Regulation of keratinocyte shape, migration and wound epithelialization by IGF-1- and EGF-dependent signalling pathways

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Summary

Adult epidermal keratinocytes migrate by crawling, a process that requires protrusion of the plasma membrane at the front of the cell and contraction of the cell body at the rear. We have found that epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) influence keratinocyte shape differently. Whereas IGF-1 stimulates membrane protrusion and facilitates cell spreading, EGF induces contraction of keratinocytes. The effects of each growth factor on keratinocyte shape are mediated by distinct signal transduction pathways: EGF stimulates the activity of the classical mitogen-activated protein kinase pathway and IGF-1 stimulates phosphatidylinositol-3-kinase. Activation of these kinases is both necessary and sufficient to induce cell shape changes upon growth factor treatment. In addition, IGF-1-stimulated keratinocyte spreading depends on the activation of Rho family proteins. In vitro assays of wound re-epithelialization show that both growth factors stimulate migration of keratinocytes, and the activity of the respective signalling pathways is required for this re-epithelialization process. When added simultaneously, IGF-1 and EGF have additive effects on wound epithelialization. Our results show that IGF-1 and EGF can influence different components of the keratinocyte migration machinery that determines the speed of wound epithelialization.

Key words: IGF-1, EGF, Cell shape, Wound epithelialization, MAP kinase, PI-3 kinase

Introduction

The outer covering of the skin, the epidermis, is exposed to the environment and is therefore particularly prone to injury. As a response to damage or disturbance of the skin's barrier function, a wound healing response is initiated. Re-epithelialization is an essential step of the wound healing process and is driven by increased proliferation and migration of keratinocytes over the wound bed. Both proliferation and migration are stimulated by the local wound milieu, which typically shows an altered composition of extracellular matrix and the presence of cytokines produced by cells of the granulation tissue and the clot (Singer and Clark, 1999).

Epidermal growth factor (EGF) is a polypeptide involved in the maturation of epithelia (Cohen and Elliott, 1963). It binds to the EGF receptor (EGFR) which is also a receptor for EGF-related cytokines such as transforming growth factor α (TGFα) (Derynck, 1986). In normal adult epidermis, EGFR is predominantly expressed in basal keratinocytes and signalling events elicited by it are known to affect their proliferation, differentiation and migration (Vassar and Fuchs, 1991) (reviewed in Nanney and King, 1996). In healing skin wounds, EGFR expression is upregulated in migrating and proliferating keratinocytes adjacent to the wound (reviewed in Nanney and King, 1996). Clinical studies of the influence of treatment of skin wounds with soluble EGF have revealed a stimulatory function of this growth factor in wound healing (Brown et al., 1989). An early event after treatment of cultured epithelial cells with EGF is remodelling of the actin cytoskeleton, which can lead to retraction of cells from the substrate surface and rounding (Chinkers et al., 1979; Peppelenbosch et al., 1993). EGF also stimulates centrifugal outwards migration of keratinocytes within colonies (Barrandon and Green, 1987).

Insulin-like growth factor 1 (IGF-1) is a peptide hormone with structural homology to proinsulin. The major source of circulating IGF-1 in postnatal life is the liver but IGF-1 can also be produced by many other tissues, where it is thought to act in a paracrine fashion. In skin, IGF-1 produced by dermal fibroblasts and macrophages (Han et al., 1987; Rappolee et al., 1988) is thought to signal to basal epidermal keratinocytes that express the IGF-1 receptor (Hodak et al., 1996). In keratinocytes, IGF-1 is thought to stimulate proliferation (Tavakkol et al., 1992) and to contribute to hair follicle morphogenesis (Liu et al., 1993; Philpott et al., 1994; Rudman et al., 1997). Mice with a targeted deletion of the IGF-1 receptor die shortly after birth from respiratory failure and show an abnormally thin and translucent epidermis with a decreased number of hair follicles (Liu et al., 1993). Until now, no function for IGF-1 in wound healing has been clearly demonstrated; there is recent evidence, however, that levels of IGF-1 are decreased in non-healing skin wounds of diabetic individuals (Blakytny et al., 2000).

Factors of the extracellular environment transduce

information by specific activation of intracellular signalling pathways. The classical mitogen-activated protein kinase (MAPK or ERK) cascade is a module of three protein kinases acting in a hierarchical order: the MAPKs (ERKs) are activated by a MAPK kinase (MEK-1) through phosphorylation of conserved threonines and tyrosines within a TXY motif (Canagarajah et al., 1997), and the MAPKK kinase (Raf) regulate MEK-1 activity. The MAPK cascade is an important regulator of cell proliferation and differentiation (Bennett and Tonks, 1997; Cowley et al., 1994; Schramek et al., 1997). We have previously shown that MEK-1 activity inhibits terminal differentiation in primary human keratinocytes (Haase et al., 2001; Zhu et al., 1999). It also exerts a crucial function in cell migration through phosphorylation of myosin light chain kinase (MLCK) (Klemke et al., 1997; Nguyen et al., 1999). Signals stimulating the activity of the MAPK cascade are 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; Miyamoto et al., 1996; Zhu et al., 1999). In several systems, including murine keratinocytes, IGF-1 is also able to stimulate ERK activation (Vasioukhin et al., 2001). The phosphorylation of ERK by MEK-1 at threonine 183 and tyrosine 185 (Rossomando et al., 1992) is necessary and sufficient for its activation and can be detected using phosphorylation specific antibodies. Exchange of serine residues 217 and 221 of MEK-1 against glutamic acid uncouples the enzyme from upstream regulating events and leads to its constitutive activation (Cowley et al., 1994).

Another important kinase involved in growth factor mediated signal transduction is phosphatidylinositol-3-kinase (PI-3K). Being a lipid kinase, this enzyme phosphorylates the inositol ring of phosphatidylinositols in their 3′ position, creating docking sites at the plasma membrane for pleckstrin homology (PH) domain containing proteins. This is thought to organize the interactions of signalling molecules spatially, thereby facilitating the formation of signalling complexes (reviewed in Czech, 2000). The PI-3K holoenzyme consists of two subunits, p85 and p110. The p85 regulatory subunit contains Src-homology 2 and 3 domains, and can bind to tyrosine kinase growth factor receptors, thus targeting the p110 catalytic subunit to the plasma membrane, where its substrates reside. Membrane targeting of p110 is sufficient for its activation because a chimeric protein consisting of a transmembrane receptor (CD2) fused to p110 exhibits constitutively activated PI-3K activity (Reif et al., 1996).

Among the factors that signal through PI-3K are platelet-derived growth factor (PDGF), insulin and IGF-1 (Kotani et al., 1994; Way and Mooney, 1993). PI-3K has been implicated in the regulation of cell proliferation, transformation, protein trafficking, actin cytoskeletal organization and apoptosis by activation of different effectors (reviewed in Toker and Cantley, 1997). One effector is the product of the *akt* oncogene, protein kinase B, which becomes phosphorylated and activated upon stimulation of PI-3K (Burgering and Coffer, 1995; Franke et al., 1995). Phosphorylation of protein kinase B/Akt at serine 473 can be detected using phosphorylation specific antibodies (Toker and Newton, 2000).

PI-3K has been shown to regulate actin cytoskeletal reorganization. This function is thought to be mediated by the small GTPase Rac, which induces protrusion of the plasma membrane and formation of lamellipodia by so far unidentified mechanisms (Nobes and Hall, 1995; Nobes and Hall, 1999; Reif et al., 1996).

Although the effects of individual growth factors on keratinocyte proliferation, differentiation and migration have been studied in culture, neither the signalling mechanisms by which these factors exert their functions nor the interactions between the respective signalling pathways are well understood. There is increasing evidence for a role of the actin cytoskeleton in the transduction of growth-factor and matrix-derived signals that regulate basic cellular functions, and it is thought that such functions could be crucial to wound epithelialization, morphogenesis, tissue modelling and regulation of proliferation (Martin, 1997). We therefore carried out experiments in which we analysed the potential of two soluble epidermotropic factors to induce actin-dependent changes of keratinocyte shape and motility, and the mechanisms involved.

Materials and Methods

Reagents and tissue samples

All reagents were purchased from Sigma (Schnelldorf, Germany) unless stated otherwise. Inhibitors for MLCK (ML-7), calpain and phospholipase C were purchased from Calbiochem (Bad Soden, Germany). Toxin B was a kind gift of K. Aktories (Freiburg, Germany). Samples of human foreskins were collected from circumcisions in the Department of Urology, University of Cologne.

Keratinocyte culture and retroviral infection

Primary human keratinocytes were isolated from foreskins and cultured on a 3T3 fibroblast feeder layer in FAD medium as described previously (Watt, 1998). 3T3 fibroblasts, strain J2, were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS). FAD medium and DMEM with low calcium concentration (FAD low Ca^{2+} and DMEM low Ca^{2+}) had essentially the same composition as FAD medium and DMEM, respectively, but contained only 50 μM Ca^{2+}. From FCS and Ham's F12 medium, Ca^{2+} was removed by incubating a volume of 50 ml with 2 g Chelex resin (Biorad, Munich, Germany) overnight on a rotating shaker followed by sterile filtration.

The constitutively active mutant of MEK1 (MAPKK1) with glutamic acid substitutions at serines 217 and 221 (Cowley et al., 1994) and cloned into pBabe puro was a generous gift of C. Marshall (Institute of Cancer Research, London, UK). The plasmid vector pEF-Bos containing the cDNA of a fusion protein between the extracellular domain of the rat CD2 receptor and the catalytic p110 subunit of PI-3K, rCD2p110 (Reif et al., 1996) was a kind gift of D. Cantrell (London). The rCD2p110 construct was subcloned into the *Xho*I site of the retroviral vector pLXSN (Clontech, Heidelberg, Germany). Correct insertion was confirmed by direct DNA sequencing. Ecotropic producer lines were generated by transfecting GP+E packaging cells (Markowitz et al., 1988b) using FUGENE (Roche, Mannheim, Germany) reagent and subsequent selection in 1 mg ml^{-1} G418 (Life Technologies, Karlsruhe, Germany) for 14 days. Supernatants were used to infect AM-12 amphotropic packaging cells (Markowitz et al., 1988a). Clones of amphotropic packaging cells producing each retroviral vector were generated and isolated as described previously (Zhu and Watt, 1996). The viral titres were as follows: 1.9×10^6 pfu ml^{-1} (MAPKK1); 4×10^5 pfu ml^{-1} (rCD2p110). Keratinocytes were infected with retrovirus by using producer cells as feeders for the first 3 days (Zhu and Watt, 1996).

Coating of cellware and spreading assay

Permanox chamber slides (Invitrogen, Karlsruhe, Germany) with two chambers (for spreading assays) or bacteriological dishes (for contraction assays) were coated with a solution of 3-5 μg ml^{-1} bovine collagen I and 20 μg ml^{-1} poly-L-lysine (PLL) or a solution of 20 μg ml^{-1} collagen I, respectively, in PBS overnight at 4°C followed by 2 hours at 37°C. Prior to plating of cells chamber slides and dishes were washed with PBS.

Feeders were removed from preconfluent cultures of infected or uninfected keratinocytes by incubation in 0.02% EDTA in PBS for 5-10 minutes. Keratinocytes were trypsinized, resuspended in 500 μl complete FAD medium and stirred into suspension medium (complete FAD medium containing 1.75% methylcellulose). Preparation of the suspension medium was as described by Adams and Watt (Adams and Watt, 1989). Cells were kept in suspension culture in a cell culture incubator for 4 hours and then harvested by diluting the suspension medium 1:10 with PBS and centrifuging. The cell pellet was washed twice in DMEM without supplements and then adjusted to a density of 10^5 cells ml^{-1}. Cells were plated onto precoated chamber slides at a density of 2×10^4 cells per chamber in the absence or presence of growth factors and inhibitors and incubated at 37°C for 40 minutes. To inhibit Rho GTPase activity, toxin B was added to the culture medium 16 hours prior to the experiment as well as to the suspension medium at a concentration of 20 ng ml^{-1}. Cells were fixed with 4% formaldehyde in PBS and permeabilized with 0.4% Triton X-100 in PBS for 5 minutes at room temperature. Slides were blocked with 50% FCS for 30 minutes at 37°C. The actin cytoskeleton was then stained with TRITC-labelled phalloidin and digital images were acquired using a Nikon Eclipse E800 microscope attached to a Basler A 113 monochrome video camera at a power of 200. For determination of cell size, a binary image was created and the area covered by each single cell was calculated using the computer program LUCIA. 200 cells per sample were analysed and the results plotted as histograms using Excel.

Determination of cell motility

Keratinocytes were harvested and plated onto bacteriological dishes coated with 10 μg ml^{-1} fibronectin (Becton Dickinson, Heidelberg, Germany) or 20 μg ml^{-1} collagen I (Sigma) at a density that allowed tracking of individual cells. Frames were taken every 2 minutes using Olympus IMT1 or IMT2 inverted microscopes driven by Broadcast Animation Controllers (BAC 900) and fitted with monochrome CCD cameras and video recorders (Sony M370 CE 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 Mathematica.

Wound healing assays

Keratinocytes were cultured on 60 mm cell culture dishes in the presence of 3T3 feeder cells or on collagen-I-coated chamber slides without feeders. When the cultures were confluent, remaining feeder cells were removed with PBS/EDTA and keratinocytes were kept in complete FAD medium containing 50 μM calcium ions (FAD low Ca^{2+}) for 24 hours. Cultures were treated with 4 μg ml^{-1} mitomycin C in DMEM containing 50 μg ml^{-1} calcium ions (DMEM low Ca^{2+}) without FCS for 2 hours, then washed in PBS and the monolayer wounded with a tip of a glass pipette. Cells were incubated in DMEM low Ca^{2+} in the absence or presence of growth factors and inhibitors for 4 hours. Cultures were fixed, permeabilized and stained with TRITC-labelled phalloidin. Digital images were acquired as described.

Western blot analysis of ERK and Akt phosphorylation

To assess the effect of growth factor treatment on activation of ERK and PI-3K, dishes of preconfluent keratinocytes were starved overnight in serum-free medium after removing their feeder layers. Cells were then stimulated with DMEM supplemented with 10 ng ml^{-1} EGF or 100 ng ml^{-1} IGF-1, or fresh medium alone for 15 minutes before lysis.

Keratinocytes were lysed in situ in modified RIPA buffer containing 5 mM EDTA, 1% Triton X-100, 20 μM leupeptin, 1 mM PMSF, 0.5 mg ml^{-1} soybean trypsin inhibitor, 0.5 mM $NaVO_3$ and 10 mg ml^{-1} *p*-nitrophenylphosphate, scraped from the dishes and sonicated for 30 seconds at full power. Lysates were centrifuged at 14,000 ***g*** for 10 minutes and the supernatant was used for western blot analysis. Equal amounts of protein were separated by SDS-PAGE and blotted onto Hybond-P PVDF membranes (Amersham, Freiburg, Germany). ERK phosphorylation was detected with antibodies specific for phosphorylated ERK1/2 (Santa Cruz Biotechnology, Heidelberg, Germany). As readout for PI-3K activity, phosphorylation of the protein kinase Akt was determined using an antibody that specifically detects phosphorylated Ser 473 of Akt (New England Biolabs, Beverly, USA). Blots were reprobed with antibodies to ERK2 (Santa Cruz Biotechnology) to check for equal loading of the lanes. Protein bands were visualized with horseradish peroxidase (HRP) coupled secondary antibodies on Hyperfilm using enhanced chemiluminescence (ECL, Amersham).

Fluorescence-activated cell sorting analysis

Keratinocytes were trypsinized, kept in suspension medium for 4 hours and then harvested. The pellet was resuspended in DMEM containing 10% FCS. Primary antibodies against EGFR (Santa Cruz Biotechnology) and IGF-1 receptor (Oncogene Science, Cambridge, USA) were diluted 1:100 and incubated on ice for 30 minutes with occasional agitation. Cells were washed twice in PBS and resuspended in DMEM containing 10% FCS and a goat polyclonal antibody against mouse IgG coupled to the dye Alexa 488 at a dilution of 1:500 (Molecular Probes, Eugene, Oregon, USA). Cells were incubated for 30 minutes, washed three times in PBS and analysed using a FACS Calibur (Becton Dickinson).

Measurement of intracellular calcium

Keratinocytes were trypsinized and kept in suspension for 4 hours, washed out and loaded with 10 μM FURA 2-AM in Ca^{2+}-free Hanks buffered salt solution (HBSS) supplemented with 15 mM HEPES for 1 hour at room temperature. All subsequent measurements were carried out in Ca^{2+}-free HBSS. Cells were washed three times and the cell density was adjusted to 10^6 cells ml^{-1}. A quartz cuvette with stirring bar was mounted in the stirred cuvette holder of a Perkin Elmer LS50B fluorescence photometer and filled with 2 ml cell suspension. The excitation wavelength was set to 380 nm, fluorescence was recorded at 509 nm. Keratinocytes were pre-incubated with inhibitors for 20 minutes. Bradykinin (10 μM) or IGF-1 (100 ng ml^{-1}) were added directly to the stirred cell suspension in the cuvette.

Results

Regulation of keratinocyte shape by IGF-1 and EGF

We established an assay that allowed quantification of cell spreading by determining the area of the chamber slide that was covered by a single cell within 40 minutes after plating. Keratinocytes were kept in suspension medium to allow for recovery of cell surface receptors after trypsin treatment. 4 hours after trypsinization, high levels of EGFR and IGF-1 receptor were detectable on the cell surface (Fig. 1A). Keratinocytes were then plated onto collagen-I-coated chamber slides in DMEM without supplements in the absence or

presence of 10 ng ml^{-1} or 100 ng ml^{-1} EGF, 10 ng ml^{-1} or 100 ng ml^{-1} IGF-1 and 4 μg ml^{-1} cytochalasin D or 0.1% DMSO as vehicle control. Analysis of the size of 200 individual keratinocytes per sample revealed that the presence of IGF-1 at 100 ng ml^{-1} and 10 ng ml^{-1} enhanced plasma membrane protrusion, as indicated by the higher number of spreading keratinocytes in those samples (Fig. 1B, top, and data not shown). In order to investigate whether the enhanced spreading upon IGF-1 treatment was driven by cytoskeletal rearrangements dependent on actin polymerization we added 4 μg ml^{-1} cytochalasin D, an inhibitor of actin polymerization, to keratinocytes when treated with 100 ng ml^{-1} IGF-1. Keratinocyte spreading was abrogated in the presence of this amount of cytochalasin D (Fig. 1B, bottom). Enhancement of cell spreading was not observed when cells were plated in DMEM in the presence of 10 ng ml^{-1} or 100 ng ml^{-1} EGF (Fig. 1B, middle, and data not shown).

We conclude that IGF-1, but not EGF, induces actin polymerization-dependent spreading of single primary human keratinocytes plated onto collagen-coated surfaces.

Treatment with EGF has been described previously to lead to cell size reduction in A431 cells (Peppelenbosch et al., 1993). We adjusted the conditions of our experiment to assess a possible influence of growth factors on cell size reduction. Keratinocytes were allowed to spread for 1 hour in DMEM without supplements. Cells were then treated with fresh DMEM without supplements or DMEM containing 10 ng ml^{-1} EGF or 100 ng ml^{-1} IGF-1. Cells were fixed after 30 minutes and cell size was determined as described. We found that cell size was reduced in a high proportion of keratinocytes treated with EGF compared with the control (Fig. 1C, top). Treatment with IGF-1 did not have this effect (Fig. 1C, bottom).

IGF-1 and EGF use distinct signal transduction pathways to regulate keratinocyte shape

EGF is known to stimulate signalling via the classical MAPK cascade (e.g. Zhu et al., 1999). When we treated preconfluent cultures of primary human keratinocytes with 10 ng ml^{-1} EGF a transient increase of MAPK phosphorylation (and hence activation) was detectable in cells analyzed after 15 minutes and 30 minutes. This stimulation was suppressed when cells were preincubated with 10 μM of the specific MEK-1 inhibitor PD98059 (Fig. 2A). EGF has also been reported to stimulate

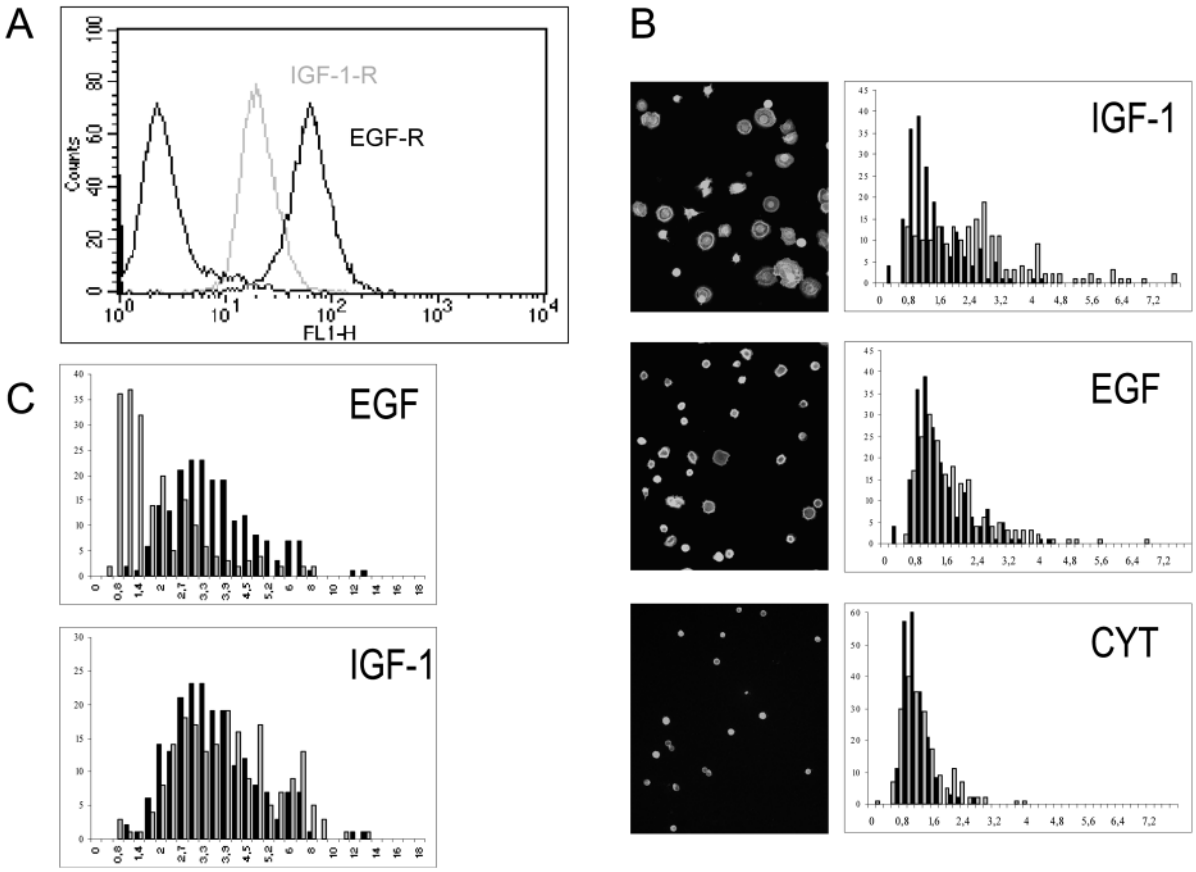

Fig. 1. Effects of IGF-1 and EGF on keratinocyte spreading and rounding. (A) FACS analysis of expression of IGF-1 receptor (grey line) and EGFR (black line) in primary human keratinocytes after suspension culture. (B) Stimulation of keratinocyte spreading by IGF-1. Histograms show size distribution of 200 keratinocytes per sample plated in the presence of 100 ng ml^{-1} IGF-1 (top), 10 ng ml^{-1} EGF (middle) or 4 μM cytochalasin D and 100 ng ml^{-1} IGF-1 (bottom). Grey bars indicate stimulated cells, black bars unstimulated cells. Left panels show microscopic pictures of stimulated keratinocytes at equal magnifications. (C) Stimulation of keratinocyte rounding by EGF. Histograms show size distribution of 200 keratinocytes per sample stimulated with 10 ng ml^{-1} EGF (top, grey bars) or 100 ng ml^{-1} IGF-1 (bottom, grey bars). Black bars in both histograms indicate unstimulated control cells.

activity of PI-3K in various cell types (Carter and Downes, 1992; Hill et al., 2000). We used western blot analysis with an antibody specific to phosphorylated Akt as a readout for PI-3K activity (Datta et al., 1996). Under our experimental conditions EGF did not stimulate activity of PI-3K in keratinocytes (Fig. 2B). Notice that, in these cultures, basal activity of MAPK is increased compared with the cells analysed in Fig. 2A. This is probably due to increased levels of paracrine growth factors in preconfluent cultures.

IGF-1 can activate PI-3K-mediated signalling in other cell types (Way and Mooney, 1993). Stimulation of preconfluent human keratinocytes with 100 ng ml^{-1} IGF-1 resulted in pronounced phosphorylation of Akt but not of MAPK. This phosphorylation was suppressed when keratinocytes were pretreated with 25 μM of a specific inhibitor of PI-3K, LY294002 (Fig. 2B).

We then tested whether the effects of EGF and IGF-1 on cell rounding and spreading that we had observed were mediated by MAPK and PI-3K, respectively. We carried out experiments in which we used either specific inhibitors or constitutively active mutants of PI-3K (rCD2p110) or MEK-1 (MAPKK1). Constitutive activation of PI-3K signalling by a fusion protein consisting of the extracellular and transmembrane domains of the rat CD2 receptor and the catalytic subunit of PI-3K (rCD2p110) has been described previously (Reif et al., 1996). Stimulation of MAPK signalling by an activating mutant of MEK-1 (MAPKK1) has been shown by Cowley et al. (Cowley et al., 1994). Both mutant kinases were expressed using retroviral gene transfer and subsequent selection with puromycin and G418, respectively. We have shown previously that expression of MAPKK1 results in constitutive activation of MAPK signalling in primary human keratinocytes (Haase et al., 2001; Zhu et al., 1999). Infection of primary keratinocytes with retroviruses encoding rCD2p110 led to expression of the fusion protein as shown by detection of a band at approximately 120 kDa in western blots with a monoclonal antibody against the extracellular domain of rat CD2 (Ox-34) (see also Reif et al., 1996). The band indicated by an arrowhead in Fig. 3A represents the rCD2p110 fusion protein; it was not present in keratinocytes infected with the empty vector neo only (Fig. 3A). We also detected expression of rCD2p110 on the surface of keratinocytes transduced with retroviruses encoding for the construct but not in keratinocytes transduced with empty vector neo. Immunofluorescent staining using the antibody Ox-34 revealed that rCD2p110 specifically localized to protruding areas of the cell membrane (Fig. 3B,C), which were characterized by a fine mesh of thin actin fibres, indicating that these protrusions were relatively young.

Treatment of keratinocytes with 25 μM or 50 μM LY294002 prior to stimulation with IGF-1 led to a reduction of cell spreading at 40 minutes or 3 hours after plating compared with

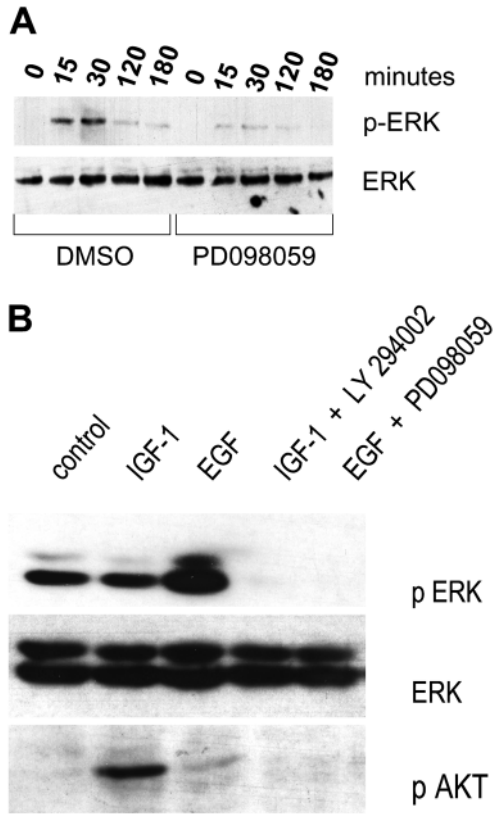

Fig. 2. Activation of PI-3K and MAPK by IGF-1 and EGF. Western blot with phosphorylation-specific antibodies against MAPK (A, top; B, top) or Akt (B, bottom) showing the effect of growth factor treatment and pharmacological inhibitors on activation of MAPK and Akt. The lower panel in A and the middle panel in B show total (phosphorylated and unphosphorylated) MAPK (ERK2) as loading control. (A) Time course of MAPK activation after stimulation with EGF in single keratinocytes treated with vehicle control (DMSO) or 10 μM PD98059. Keratinocytes were stimulated for the indicated times. (B) Activation of MAPK and Akt in preconfluent cultures of keratinocytes. Cells were pretreated with DMSO or the inhibitors PD98059 (10 μM) and LY294002 (25 μM), respectively, and then stimulated with growth factors for 15 minutes.

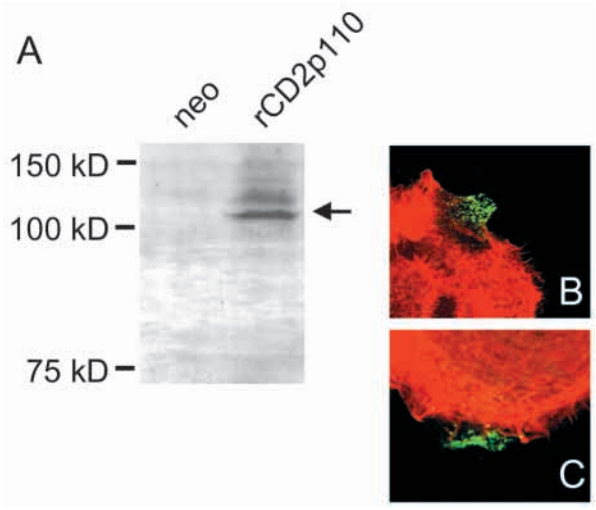

Fig. 3. Expression of a constitutively active PI-3K mutant (rCD2p110) in primary human keratinocytes. (A) Western blot analysis with the anti rat CD2 antibody Ox34 of keratinocytes infected with retroviruses encoding rCD2p110 or control virus (neo). Arrow points to rCD2p110 protein. Equal loading of the lanes was checked by Coomassie blue staining. (B,C) Immunofluorescent staining with Ox34 (green) and TRITC-phalloidin (red). Green staining shows localization of rCD2p110 at protruding areas of the cell membrane.

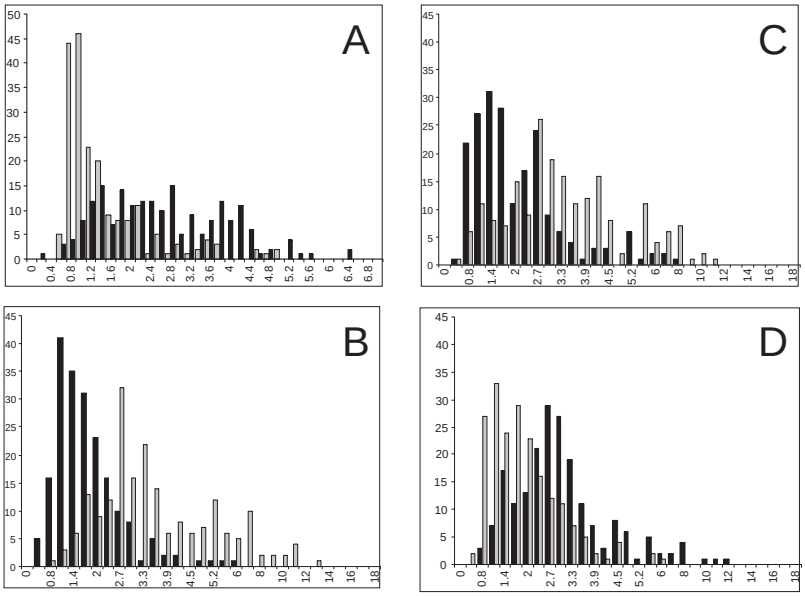

Fig. 4. Activation of PI-3K and MAPK are necessary and sufficient to induce keratinocyte shape changes. Histograms show size distribution of 200 keratinocytes per sample. (A) Keratinocytes plated in the presence of 100 ng ml^{-1} IGF-1 and pretreated with 25 μM LY294002 (grey bars) or the vehicle control 0.1% DMSO (black bars). (B) Keratinocytes infected with rCD2p110 (grey bars) or empty vector neo (black bars) plated in the absence of growth factors. (C) Keratinocytes stimulated with 10 ng ml^{-1} EGF and pretreated with 10 μM PD98059 (grey bars) or the vehicle control 0.1% DMSO (black bars). (D) Keratinocytes infected with MAPKK1 (grey bars) or empty vector puro (black bars) in the absence of growth factors.

control cells pretreated with DMSO only (Fig. 4A and data not shown). Conversely, activation of PI-3K signalling by expression of rCD2p110 resulted in enhanced spreading of keratinocytes compared with the neo control (Fig. 4B).

We next tested whether the effect of EGF on keratinocyte size reduction was mediated by MAPK. When spreading keratinocytes were treated with 10 μM PD98059 prior to EGF stimulation, the reduction in cell size was less pronounced than in keratinocytes pretreated with DMSO only (Fig. 4C). Conversely, keratinocytes expressing MAPKK1 were smaller than controls (Fig. 4D). From these experiments, we conclude: (1) that activation of PI-3K is sufficient to induce spreading of human keratinocytes and partly mediates IGF-1-stimulated spreading; and (2) that activation of MAPK is sufficient to induce rounding of human keratinocytes and the reduction of keratinocyte size induced by EGF is mediated by MAPK.

Crucial downstream effectors of IGF-1-stimulated, PI-3K-dependent Schwann cell motility are the small GTP-binding proteins Cdc42 and Rac (Cheng et al., 2000). We therefore tested the involvement of Rho GTPases in IGF-1-stimulated spreading by inhibiting their function with the exotoxin B of the bacterium *Clostridium difficile* (toxin B) (Just et al., 1995). Preincubation of primary human keratinocytes with toxin B inhibited IGF-1-induced spreading (Fig. 5G). We conclude that Rho GTPase function is essential for spreading of keratinocytes.

Another signalling pathway known to regulate cell movements involves phospholipase Cγ (PLCγ) (Chen et al., 1994). Activity of PLC results in the enzymatic hydrolysis of phosphatidylinositol-4,5 bisphosphate and the generation of inositol-1,4,5 trisphosphate, which releases Ca^{2+} from intracellular stores (Berridge and Irvine, 1984). Intracellular Ca^{2+} concentration after agonist stimulation can therefore be used as a measure of PLC activity. We used a fluorimetric method with the Ca^{2+}-binding fluorescent dye FURA-2 to test inhibition of PLC activity by the inhibitor U73122. U73343, an isomer of U73122 that is less efficient at inhibiting PLC, was used as a control. Keratinocytes were loaded with FURA-2 and intracellular fluorescence of suspended keratinocytes in Ca^{2+}-free Hanks buffered salt solution was measured at 509 nm. Under these conditions, stimulation with 100 ng ml^{-1} IGF-1 did not result in a detectable increase of the intracellular calcium concentration (Fig. 5B). Stimulation with 10 μM bradykinin resulted in a rapid increase in fluorescence (Fig. 5A). Cells were then pre-incubated with different concentrations of U73122 and U73343, and stimulated with 10 μM bradykinin. Although incubation with 1 μM or 5 μM of both U73122 and U73343 reduced the amplitude of fluorescence after bradykinin stimulation, this suppression was more pronounced in cells pre- incubated with U73122 (Fig. 5C-F).

Using inhibitor concentrations of 1-10 μM U73122 and U73343, we tested whether PLC contributes to IGF-1-stimulated spreading in human keratinocytes. In four out of five experiments, there was consistent inhibition of cell spreading at concentrations from 1 μM to 10 μM U73122 (Fig. 5H). At concentrations of 1-4 μM, U73343 had no detectable influence on the degree of IGF-1-induced cell spreading (Fig. 5I). At 10 μM, both substances inhibited spreading to a similar degree, suggesting that reduced spreading at this inhibitor dose was not due to specific inhibition of PLC (data not shown). Inhibitors were not toxic as determined by analysis of lactate dehydrogenase (LDH) release (data not shown). We conclude, based on experiments relying on pharmacologic inhibition, that PLCγ activity is required for IGF-1-stimulated spreading of human keratinocytes.

Conceivable mechanisms for MAPK-mediated keratinocyte contraction include stimulation of myosine light chain kinase (MLCK) (Klemke et al., 1997) or the activation of calpain, a protease implicated in the degradation of focal adhesion components (Glading et al., 2000). When we stained keratinocytes with antibodies against β1 integrin and vinculin in order to visualize focal adhesions, we noticed that MAPKK1 expressing cells seemed to have fewer focal adhesions than control cells (data not shown). We used the MLCK inhibitor ML-7 and the calpain inhibitor MDL28170 at concentrations

of 1-10 μM in order to test the involvement of these enzymes. Under these conditions, the inhibitors showed no influence on EGF-stimulated keratinocyte contraction (data not shown).

MAPK mediates EGF-stimulated keratinocyte motility

EGF and insulin are among the growth factors that stimulate keratinocyte migration (Barrandon and Green, 1987; Benoliel et al., 1997). MAPK has been shown to regulate the motility of several cell types by direct phosphorylation of MLCK (Klemke et al., 1997; Nguyen et al., 1999). We therefore analysed the influence of MEK-1 mutants and the specific MEK-1 inhibitor PD98059 on keratinocyte motility. To exclude toxic effects of the chemical inhibitor PD98059 on keratinocytes during the prolonged observation period for cell migration (24 hours), we also used a dominant negative mutant of MEK-1, *MANA* (Cowley et al., 1994), to inhibit MAPK activity. The effect of this construct on suppression of MAPK activity has been shown previously (Cowley et al., 1994; Haase et al., 2001; Zhu et al., 1999).

Keratinocyte motility was monitored by time lapse video microscopy on fibronectin and collagen coated dishes. Similar results were obtained for both substrata. Data shown in Fig. 6A,B were obtained with fibronectin coated dishes. Data sets shown in Fig. 6C,D were obtained with collagen coated dishes (two experiments) and fibronectin coated dishes. Constitutive activation of MAPK by expression of MAPKK1 led to an increase in the total distance moved by single cells (Fig. 6A,B) and a twofold increase in average cell speed (Fig. 6C). Fig. 6C shows the results of four separate experiments in which the speeds of cells expressing the empty vector, puro, or MAPKK1 were 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^{-1}) and MAPKK1 expressing (32.8±1.2 μm h^{-1}) keratinocytes was statistically significant (P<0.01).

Consistent with previous findings (Barrandon and Green, 1987), incubation of keratinocytes with 10 ng ml^{-1} EGF led to a threefold increase in cell speed (P<0.001; compare puro expressing cells in Fig. 6C,D). In the presence of 10 ng ml^{-1} EGF, inhibition of MAPK activity with 25 μM PD98059 or by introduction of *MANA* reduced keratinocyte motility. The histogram in Fig. 6D shows the speed of all cells analysed in five independent experiments in which MEK-1 activity was inhibited. 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 in these experiments was always completely random (Fig. 6A,B) and neither activation nor inhibition of MAPK signalling had any influence on the directionality of cell movement.

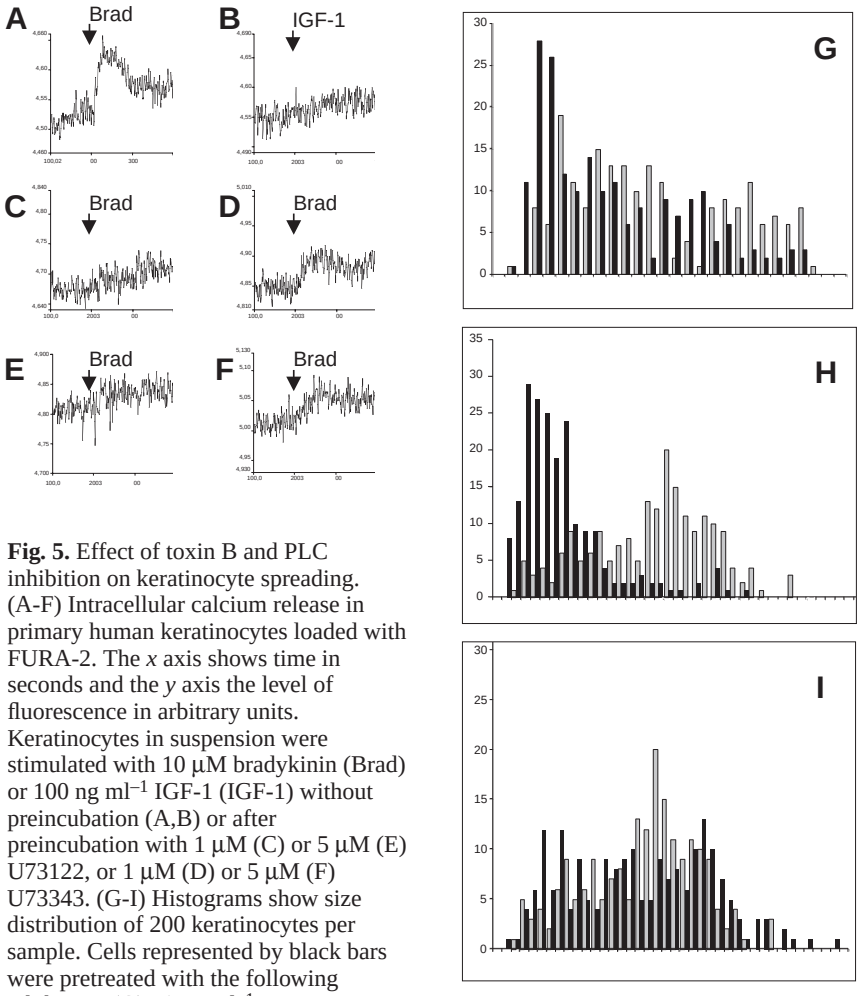

Fig. 5. Effect of toxin B and PLC inhibition on keratinocyte spreading. (A-F) Intracellular calcium release in primary human keratinocytes loaded with FURA-2. The *x* axis shows time in seconds and the *y* axis the level of fluorescence in arbitrary units. Keratinocytes in suspension were stimulated with 10 μM bradykinin (Brad) or 100 ng ml^{-1} IGF-1 (IGF-1) without preincubation (A,B) or after preincubation with 1 μM (C) or 5 μM (E) U73122, or 1 μM (D) or 5 μM (F) U73343. (G-I) Histograms show size distribution of 200 keratinocytes per sample. Cells represented by black bars were pretreated with the following inhibitors: (G) 20 ng ml^{-1} toxin B overnight; (H) 3 μM U73122 for 20 minutes; (I) 3 μM U73343 for 20 minutes. Grey bars show cells pretreated for the same time with the relevant vehicle control. All cells were plated in the presence of 100 ng ml^{-1} IGF-1.

IGF-1 enhances keratinocyte random migration independently of PI-3K activity

We also investigated the influence of IGF-1 on keratinocyte motility. In three independent experiments, treatment of primary human keratinocytes with 100 ng ml^{-1} IGF-1 stimulated random cell migration by a factor of 1.5 to 2 (Fig. 6E). Two experiments were carried out with fibronectin coated dishes and one experiment with collagen coated dishes, yielding similar results. We further tested the requirement of PI-3K activity for spontaneous and IGF-1 induced migration. Treatment with 25 μM LY294002 slowed down spontaneous keratinocyte movements in the absence of IGF-1 but did not reduce IGF-1 stimulated migration even when added at a concentration of 50 μM (Fig. 6E,F and data not shown). During the 24 hour period of observation, no signs of cytotoxicity were detected in the keratinocytes. We conclude that PI-3K activity

can regulate keratinocyte motility but it is not required for IGF-1 stimulated keratinocyte migration in vitro.

Co-operative regulation of wound epithelialization by IGF-1 and EGF dependent pathways

Epidermal wound healing is a typical situation during which keratinocyte shape and migration are regulated and both EGF and IGF-1 levels are known to be increased in skin wounds (Singer and Clark, 1999). Because cell shape and random cell migration represent only single functions of keratinocytes within the complex process of wound epithelialization, we compared the effects of EGF and IGF-1 in a wound epithelialization model of a keratinocyte monolayer in vitro. Keratinocytes were grown on cell culture dishes in the presence of feeders (three experiments) or on collagen coated dishes in the absence of feeders (two experiments). Similar results were obtained under both conditions. In order to exclude effects of either growth factor on cell-cell adhesion and on cell proliferation, confluent sheets of keratinocytes in FAD low Ca^{2+} were treated with 4 μg ml^{-1} mitomycin C for 2 hours. This treatment prevented the formation of Ca^{2+}-dependent cell-cell contacts and abrogated proliferation of keratinocytes (data not shown). We then wounded the keratinocyte monolayer with a glass pipette tip, creating wounds of constant size, and monitored wound closure over 4-6 hours. As expected, treatment with 100 ng ml^{-1} or 10 ng ml^{-1} EGF accelerated wound closure compared with the untreated control (Fig. 7A,E and data not shown). This was almost completely inhibited by treatment with 10 μM of PD98059 (Fig. 7E,F). Addition of IGF-1 at 100 ng ml^{-1} also accelerated wound closure (Fig. 7A,C) and this effect could be abrogated by inhibition of PI-3K activity using 25 μg ml^{-1} LY294002 (Fig. 7C,D). When EGF and IGF-1 were added simultaneously to the wounded cultures, re-epithelialization of the denuded area was faster than with either growth factor alone (Fig. 7B).

We also investigated whether activation of MAPK or PI-3K were sufficient to stimulate wound epithelialization using confluent monolayers of keratinocytes infected with MAPKK1, rCD2p110 or empty vector controls on collagen coated dishes. Constitutive activation of MAPK signalling by expression of MAPKK1 accelerated wound closure dramatically (Fig. 7G,H). Activation of PI-3K by expression of rCD2p110 also stimulated re-epithelialization, but this effect was less pronounced than the effect of MAPK activation (Fig. 7I,J).

Using pharmacological inhibitors, we further analysed the contribution of different signalling molecules to IGF-1 and EGF stimulated wound epithelialization. Consistent with the results obtained in the spreading assay, preincubation with toxin B (an inhibitor of Rho activity) strongly inhibited IGF-1 induced wound closure (Fig. 8A,B). Whereas addition of MDL28170 and U73122 had no inhibitory effect, ML-7 delayed EGF stimulated wound closure at concentrations of 5 μM and 10 μM (Fig. 8C-E).

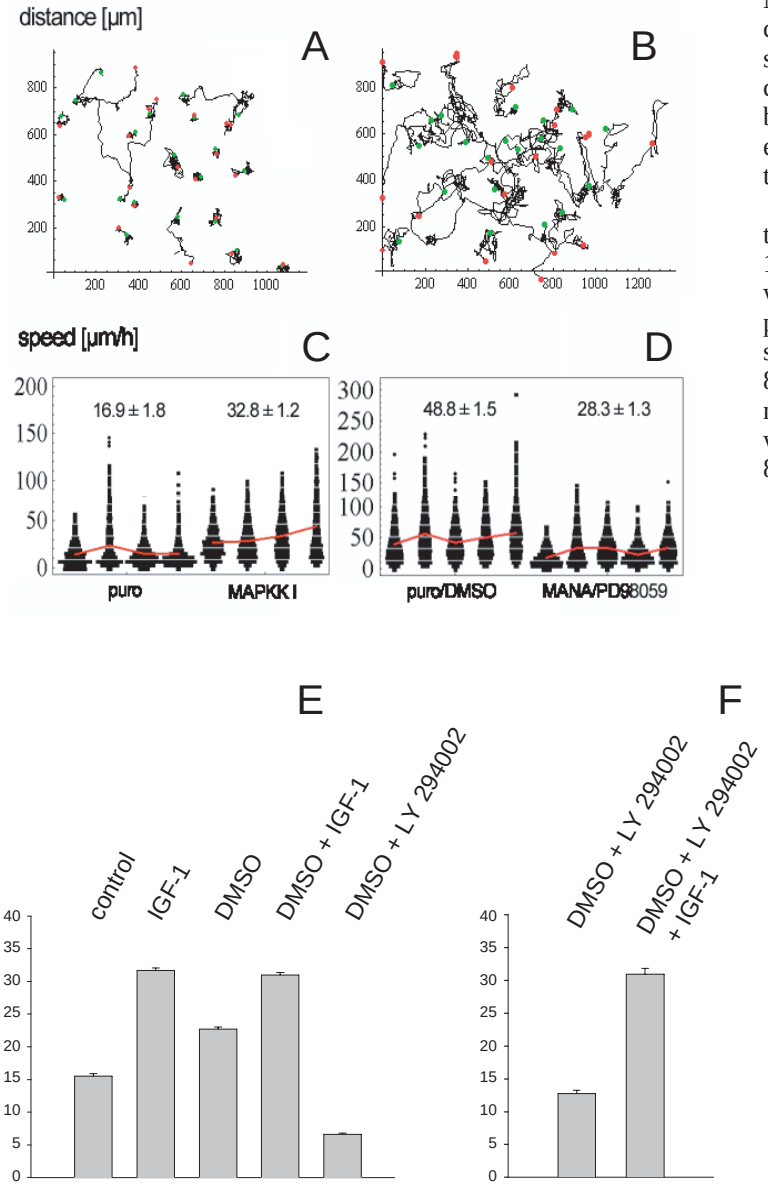

Fig. 6. Effects of MEK-1 activity, IGF-1 and PI-3K activity on keratinocyte motility. (A,B) Trajectories of cells expressing puro (A) or MAPKK1 (B) followed for 24 hours, showing 20 cells per sample. Origin and end point for each cell are indicated by coloured dots (green/red). (C,D) Histograms showing speed of cell movement. Each bar represents a separate experiment, with 20 cells being 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. Mean and standard error 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 PD98059 in the presence of 10 ng ml^{-1} EGF. (E) Migration speed (μm h^{-1}) of keratinocytes left untreated or treated with 0.1% DMSO or 25 μM LY294002 and left unstimulated or stimulated with 100 ng ml^{-1} IGF-1. (F) Keratinocytes were preincubated with 0.1% DMSO and 25 μM LY294002, and left unstimulated or were stimulated with 100 ng ml^{-1} IGF-1. A similar result was obtained when 50 μM LY294002 were used. Bars in E,F show mean±s.e.m.

Discussion

Functions of epidermal keratinocytes are thought to be regulated by a multitude of extracellular influences, such as contact to extracellular matrix molecules and soluble factors that act in an autocrine or paracrine fashion (Jacinto et al., 2001; Martin, 1997; Singer and Clark, 1999). Keratinocyte proliferation and migration are essential for the formation of skin appendages and epidermal repair after wounding, and many growth factors investigated so far have been shown to stimulate these functions. This apparent uniformity of growth factor effects on keratinocyte proliferation and migration raises the question of whether there are specific contributions of individual factors to particular keratinocyte functions. We have therefore analysed and compared signalling pathways stimulated either by IGF-1 or EGF for their potential to regulate the shape of individual keratinocytes, to stimulate random migration and to facilitate wound epithelialization in vitro.

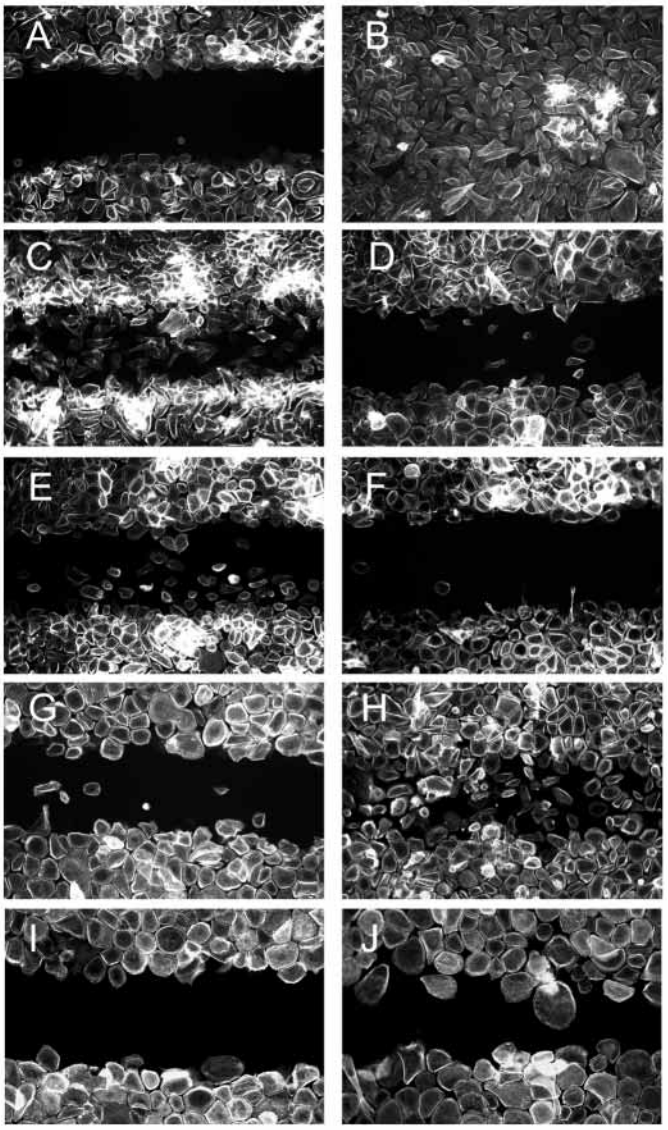

Fig. 7. Effect of growth factor signalling on wound epithelialization. Mitomycin-C-treated keratinocytes in FAD low Ca^{2+} were wounded and incubated for 6 hours in the presence or absence of growth factors. (A-F) Normal human epidermal keratinocytes were left untreated (A) or were treated with: (B) 10 ng ml^{-1} EGF+ 100 ng ml^{-1} IGF-1; (C) 100 ng ml^{-1} IGF-1; (D) 100 ng ml^{-1} IGF-1 μM and 25 μM LY294002; (E) 10 ng ml^{-1} EGF; (F) 10 ng ml^{-1} EGF and 10 μM PD98059. (G-J) primary human keratinocytes infected with retroviral vectors encoding: (G) empty vector puro; (H) MAPKK1; (I) empty vector neo; (J) rCD2p110.

In our study we have identified a new function of IGF-1 in epidermal keratinocytes: IGF-1 regulates keratinocyte shape by stimulating plasma membrane protrusion and spreading. This action of IGF-1 results in the acceleration of wound epithelialization in vitro and it requires activity of PI-3 kinase and of Rho GTPases (Ridley and Hall, 1992; Ridley et al., 1992). In view of the importance of regulated shape changes of epidermal cells during *Drosophila* development (Harden et al., 1995) this mechanism could also serve as a potential explanation for the disturbance of hair follicle formation in the skin of IGF-1-receptor deficient mice (Liu et al., 1993).

EGF, in contrast to IGF-1, induces rounding of the cell body, leading to a decrease of the cell-substratum contact area. Similarly, in A431 cells EGF has been shown to induce cytoskeletal rearrangements that lead to cell contraction and rounding (Peppelenbosch et al., 1993). Considering these results, it might seem contradictory that treatment of keratinocyte colonies with EGF should lead to enlargement of these colonies within minutes (Barrandon and Green, 1987). This enlargement, however, does not directly reflect cytoskeletal changes induced by EGF but is caused by translocation of keratinocytes from the colony centre to its periphery because of increased migration. The most likely interpretation, combining both observations, is that cell body contraction is part of the EGF stimulated migration process.

The observed differences in the regulation of keratinocyte shape led us to investigate intracellular pathways that mediate EGF and IGF-1 functions in more detail. As has been described previously in keratinocytes and in other cell types, EGF treatment resulted in activation of the classical MAPK pathway (Gotoh et al., 1990; Zhu et al., 1999). Treatment of cultured primary human keratinocytes with IGF-1-stimulated phosphorylation of the protein kinase Akt, indicating activation of PI-3 kinase, but not the classical MAPK pathway. This result is different from data obtained in murine keratinocytes, in which IGF-1 treatment led to an increase in MAPK activity (Vasioukhin et al., 2001). This difference might reflect species-specific responses to IGF-1 or variations in culture conditions. Although treatment with IGF-1 did not stimulate MAPK activity above background levels, pretreatment with LY294002, a specific inhibitor of PI-3K, completely abrogated MAPK activity (Fig. 2B). This might indicate that activity of PI-3K could be required by keratinocytes in order to enable MAPK activation by autocrine signalling. Inhibition of basal MAPK activity by LY294002 could also account for the reduction of spontaneous, IGF-1 independent migration observed in keratinocytes treated with this inhibitor (Fig. 6E).

Using pharmacological inhibitors and mutants of MEK-1, we found that activity of the MAPK cascade is both required and sufficient to induce keratinocyte rounding. In a cell migration model proposed recently (Cheresh et al., 1999), MAPK acts as an opponent of p130CAS, cCrkII and the small GTPase Rac, which are known to stimulate ruffling, by regulating the actin-myosin motor that generates forces for cell

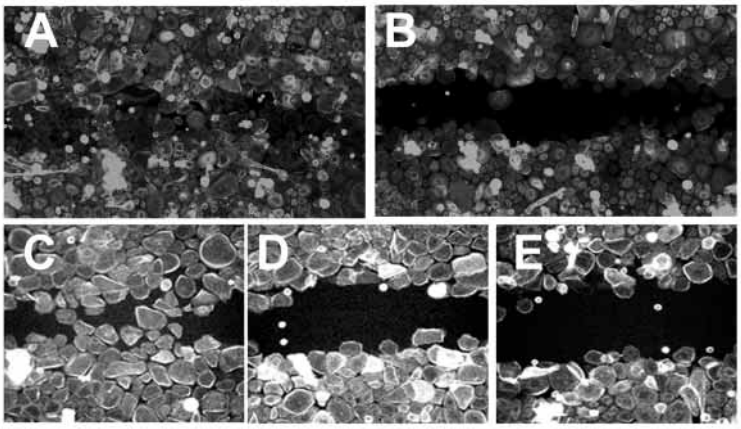

Fig. 8. Effect of toxin B and ML-7 on wound epithelialization in vitro. Mitomycin-C-treated keratinocytes in FAD low Ca^{2+} were wounded and incubated for 6 hours in the presence of growth factors. Wounded monolayers were stimulated with 100 ng ml^{-1} IGF-1 (A,B) or 10 ng ml^{-1} EGF (C-E) in the presence of 20 ng ml^{-1} toxin B (B) and 5 μM (D) or 10 μM (E) ML-7 or the respective vehicle controls (A,C).

contraction. Proposed mechanisms for the stimulatory effect of MAPK on cell motility include phosphorylation and activation of MLCK (Klemke et al., 1997), and activation of calpain, a protease implicated in the degradation of focal adhesion components (Glading et al., 2000). We did not find evidence for the involvement of these enzymes; the mechanism of MAPK mediated keratinocyte cell rounding therefore remains unidentified here. One possibility that we have not addressed in our study is the stimulation of matrix metalloproteinases through sustained activation of MAPK (McCawley et al., 1999), which could both result in degradation of matrix molecules and enhance the release of autocrine ligands for the EGFR from the keratinocyte surface (Fan and Derynck, 1999). Although MLCK activity was not required for EGF induced cell rounding, the MLCK inhibitor ML-7 prevented wound epithelialization stimulated by EGF. This indicates that other MLCK dependent cellular functions are required for the stimulation of wound closure by EGF.

In contrast to MAPK, activation of PI-3K stimulated membrane protrusion, whereas its inhibition delayed keratinocyte spreading. PI-3K has been described to induce cytoskeletal rearrangements by stimulation of Rac activity (Nobes et al., 1995) and we show here the involvement of small GTPases of the Rho family in IGF-1-stimulated keratinocyte spreading. Consistent with a putative role of Rac, rCD2p110 localized to protruding areas of the cell membrane, where Rac is normally active (Del Pozo et al., 2002; Nobes and Hall, 1995).

In addition, we found a requirement for PLC activity for keratinocyte spreading. Although IGF-1 did not stimulate PLC activity in suspended keratinocytes, U73122 inhibited keratinocyte spreading when added at concentrations that specifically suppress PLC activity. Efficient activation of PLC by growth factors has been reported to depend on cell adhesion (McNamee et al., 1993) and it is therefore possible that PLCγ is activated by IGF-1 in adherent, but not in suspended, keratinocytes.

In adult skin, keratinocytes migrate by crawling (Fenteany et al., 2000) (reviewed in Jacinto et al., 2001). This form of movement requires extension of the plasma membrane on one hand and contraction of the cell body on the other. The relationship between cell shape changes and migration speed is so far unclear. It has been suggested for different cell types, however, that activation of PI-3K and Rac, which promote cell spreading, is sufficient to stimulate motility (Keely et al., 1997). The fact that inhibition of PI-3K can delay IGF-1-induced keratinocyte spreading but not inhibit IGF-1-stimulated motility suggests, however, that IGF-1 uses multiple intracellular pathways to exert its effects on keratinocyte shape and motility. This is further supported by the observed inhibition of cell spreading with the PLC inhibitor U73122 and by results of others showing the involvement of NF-κB-dependent signalling in the regulation of insulin-stimulated motility of keratinocytes (Benoliel et al., 1997). The finding that inhibition of PI-3K activity can reduce keratinocyte motility in the absence of exogenous growth factors (Fig. 6E) could reflect the requirement for PI-3K activity to maintain a basal level of MAPK signalling (Fig. 2B).

Although the PI-3K inhibitor LY294002 failed to affect IGF-1-stimulated random motility of keratinocytes, it was able to inhibit spreading of keratinocytes on collagen I and to delay wound closure in vitro. These findings raise the possibility that the speed of random keratinocyte migration is not the only determinant of wound epithelialization speed and suggest that changes in the keratinocyte actin cytoskeleton are crucial for the stimulation of wound epithelialization by IGF-1. Because wound epithelialization in vivo starts from the wound edge, it is conceivable that disturbances of cell spreading could delay or prevent the start of epithelial sheet locomotion over the wound. This might occur in chronic wounds of diabetic individuals, in which IGF-1 amounts are reduced (Blakytny et al., 2000) and/or decreased levels of insulin hamper optimum keratinocyte migration (Benoliel et al., 1997). The biological significance of cytokine-induced cytoskeletal rearrangements has been illustrated in β5-integrin-transfected CS-1 melanoma cells in which IGF-1 leads to the co-localization of αvβ5 integrin with α-actinin and results in enhanced tumour cell metastasis in vivo (Brooks et al., 1997).

The speed of epidermal wound healing is apparently regulated by an interplay of mechanisms involving different signalling molecules. Our data provide evidence for the existence of at least two relevant signalling pathways that are able to stimulate wound epithelialization in an additive manner: an EGF-stimulated, MAPK-dependent pathway and an IGF-1-stimulated, PI-3K/Rac-dependent pathway. Further dissection of these pathways will provide deeper insight into the mechanisms that regulate skin wound epithelialization.

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