keratinocytes that also expressed β1 integrins and was situated in the proximal third of the rete ridges. This staining pattern is comparable with the distribution of activated MAPK in basal and suprabasal keratinocytes in the proximal areas of hyperproliferative mouse epidermis in α-catenin knock out mice (Vasioukhin et al. 2001).

There are two conceivable alternative hypotheses for how suprabasal integrin expression could trigger epidermal hyperproliferation: either the presence of integrins on the surface of suprabasal cells provides a signal that the underlying basal cells respond to or else the integrins signal to the suprabasal cells themselves, possibly leading to unscheduled synthesis of growth factors or cytokines (Carroll et al. 1995; Romero et al. 1999). Basal keratinocytes are not stimulated to proliferate when exposed to integrin-positive suprabasal keratinocytes in vitro (Romero et al. 1999) and so at present there is no evidence in favour of the first hypothesis. This has led me to consider the alternative hypothesis, and therefore to examine signal transduction by suprabasal integrins. It is not clear how an integrin that is expressed in suprabasal layers of the epidermis could be engaged in vivo since its ligands have so far not been found in

the suprabasal compartment. Nevertheless, when brought into contact with ligands β1 integrins expressed in suprabasal keratinocytes seem to function like integrins expressed on basal keratinocytes with regards to MAPK activation (Figure 5.2 c).

It is conceivable that a so far unidentified integrin ligand could be present in suprabasal keratinocytes of psoriatic lesions and engage integrins here. Although there is no evidence for suprabasal accumulation of the major basement membrane proteins in hyperproliferative epidermis (Hertle et al. 1992) type XIII collagen is expressed in all the living epidermal layers, raising the possibility that suprabasal integrins are ligated (Peltonen et al. 1999), see also (Koch et al. 1999).

Alternatively, there could be a mechanism by which integrins support the activation of MAPK without being engaged by ligand. The data obtained in β1 transgenic, phenotypically normal skin suggest that aberrant integrin expression alone is not sufficient to activate MAPK but that this response requires an additional factor. It is likely that cytokines like the interleukins 1 or 6, which are found to be increased in inflammatory, hyperproliferative skin conditions (Schon 1999) could play such a role. In the absence of more data clarifying the mechanism of integrin signalling to MAPK, however, this remains pure speculation. Since MAPK activity is jointly regulated by EGF and integrins in keratinocytes a role of MAPK in the development of psoriasis is not unlikely. By means of immunostaining I was able to detect MAPK in the nuclei of keratinocytes in skin samples from patients with psoriasis and in samples of healing mouse skin and thereby provided the first evidence for activation of MAPK in these situations.

CHAPTER 6
GENERAL DISCUSSION

In my PhD project I have investigated integrin signalling to mitogen activated protein kinase in human epidermal keratinocytes. The results of these investigations have allowed me to identify functions of this signalling pathway in the regulation of keratinocyte proliferation, migration and differentiation. They also suggest that MAPK signalling may contribute to the regenerative response of the skin after injury and is possibly involved in pathogenetic mechanisms leading to hyperproliferative skin disease. In this Chapter I will discuss the limitations of my work and the questions raised by it, and I will speculate on the implications my results have for Dermatology.

6.1. Integrin mediated activation of mitogen activated protein kinase

Contact of primary human keratinocytes to extracellular matrix led to pronounced stimulation of MAPK signalling. This result was not unexpected since it had been shown before that plating of NIH-3T3 fibroblasts caused activation of MAPK signalling. Probably more relevant for skin biology is the finding that in the presence of 10 ng/ml EGF, a concentration that is present in normal keratinocyte culture medium, MAPK signalling was greatly enhanced upon keratinocyte contact with extracellular matrix molecules. It is known that the receptor for growth factors of the EGF family is constitutively expressed in the basal layer of the epidermis and fulfils important functions in skin physiology (Nanney and King 1996). Whereas the separate analysis of growth factor signalling and integrin signalling is undoubtlessly useful, it is difficult to imagine a situation in the epidermis in vivo in which integrin and growth factor signalling would not mutually influence each other. Thus, the well established inhibitory role of integrins in keratinocyte differentiation (Adams and Watt 1989; Jones and Watt 1993; Watt et al. 1993) may be related to their permissive function in growth factor signalling. Mechanisms for an interaction between integrins and growth factor receptors have been proposed: (Lin et al. 1997; Renshaw et al. 1997; Moro et al. 1998), and they involve, at least partly, close physical contact between integrins, growth factor receptors and downstream signalling molecules (Fincham et al. 1996; Fincham et al. 2000). The finding that targeting of MAPK to focal adhesions requires the activity of myosin light chain kinase (MLCK) and myosin demonstrates the importance of

elements of the actin cytoskeleton in this process of spatial organisation (Fincham et al. 2000; Fincham et al. 2000). The relevance of this is further illustrated by the fact that disruption of α-catenin, an actin binding protein with important regulatory functions for the cytoskeleton (Vasioukhin et al. 2001) leads to abnormal growth factor responses in epidermal keratinocytes. My result that CD8β1 is able to support MAPK activation when cells are plated onto OKT 8 antibody fits very well into a picture in which integrins can regulate the activity of signalling pathways via actin cytoskeletal functions.

In view of increasing evidence for a finely tuned spatial regulation of signalling molecules it is important to be aware of the limitations of in vitro models for signal transduction research. For practical reasons cells are studied in the unnatural conditions of a cell culture dish. Basic work on signal transduction is frequently carried out in immortalised cell lines. Since immortalised cells often differ significantly in shape from primary cells (e.g. HaCaT cells, an immortalised line of human keratinocytes that spread much less than primary human keratinocytes, (Boukamp et al. 1988)) it is questionable whether the mechanisms of spatial organisation are fully maintained. Even primary murine keratinocytes show alterations in cytoskeletal regulation after several passages in vitro (Vasioukhin et al. 2000) and it is possible that this has an influence on the activity of signalling pathways.

For the reasons given above and in Chapter 3 it seems likely that the mechanism by which CD8β1 expression leads to inhibition of MAPK phosphorylation involves cytoskeletal regulation. Following this hypothesis it appears interesting to investigate the effect of CD8β1 on actin cytoskeleton dependent functions like cell spreading and motility and on the spatial distribution of signalling molecules of the EGF receptor signalling pathway. Furthermore, since binding sites for proteins with regulatory functions for the cytoskeleton have been mapped within the β1 integrin tail (Reszka et al. 1992) it would be interesting to mutate these sites and analyse the effect of such mutations on MAPK activation. This would eventually allow to identify immediate downstream signalling partner(s) of the β1 integrin tail in the pathway that regulates MAPK activity. I have initiated such a project by introducing mutations into the cytoplasmic domain of CD8β1 that are, when introduced into the β1 integrin chain known to interfere with its function (Reszka et al. 1992; Levy et al. 2000). Involvement of specific sites of the β1 cytoplasmic domain in signal transduction to MAPK should

be reflected by differences between the mutants and CD8β1 in the activity of the signalling pathway.

6.2. Regulation of keratinocyte adhesion by CD8β1 and MAPK signalling

The reduction in MAPK activation caused by CD8β1 made it desirable to modulate MAPK activity independently of extracellular factors. An established method to achieve that was the expression of a constitutively active mutant of a protein involved in the pathway. There are constitutively active mutants of Ras, Raf and MAPKK1 (Roop et al. 1986; Cowley et al. 1994; Ravi et al. 1998). Since Ras is known to activate several other downstream signalling pathways apart from MAPK (Marshall 1999) and constitutively active Raf exists as a deletion mutant lacking domains that are potentially important for its localisation I chose the most direct way of activating MAPK, by expression of a constitutively active MAPKK1. This mutant kinase contains only two point mutations that correspond to the phosphorylation sites for Raf and has no other alterations in its sequence. Therefore, constitutively active MAPKK1 is most likely to be regulated in the same way as endogenous MAPKK1, apart from phosphorylation by Raf (Alessi et al. 1994). Since no other upstream regulating kinase than MAPKK is known for MAPK this is also the most specific way to stimulate MAPK signalling.

Infection of primary keratinocytes with constitutively active MAPKK1 resulted in a stimulation of MAPK phosphorylation both in adherent and in suspended cells (see Figure 3.3) indicating that the mechanisms of adhesion dependent MAPK activation lie upstream of MAPKK1 (Lin et al. 1997; Renshaw et al. 1997). The MAPK activity obtained after infection was different from the strong and transient signal observed after plating to ECM: activation was less pronounced, did not weaken with time and was not dependent on integrin engagement. Whereas the consequences of the activation strength of MAPK have not been systematically investigated analyses of activation kinetics have shown that transient or sustained stimulation of signalling can have very different effects on cell behaviour (Marshall 1995). This is of high relevance for the interpretation of my results; one has to bear in mind that plating cells onto ECM represents a short moment of a maximal adhesion response that does not occur in vivo. Constant remodeling of cell- matrix adhesion and cell shape, including formation of new adhesion sites and dissolution of others, are characteristic features of keratinocytes in culture as evident from my time lapse video recordings. Adhesion mediated

stimulation and suspension induced abrogation of MAPK activity may therefore be ongoing processes affecting different regions within an individual cell but yielding a certain net level of MAPK activity. Increasing this net level led to different effects in keratinocytes expressing CD8β1 as compared to the CD8 controls.

Since in my experimental system changes of MAPK activity and cell behaviour always occur simultaneously it is not possible to identify a hierarchy of the observed effects. Thus, increased MAPK activity after introduction of MAPKK1 may directly lead to enhanced signalling to downstream partners, e.g. Elk-1, which then could influence stem cell properties. On the other hand β1 integrin functions are restored simultaneously and all the effects observed could therefore be the consequence of repaired integrin signalling. How does MAPK activation restore integrin functions? One possible mechanism could be the increase of β1 integrin surface levels that I have observed after introduction of MAPKK1 into CD8β1 expressing cells (see Chapter 3).

Surface levels of integrins could be regulated on a transcriptional level or by mechanisms affecting integrin trafficking (Hotchin et al. 1995). Recently a role for protein kinase C α in regulating integrin traffic has been proposed (Ng et al. 1999). Protein kinase C can regulate MAPK activity (Howe and Juliano 1998) and one could speculate that MAPK might be an element of a pathway that regulates the availability of integrin receptors on the cell surface. This would also explain why expression of MANA led to a reduction of integrin surface levels.

Recently published data on a role of MAPK in the regulation of cell spreading tempt me to speculate upon another possibility: in fibroblasts activated MAPK is found in focal adhesions and its activity is required for cell spreading (Fincham et al. 2000). CD8β1, which localises to focal adhesions (Bishop et al. 1998), could inhibit cell spreading, possibly through decreased MAPK activation. This would probably lead to a reduced number of adherent cells in my adhesion assays since keratinocytes with a smaller contact area to the underlying dish would be more likely to detach during the washing steps at the end of the assay. Restoring cell spreading by MAPK activation would then lead to a more stable adhesion. Work by (Bishop et al. 1998) has shown that CD8β1 expressing keratinocytes are able to spread, however there is no quantitative comparison between those and control cells. Though these considerations form a rationale for further experiments investigating the role of CD8β1 and MAPKK1 in keratinocyte spreading and translocation of active MAPK to focal adhesions one has to bear in mind

that focal adhesions formed by keratinocytes have a different morphology than those of fibroblasts and possibly the regulatory mechanisms involved are different.

6.3. Regulation of keratinocyte motility and spreading by MAPK

Because the role of EGF and related growth factors in keratinocyte motility is well established (Martin 1997) and mechanisms are emerging by which MAPK can influence cell shape and migration it is not completely surprising that MAPK activity seems to be required in order for a keratinocyte to migrate in vitro. An interesting molecule in this context is myosin light chain kinase (MLCK) which is a direct target of MAPK and regulates cell motility (Klemke et al. 1997; Nguyen et al. 1999) and is apparently required for the translocation of activated MAPK to focal adhesions in fibroblasts (Fincham et al. 2000). It would be interesting to analyse the role of MLCK in keratinocytes by determining its phosphorylation and inhibiting its function. When I stained keratinocytes in culture with an antibody against phosphorylated MAPK I did not observe staining of focal adhesions or lamellipodia. This might be due to technical problems or it might indicate that focal adhesion assembly and membrane protrusion are differently regulated in keratinocytes and fibroblasts.

Fincham et al have localised active MAPK to newly forming focal adhesions and to the protruding lamellipodium of fibroblasts indicating a role for MAPK in spreading and the assembly of focal adhesions. When I plated keratinocytes infected with MAPKK1 to matrix coated dishes I noticed that they were smaller than control cells (see Figure 4.8) and they also appeared to have fewer focal adhesions when stained with antibodies against β1 integrins or vinculin. I therefore hypothesised that activation of MAPK might stimulate disintegration of focal adhesions or inhibit their formation. This would be a conceivable mechanism for increasing speed of motility since cell migration requires not only the assembly of cell matrix contacts but also their dissolution and there is evidence that migration speed is determined by the rate of disintegration of cell matrix contacts at the rear edge of the cell (Palecek et al. 1998). A reduced number of focal adhesions could possibly also account for the more rounded shape of the keratinocytes. I attempted to follow the kinetics of focal adhesion assembly and disintegration in keratinocytes in culture after microinjection with rhodamine labelled vinculin but this was not successful since the rhodamine signal was too weak for a long term observation and the labelled protein seemed to be degraded rapidly. It would clearly be interesting to

use improved techniques to follow the idea of a function for MAPK in the breakdown of focal adhesions. Constructs of MAPK or focal adhesion proteins tagged with fluorescent proteins may prove useful for such an expedition.

Although investigations on focal adhesion dynamics are attractive, effects of MAPK activity on focal adhesion turnover might be entirely secondary. Fully developed focal adhesions are not essential for cell movement as in the case of fast moving fish keratocytes that only form close contacts (Lee et al. 1993). Apart from exerting direct effects on the contractile apparatus of the cell MAPK can regulate the secretion of matrix metalloproteinases (Zeigler et al. 1999). Collagenase I activity is required for keratinocyte migration and appears to be regulated by EGF (Pilcher et al. 1997; Pilcher et al. 1999). It will clearly be interesting to analyse the role of MAPK in the synthesis and activation of collagenase I in keratinocytes.

6.4. Regulation of keratinocyte proliferation and differentiation by MAPK

MAPKK1 increases the proliferative potential of keratinocytes expressing CD8β1 and MANA reduces the proliferative potential of normal keratinocytes. Again, MAPK could act either through regulating transcription events or by restoring integrin function or both. There is strong evidence that MAPK signalling can directly regulate the cell cycle machinery (Lenormand et al. 1993) though this has not been thoroughly investigated in keratinocytes. In this context it is important to distinguish between proliferative activity and proliferative potential of keratinocytes. Whereas the former measures the growth rate determined by the cell cycle time, proliferative potential is a parameter characterising the ability of a keratinocyte to self renew and to permanently cover a surface with a stratified epithelium. Proliferative potential is determined by the stage of differentiation to which a keratinocyte has progressed and is reflected in culture by the colony forming efficiency. Though high proliferative activity and high proliferative potential are probably not mutually exclusive the proliferative potential of a keratinocyte does not necessarily correlate with its proliferative activity.

Considering data by (Barrandon and Green 1985;1987) which show that keratinocyte shape and the ability to migrate are determinants of their colony forming ability it is likely that actin cytoskeleton dependent keratinocyte functions can contribute to their proliferative potential. The possible role of MAPK dependent cytoskeletal changes in restoring adhesion of CD8β1 expressing cells (see 6.2.) could also be relevant to the

increase in colony forming efficiency seen after introduction of MAPKK1. Would this then mean that proliferative potential always correlates with the ability of keratinocytes to spread? Probably not, since I have also observed that keratinocytes with compromised MAPK activity can spread but have a decreased proliferative potential (Figure 4.8; Table 3.2). In fact, cells expressing MANA or treated with PD098059 appeared larger than controls or MAPKK1 infected keratinocytes that did not express CD8β1 (=MAPKK1 infected normal keratinocytes) (Figure 4.8). This is in agreement with earlier findings that large keratinocytes directly isolated from human epidermis show lower colony forming efficiency than small cells (Barrandon and Green 1985). Interestingly, MAPKK1 expressing normal keratinocytes appeared smaller than cells infected with puro alone or MANA expressing cells (Figure 4.8).

When introduced into normal primary human keratinocytes MAPKK1 stimulated and MANA reduced cell proliferation (Figure 4.1). The differences in proliferative activity compared to control keratinocytes are significant, but not dramatic. One reason for this may be that the keratinocytes I used were primary cells which probably have a higher potential to restore their signalling balance. For example, introduction of MAPKK1 could possibly lead to higher activity of a negative feedback loop involving MAPK phosphatases (Sun et al. 1993; Ward et al. 1994) though I have not tested this hypothesis. Nevertheless MAPKK1 led to a long lasting increase of MAPK activity to a submaximal level. Another possibility is therefore that keratinocytes in culture are already strongly stimulated to proliferate by the culture conditions and that it therefore is more difficult to achieve a further increase. This would not explain the relatively weak effect of MANA on inhibition of proliferation. There are other signalling pathways that stimulate proliferation of keratinocytes in culture, e.g. signalling via adenylate cyclase that is permanently stimulated in cultured keratinocytes by addition of cholera toxin to the medium, which inhibits the function of the inhibitory GTP binding protein G_i. This pathway would probably not be affected by introduction of MANA. It should also be mentioned that the MANA construct is less efficient in inhibiting MAPK than MAPKK1 is in stimulating it (Cowley et al. 1994). This is because in MANA only one serine residue (Ser 221) has been mutated whereas in MAPKK1 both phosphorylation sites (Ser 217, 221) were substituted.

The increase in proliferative activity upon introduction of MAPKK1 could be the consequence of decreased cell cycle time and this would be in agreement with results obtained in other cells showing an effect of MAPK activity on transition of cells through the restriction point (Lenormand et al. 1993). I have no evidence to support or refute this hypothesis since I have not measured cell cycle times of transduced keratinocytes; this would be a useful experiment. The data presented in Table 4.1 show that introduction of MAPKK1 is not sufficient for cell cycle progression and that there is a higher proportion of cycling adherent keratinocytes in cultures expressing MAPKK1. This suggests that cell cycle progression requires an additional, adhesion dependent signal and recent work by (Olson et al. 1998) suggests that this signal might be provided by small GTPases of the Rho family.

Although MAPKK1 was not able to induce anchorage independent growth of keratinocytes it could delay the onset of expression of differentiation markers in suspension culture (Figure 4.2) and in organotypic culture (Figures 4.3, 4.6). This raises the possibility that the primary function MAPK exerts in this experimental system is the delay of onset of terminal differentiation. How could this lead to increased proliferation in the cultures? Because MAPK suppresses the onset of terminal differentiation it could allow more divisions of keratinocytes before they enter the differentiation program. CD8 expressing keratinocytes infected with MAPKK1 or puro did not show a difference in the proportion of stem cell colonies and abortive colonies. However, MAPKK1 could increase the number of cell divisions of transit amplifying keratinocytes prior to their entry of the terminal differentiation program. In theory expansion of the epidermal transit amplifying compartment would lead to a rapid and dramatic increase of the cell number in the epidermis. It is therefore a suitable model to reflect on the onset of a psoriatic skin lesion which often develops rapidly and leads to an enormous increase in epidermal cellularity. In psoriasis the increased number of cycling keratinocytes requires space and therefore causes the typical elongation of the rete ridges (Iizuka et al. 1996; Iizuka et al. 1997;1999). Interestingly, in epidermis reconstituted by MAPKK1 expressing cells (Figure 4.3) I have observed histological changes that could represent a similar phenomenon. One additional round of divisions of transit amplifying cells would increase the epidermal cell number by the power of two, which would be sufficient to cause the observed effects.

6.5. MAPK activation in wound healing and hyperproliferative skin disease

On the basis of my in vitro experiments I have developed the hypothesis that MAPK is involved in the regulation of wound healing and in the pathogenesis of hyperproliferative skin disease. In my studies on MAPK localisation in wounded and diseased tissue I obtained evidence in order to support this hypothesis. Further experimental proof is now required.

It is well accepted that MAPK translocates to the cell nucleus upon activation and nuclear localisation of MAPK has been used as a readout for its activation in other cases (Arendt et al. 1995; Lenormand et al. 1998; Tarnawski et al. 1998; Brunet et al. 1999). I have observed nuclear staining of MAPK in skin samples of healing mouse wounds and in psoriatic skin samples using two different antibodies against ERK. In both cases staining could be blocked by preincubation with a peptide corresponding to the immunisation epitope. I have also tried to stain tissue sections with an antibody that recognises phosphorylated ERK using different fixation conditions, without success. It is likely that the phosphorylation specific epitope of MAPK is more sensitive to degradation since it can be destroyed by dephosphorylation. MAPK specific phosphatases exist within the cytoplasm (Sun et al. 1993; Ward et al. 1994) and it is known that MAPK can also be dephosphorylated by other phosphatases (Pettiford and Herbst 2000). Furthermore, since the tissue that I used for staining was not taken with the intention to analyse protein phosphorylation it may not have been treated in the most careful way in order to prevent dephosphorylation. At the moment visualisation of MAPK activity in situ depends on the use of a phosphorylation specific antibody since there is no other suitable method available.

In a very recent paper by (Vasioukhin et al. 2001) the authors have observed nuclear staining of phosphorylated MAPK in keratinocytes of hyperproliferating mouse epidermis without specifying, however, which antibody they used. This paper demonstrates that MAPK activation can play a causal role in the development of a hyperproliferative skin phenotype which in this case was induced by targeted deletion of the α-catenin gene. The results also show that the primary reason for activation of the MAPK pathway in skin does not necessarily have to lie in the signalling pathway itself but that disturbing other regulatory influences can give rise to an altered growth factor response followed by MAPK activation and hyperproliferation. α-catenin, like β1

integrins, can connect to the actin cytoskeleton and this makes it more likely that suprabasal integrin expression in hyperproliferating epidermal keratinocytes could alter the response to growth factors or cytokines of the local environment and thus contribute to the change of keratinocyte behaviour. Work by Romero et al. (1999) and my results demonstrate that suprabasally expressed integrins are functional when brought into contact with their ligands. Although thus far engagement of suprabasal integrins has not been demonstrated it is possible that integrin ligands are present in the suprabasal layers, as in the case of type XIII collagen which is expressed in all the living epidermal layers (Peltonen et al. 1999).

The work by Vasioukhin et al demonstrates a correlation between MAPK and epidermal hyperproliferation, however it does not solve the question whether MAPK activation is sufficient to induce hyperproliferative skin disease. To address this problem it would be useful to modulate MAPK activity in the epidermis in vivo. This could be achieved by expressing mutant versions of MAPKK1 in transgenic mice under the control of an epidermis specific promoter, e.g. the promoter for keratin 14 (Saitou et al. 1995). Activation and inhibition of MAPK would then allow to analyse the role of this signalling pathway in the development of hyperproliferative skin disease and wound healing more specifically.

Understanding the mechanisms that regulate MAPK signalling in epidermis and the consequences of its activation could have important clinical implications. In chronic skin wounds where reepithelialization is thought to be inhibited by factors present in the wound milieu (Martin 1997), MAPK activity could be impaired due to an abnormal composition of the wound matrix and possibly the absence or presence of certain growth factors, cytokines and proteases. If so, this would make the MAPK cascade a target for pharmacological or biological intervention. Temporary stimulation of MAPK activity, e.g. by pharmacological agents or adenoviral infection with an activating mutant could overcome the signalling defect and initiate wound epithelialisation.

Conversely, if an uncontrolled increase of proliferative potential due to increased activity of the MAPK cascade is a pathogenic mechanism involved in the development of hyperproliferative skin disease, inhibition of MAPK signalling could be an efficient therapeutic approach. A number of specific pharmacological inhibitors is now available. A recent study in which a new and very potent inhibitor of MAPK activity, PD 184352 has proven successful in the treatment of experimentally induced tumours in mice,

suggests that such a concept might be indeed very promising (Sebolt-Leopold et al. 1999). It would be very interesting to test this inhibitor also in in vivo models of hyperproliferative skin disease, e.g. in mice with suprabasal β1 integrin expression (Carroll et al. 1995), in amphiregulin overexpressing mice (Cook et al. 1999)or in skin specific α-catenin knock out mice (Vasioukhin et al. 2001).

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