Glycogen synthase kinase-3 regulates formation of long lamellipodia in human keratinocytes

Leeni Koivisto[1], Keyhan Alavian[1], Lari Häkkinen[1], Steven Pelech[2], Christopher A. McCulloch[3] and Hannu Larjava[1,*]

[1]University of British Columbia, Faculty of Dentistry, Department of Oral Biological and Medical Sciences, 2199 Wesbrook Mall, Vancouver, BC V6T 1Z3, Canada
[2]Kinexus Bioinformatics Corporation, Suite 401, 2389 Health Sciences Mall, Vancouver, BC V6T 1Z4, Canada
[3]University of Toronto, Faculty of Dentistry, Department of Biological and Diagnostic Sciences, 150 College Street, Toronto, ON M5S 3E2, Canada
*Author for correspondence (e-mail: larjava@interchange.ubc.ca)

Accepted 28 May 2003
Journal of Cell Science 116, 3749-3760 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00693

Summary

During wound healing, keratinocytes initiate migration from the wound edge by extending lamellipodia into a fibronectin-rich provisional matrix. While lamellipodia-like structures are also found in cultured keratinocytes exposed to epidermal growth factor (EGF), the signaling pathway that regulates the formation of these structures is not defined. In cultured human keratinocytes seeded on fibronectin, we found that protein-serine/threonine kinase inhibitors including staurosporine, induced concentration-dependent formation of extended lamellipodia (E-lams). The formation of E-lams was inhibited by the protein-tyrosine kinase inhibitors herbimycin A and genistein and augmented by the protein-tyrosine phosphatase inhibitor sodium orthovanadate. Staurosporine treatment induced relocation of tyrosine phosphorylated phospholipase C-γ1 (PLC-γ1) to the tips of lamellipodia where actin assembly was initiated. Consistent with an involvement of PLC-γ1 in E-lam formation, intracellular free calcium (Ca^{2+}) was elevated during the formation of E-lams and conversely, E-lam formation was blocked by intracellular Ca^{2+} chelation with BAPTA/AM, but not by extracellular reduction of Ca^{2+} by EGTA. Notably, glycogen synthase kinase-3α/β (GSK-3α/β) was activated by staurosporine as evidenced by reduced phosphorylation on Ser-21/9. Suppression of GSK-3 activity by $LiCl_2$ or by a specific chemical inhibitor, SB-415286, blocked E-lam formation but without altering cell spreading. Furthermore, GSK-3 inhibitors blocked both staurosporine- and EGF-induced keratinocyte migration in scratch-wounded cultures. We propose that GSK-3 plays a crucial role in the formation of long lamellipodia in human keratinocytes and is potentially a central regulatory molecule in epithelial cell migration during wound healing.

Key words: Fibronectin, Wound healing, Calcium, EGF, Migration

Introduction

After disruption of epithelia by injury, migration of epithelial cells is rapidly initiated to re-establish tissue integrity (Stenn and Malhorta, 1992; Woodley, 1996). Keratinocyte migration on the fibronectin-rich, provisional wound matrix is initiated by the formation of long lamellipodia that extend into the wound, a step that is considered crucial for epidermal wound closure (Odland and Ross, 1968; Larjava et al., 1996; Woodley, 1996). Similar cellular structures reflect invasive, migratory behavior of border cells during *Drosophila melanogaster* oogenesis (Fulga and Rørth, 2002). Lamellipodia formation and cell migration are dependent on the coordinated assembly of actin filaments and microtubules (Larjava et al., 1996; Mikhailov and Gundersen, 1998; Small et al., 1999). Actin assembly in turn relies on the activity of Rac1, a small GTPase of the Rho family (Nobes and Hall, 1995; Fenteany et al., 2000). Migration of keratinocytes on the provisional matrix also requires integrins (Grinnell, 1992; Hughes and Pfaff, 1998; Larjava et al., 2002), transmembrane receptors for matrix proteins whose binding activity is controlled in part by alterations of affinity. The pathways involved in the formation of extended lamellipodia in keratinocytes remain virtually unexplored.

Structures resembling the cytoplasmic extensions of keratinocytes in wounds have been observed previously in cultured mouse epidermal cells and megakaryoblastic leukemia cells following exposure to the protein kinase inhibitor, staurosporine (Sako et al., 1988; Yamazaki et al., 1999). Similarly, rat pheochromocytoma PC12 cells exhibit neurite outgrowth after stimulation with staurosporine (Rasouly et al., 1994; Rasouly et al., 1996). Although the signaling pathways underlying these phenomena are not defined, there may be a common mechanism that also controls the formation of cellular extensions in keratinocytes. Accordingly, staurosporine may provide a useful tool to study the cellular signaling events that regulate extended lamellipodia formation.

Staurosporine is a broad-spectrum protein-serine/threonine kinase inhibitor whose function is dependent on interactions with the ATP-binding site of its target kinases. As staurosporine can either stimulate or inhibit multiple protein kinases in a cell-type specific manner, multiple potential targets may be involved in the formation of cytoplasmic extensions. For example, molecular targets of staurosporine that can regulate cell shape include protein kinases C (PKC[1]), A, G and B/Akt as well as Ca^{2+}/calmodulin-dependent kinase, myosin

light chain kinase, p21-activated kinase 2, 3′-phosphoinositide-dependent protein kinase 1 and Rho-associated kinase (Tamaoki et al., 1986; Herbert et al., 1990; Feng et al., 1999; Masure et al., 1999; Zeng et al., 2000; Hill et al., 2001). Moreover, the effects of staurosporine appear to be strongly influenced by the cell type that is studied, the composition of the matrix and the concentration of staurosporine. Consequently, different studies employing staurosporine often present contradictory conclusions. For example, staurosporine can either inhibit or activate PKC in keratinocytes, depending on the nature of the experimental model (Sako et al., 1988; Dlugosz and Yuspa, 1991; Matsui et al., 1992; Matsui et al., 1993; Jones and Sharpe, 1994; Sudbeck et al., 1994; Stanwell et al., 1996).

In this study, several different protein-serine/threonine kinase inhibitors, including staurosporine, promoted dose-dependent spreading and extended lamellipodia (E-lam) formation in keratinocytes seeded on fibronectin. Our major finding is that staurosporine and epidermal growth factor can induce E-lam formation, and that ultimately, the activity of glycogen synthase kinase-3 (GSK-3) is required for epithelial cell migration and E-lam formation.

Materials and Methods

Reagents

Staurosporine, herbimycin A, genistein and sodium orthovanadate were obtained from Sigma (St. Louis, MO, USA). LY-294002, H-7, K-252a, BAPTA-AM, U-73122 and thapsigargin were obtained from Biomol (Plymouth Meeting, PA, USA). Tyrphostin AG1478 was purchased from Calbiochem (La Jolla, CA, USA). SB-415286 was a generous gift from GlaxoSmithKline (Research Triangle Park, NC, USA).

Electron microscopy of 3-day-old wounds

Wounds were created in the palatal gingiva of human volunteers with a scalpel (2-3 mm deep, 2 mm wide and 8 mm long). The protocol was approved by the Clinical Research Ethics Board of the University of British Columbia. Tissue biopsies from 3-day-old wounds were fixed in 2% glutaraldehyde for 2 hours, impregnated with 1% osmium tetraoxide for 30 minutes, 2% uranylacetate for 30 minutes, alcohol-dehydrated and embedded in Epoxy-resin (Epon 812). For transmission electron microscopy, 500 nm thick sections were cut with a glass knife (Microtome MT 6000 Sorvall, Dupont, Wilmington, DE) and stained with 1% Toluidine Blue/1% borax for 5 minutes on a hot plate. Sections (80 nm thick) were made with a diamond knife and placed on nickel grids, stained with 5% uranylacetate for 20 minutes and with lead citrate for 5 minutes, and then viewed and photographed with a transmission electron microscope (Philips 300, Philips, Eindhoven, Holland).

Cell culture

The immortalized epidermal keratinocyte cell line HaCaT (a generous gift from Dr Norbert Fusenig, German Cancer Center, Heidelberg, Germany) was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL Life Technologies, Rockville, Maryland, USA) supplemented with 23 mM sodium bicarbonate, 20 mM Hepes, antibiotics (50 μg/ml streptomycin sulfate, 100 U/ml penicillin) and 10% heat-inactivated fetal calf serum (FCS; Gibco BRL). The HaCaT cell line models many of the properties of normal epidermal keratinocytes, is not invasive and can differentiate under appropriate experimental conditions (Boukamp et al., 1988). Cells were always subcultured for 2 days before experiments to maintain consistency throughout the study. Primary mouse keratinocytes were isolated and maintained as described previously (Häkkinen et al., 2002).

To study whether growth factors could induce lamellipodia formation in HaCaT keratinocytes, cells were seeded on 6-well plates (30,000 per well) in complete growth medium. The next day, cells were changed to DMEM containing 1% FCS and epidermal growth factor (EGF; Invitrogen), transforming growth factor β1 (TGFβ1; Chemicon, Temencula, CA, USA), tumor necrosis factor α (TNFα; Chemicon) or keratinocyte growth factor (KGF; Upstate, Lake Placid, NY, USA). The morphology of the cells was recorded by digital photography after 48 hours in culture.

Cell spreading assays

Cell spreading experiments were done essentially as described before (Koivisto et al., 1999). Cell culture plates were coated with fibronectin (2 $\mu g/cm^2$; from bovine plasma; Chemicon). Unoccupied sites were blocked with 1% heat-denatured bovine serum albumin. HaCaT cells (30,000/cm^2) were seeded on plates in serum-free DMEM containing 50 μM cycloheximide to prevent de novo protein synthesis. Inhibitors were added either at the moment of seeding, or cells were allowed to attach and spread for 120 minutes prior to adding the agents, depending on the nature of the experiment to be performed. To quantify the effect of the drugs on cell spreading and E-lam formation, cells were fixed by carefully adding formaldehyde (8% formaldehyde and 10% sucrose in phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+}). The wells were filled with distilled water to the top and covered with a glass plate for observation by phase-contrast microscopy. Four representative fields from each replicate well were studied. The percentages of spread cells and cells with E-lams of total cell number in each field were counted. Cell with lamellar cytoplasm were considered as spread. Alternatively, cells were collected and lysed at certain time points and analyzed for protein phosphorylation.

To examine the more detailed morphology of spreading keratinocytes, HaCaT cells were allowed to spread on fibronectin-coated coverslips in the presence or absence of staurosporine followed by fixation with glutaraldehyde (2.5% in 0.1 M PBS) and preparation for scanning electron microscopy (Aoki and Tavassoli, 1981; Firth et al., 1997).

Immunoblotting

Cells were allowed to spread on fibronectin and treated with staurosporine as described above and lysed at designated time points in Tris-buffered saline (pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 mM EGTA, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 100 mM n-octyl β-D-glucopyranoside and 0.5% Nonidet P-40. Cell lysates were separated by SDS-PAGE, transferred onto Hybond ECL membrane (Amersham, Little Chalfont, Buckinghamshire, UK) and immunoblotted with antibodies recognizing phospho-GSK-3α/β (Y^{279}/Y^{216}; BioSource International, Camarillo, CA, USA or S^{21}/S^{9}; Cell Signaling Technology, Beverly, MA, USA) or total GSK-3 (0011-A; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Peroxidase-conjugated IgGs (Santa Cruz Biotechnology) were used as secondary antibodies. Detection was performed with Amersham's ECL kit. Cell lysates with or without staurosporine treatment were also probed for the quantitative expression of 33 known phosphoproteins using validated commercial antibodies in the Kinetworks™ Phospho-Site Screen (KPSS 1.0), immunoblotting analysis provided by Kinexus Bioinformatics Corporation (Vancouver, Canada). For this screen, cells were treated with staurosporine either for 10 minutes, which preceding E-lam formation, or for 60 minutes, by which time the E-lams have formed. Untreated control samples were collected at the same time points.

Immunocytochemistry

Cells were allowed to spread on fibronectin-coated SonicSeal Slide™ wells (Nunc, Naperville, IL, USA), treated with staurosporine as described above, formaldehyde-fixed and permeabilized with PBS containing 0.5% Triton X-100. Immunolocalization was performed as described previously (Larjava et al., 1990) using antibodies recognizing phospholipase Cγ-1 phosphorylated on Tyr-783 (Sigma) or GSK-3 (0011-A; Santa Cruz Biotechnology). Species-specific Alexa Fluor™ 546 IgG molecules (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Alternatively, anti-mouse Vectastain® ABC and Vector® VIP peroxidase substrate kits (Vector laboratories Inc., Burlingame, CA, USA) were used for immunodetection of GSK-3. Rhodamine-labeled phalloidin (Sigma) was used to detect actin filaments in acetone-fixed samples.

Measurement of intracellular Ca^{2+}

Cells were plated and allowed to spread on fibronectin-coated coverslips as described above and loaded with fura 2/AM (2 μM; Molecular Probes). Single cells were measured with an inverted microscope optically interfaced to a Photon Technology Incorporated (London, ON) ratio fluorimeter. The slit widths were set at 3 nm and excitation was set at alternating wavelengths (100 Hz) of 346 and 380 nm. Measurements were conducted at room temperature in serum-free PBS containing 20 mM Hepes buffer to maintain the pH at 7.4. A stable baseline Ca^{2+} ratio was obtained for ~50 seconds after which 50 nM staurosporine was added. All cells were measured for 500 seconds.

Cell migration

For scratch wound migration assays, HaCaT cells were seeded on 24-well plates (1.7×10^5 cells per well) in DMEM containing 10% FCS. The cells were grown to confluence for 2 days after which the cultures were scratch-wounded with a pipette tip. Loose cells were removed by washing with PBS, and the cells were allowed to migrate for 24 hours in Minimum Essential Medium (EMEM; Bio-Whittaker, Walkersville, MD, USA) supplemented with 1% FCS in the presence of staurosporine (50 nM) or EGF (10 ng/ml) and inhibitors. The inhibition in cell migration produced by GSK-3 inhibitors was quantified by comparing the number of cells within the wound area of inhibitor-treated samples with the number of cells in control wounds. Eight representative fields from each wound were studied.

Statistical analysis

All experiments were repeated at least three times. Each of the spreading assays used two to four replicate wells, and four fields of cells for each well were counted (n=8-16). Statistical significance was set at $P<0.05$, and the difference between two groups was calculated using unpaired, two-tailed Student's t-test.

Result

Keratinocytes form lamellipodia in mucosal wounds that can be simulated in vitro

Keratinocytes in 3-day-old human mucosal wounds formed long, finger-like projections, which flared at their termini, into the loose matrix of the wound bed (Fig. 1A). No hemidesmosomes or basement membrane were observed underneath the advancing cells (Fig. 1A). Primary mouse keratinocytes that were cultured in the presence of fibroblast-conditioned medium and EGF (Häkkinen et al., 2002) also spontaneously formed long, thin lamellipodia (Fig. 1B), similar to those seen in human wounds. We refer to these structures as

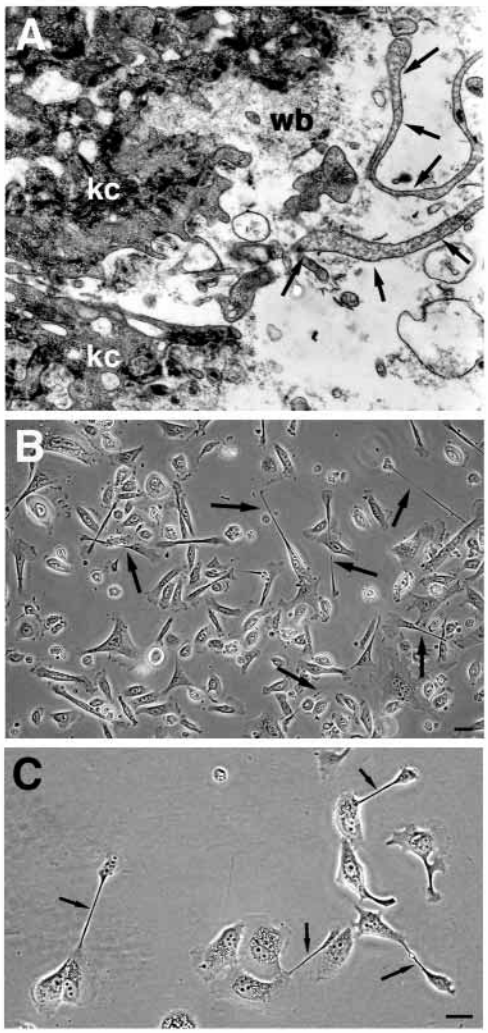

Fig. 1. Keratinocyte E-lam formation in mucosal wounds and in vitro. (A) Electron micrograph of migrating keratinocytes in 3-day-old human mucosal wounds. Arrows mark cellular extensions formed by wound keratinocytes. wb, wound bed; kc, keratinocyte. (B) A photograph of primary mouse keratinocytes grown in medium containing fibroblast conditioned medium and EGF (4 ng/ml). Arrows mark E-lams. (C) HaCaT keratinocytes were treated with EGF (4 ng/ml) in DMEM containing 1% FBS for 48 hours and then photographed. Arrows mark E-lams. Scale bar: 10 μm.

'extended lamellipodia', or 'E-lams'. Compared to classical lamellipodia, E-lams contain an additional thin cellular process resembling an arm. At the tip of the arm, a veil-like lamellipodium was formed (Fig. 1B). To test whether growth factors and cytokines commonly present in wound fluids (EGF, TGFβ1, TNFα, and KGF) could induce E-lam formation in cultured human keratinocytes, HaCaT cells were grown in the presence of these cytokines for 48 hours. Only EGF was capable of inducing E-lam formation in HaCaT cells (Fig. 1C). The observed E-lam formation in cultured HaCaT cells was in part dependent on EGF receptor (EGFR)-mediated signaling, because tyrphostin AG1478 (2 μM), a highly selective inhibitor of EGFR tyrosine kinase activity (Levizki and Gazit, 1995; Osherov and Levitzki, 1997), prevented EGF-induced E-lam formation (data not shown). As EGF-induced E-lam formation

was a relatively slow process (no E-lams were observed before 24 hours), this phenomenon probably involves numerous pathways and alterations in patterns of gene expression and protein synthesis. Accordingly, we elected to search for model systems that produced more definitive and rapid formation of E-lams.

Staurosporine induces extended lamellipodia and cell spreading in human keratinocytes on fibronectin

To focus on a model that exhibited more rapid induction of E-lams and that would permit study of key signaling events in E-lam formation, we tested whether the protein-serine/threonine kinase inhibitor staurosporine could accelerate E-lam formation in keratinocytes. Staurosporine has been shown to promote lamellipodia extension in other cell types (Sako et al., 1988; Rasouly et al., 1994; Rasouly et al., 1996; Yamazaki et al., 1999). Accordingly, HaCaT cells were seeded on to fibronectin-coated substrata in the presence of staurosporine, a treatment that induced keratinocytes to form morphologically similar E-lams as seen in EGF-treated keratinocytes. The ability of staurosporine to induce formation of E-lams was not restricted to HaCaT keratinocytes as the same phenomenon was observed with primary mouse and human epidermal keratinocytes (data not shown). In contrast to EGF, staurosporine induced E-lams within 60 minutes in the absence of de novo protein synthesis (Fig. 2Ab). Staurosporine, therefore, possibly bypassed the requirement for EGFR signaling, as neither EGF (30 ng/ml) nor tyrphostin AG1478 (2 μM) exerted any effect on staurosporine-induced E-lam formation (data not shown). Staurosporine also induced a dose-dependent increase in spreading of keratinocytes on fibronectin. Maximal induction of spreading was achieved with 10 nM staurosporine. E-lam formation was, however, typically initiated at concentrations above 30 nM staurosporine (Fig. 2B). Staurosporine was able to induce E-lams to the same extent in spreading cells and in cells that had already spread (Fig. 2C). This characteristic allowed us to investigate signaling mechanisms of E-lam formation that are independent of cell spreading. Scanning electron microscopy was used to visualize staurosporine-induced morphological changes at higher resolution. In addition to E-lam formation, cells treated with 50 nM staurosporine exhibited fine filopodia that projected from the lamellipodia (Fig. 2A).

To confirm that E-lam formation was associated with the inhibition of protein-serine/threonine kinases, two other broad-spectrum protein-serine/threonine kinase inhibitors, H7 and K252a, were tested for their ability to induce E-lam formation in cells plated on fibronectin. Both inhibitors were able to induce cell spreading and E-lam formation, although they were less potent than staurosporine, indicating that E-lam formation was indeed related to inhibition of protein-serine/threonine kinases. For H7, maximal cell spreading was induced at 400 nM, whereas E-lams did not start to form until concentrations above 2 μM were used (data not shown). For K252a, both maximal cell spreading and the onset of E-lam formation were reached at 50 nM (data not shown). The maximal incidence of E-lam formation with H7 and K252a never exceeded 20% of the cells (data not shown). At 50 nM staurosporine, E-lams that were morphologically similar to those induced by EGF occurred in 15-25% of cells. Higher concentrations of staurosporine increased the proportion of cells exhibiting E-lams but their morphology became more irregular (Fig. 2B and data not shown). Accordingly, this 50 nM concentration was used in further studies that focused on the signal transduction

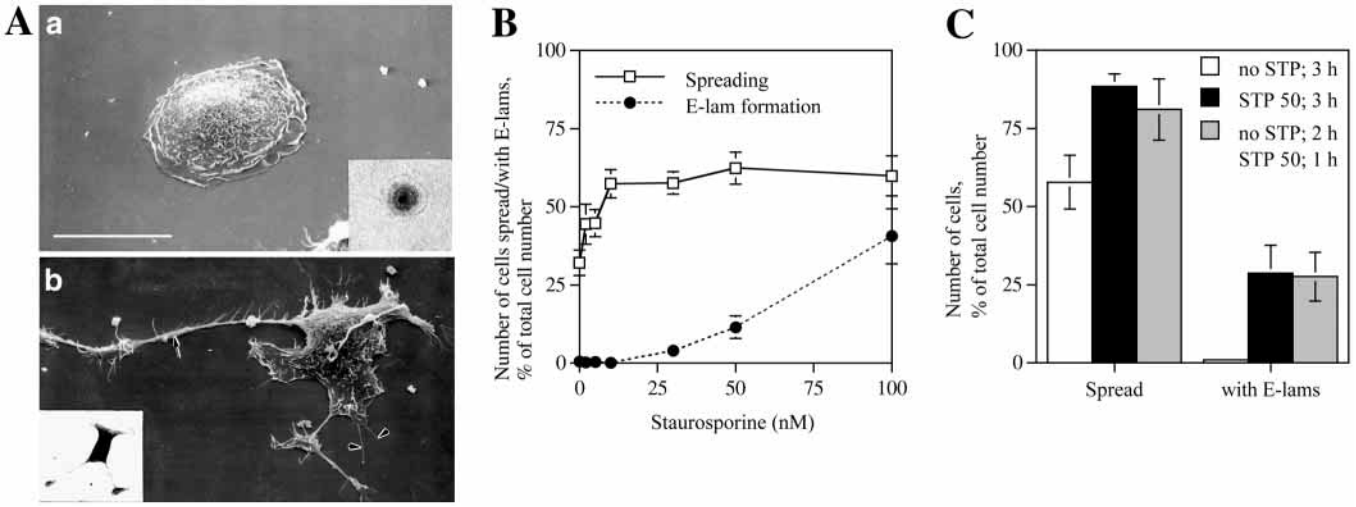

Fig. 2. Staurosporine-induced E-lam formation in HaCaT keratinocytes. (A) The morphology of HaCaT cells spreading on fibronectin in absence (a) or in presence (b) of staurosporine as visualized by scanning electron microscopy. Extended lamellipodia are marked with arrows and filopodia with arrowheads. Scale bar: 10 μm. Equivalent phase contrast pictures (insets) from crystal violet stained cells were taken using 20× objective. (B) The cells were allowed to spread on fibronectin for 60 minutes in presence of staurosporine (0-100 nM). The cells were fixed and four representative fields in triplicate wells were examined by phase-contrast microscopy using a 20× objective (n=12). The percentage of cells that were spread or forming E-lams out of the total cell number within each field was calculated (mean±s.d.). (C) The cells were allowed to spread on fibronectin for 2 hours and then treated with 50 nM staurosporine for 1 hour or left untreated. For comparison, some of the cells were treated with staurosporine for 3 hours. The cells were fixed, and the percentage of cells that were spread or forming E-lams of the total cell number was calculated as in B (mean±s.d., n=12).

mechanisms regulating lamellipodia formation in keratinocytes.

To determine whether the formation of E-lams also required changes in tyrosine phosphorylation in addition to the inhibition of protein-serine/threonine kinases, cells were allowed to spread on fibronectin and then treated with staurosporine (50 nM) in combination with the nonselective protein-tyrosine kinase inhibitors (herbimycin A; 10 μM, genistein; 200 μM) or a protein-tyrosine phosphatase inhibitor (sodium orthovanadate; 500 μM). Both herbimycin A (Fig. 3E,F) and genistein potently inhibited E-lam formation (Fig. 3G,H), indicating that E-lam formation was dependent on tyrosine phosphorylation that is mediated by protein-tyrosine kinases other than EGFR tyrosine kinase. As indicated above, E-lam formation was unresponsive to the inhibition of EGFR tyrosine kinase. In agreement with the importance of tyrosine phosphorylation in E-lam formation, sodium orthovanadate increased the proportion of cells forming E-lams from ~20% to ~60% (Fig. 3B,D). In control cells without staurosporine, sodium orthovanadate caused cell retraction (Fig. 3A,C).

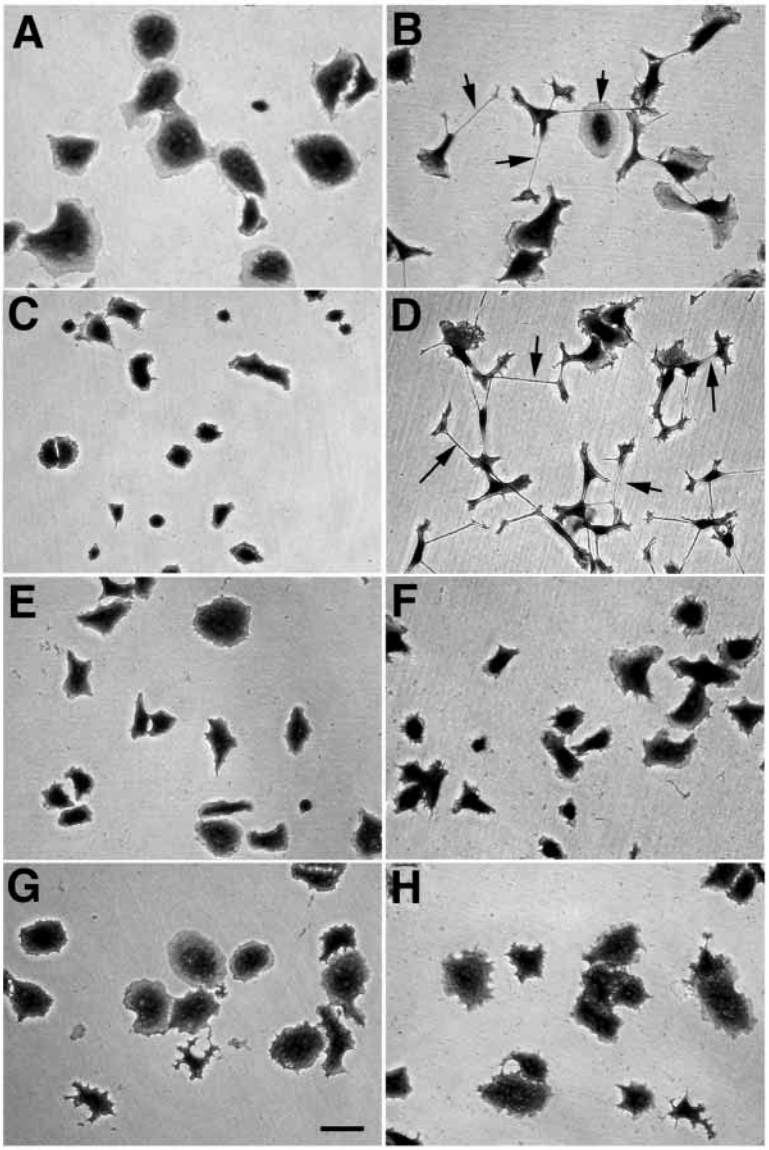

Fig. 3. The effect of tyrosine kinase and phosphatase inhibitors on E-lam formation. The cells were allowed to spread on fibronectin for 120 minutes and then not treated (A,B), or treated with 500 μM sodium orthovanadate (C,D), 10 μM herbimycin A (E,F) or 200 μM genistein (G,H) in the presence (B,D,F,H) or absence (A,C,E,G) of 50 nM staurosporine for 60 minutes. The cells were fixed, stained with crystal violet and photographed. E-lams are marked with arrows. Scale bar: 10 μm.

Staurosporine induces GSK-3 activation

To examine changes in protein phosphorylation that are involved in E-lam formation, cells treated with 50 nM staurosporine were analyzed by Kinexus Kinetworks™ Phospho-Site Screen (data not shown). The results indicated that staurosporine may increase GSK-3 activation. Notably, GSK-3 is a major regulator of the cytoskeleton in other cell types (Wagner et al., 1996). The activity of GSK-3α/β is mainly regulated by inhibitory phosphorylation on serine residues (S^{21}/S^{9}) (Grimes and Jope, 2001). Accordingly, we found that staurosporine strongly induced dephosphorylation of GSK-3α/β on serine 21/9 (Fig. 4A,B). The reduction of GSK-3 serine phosphorylation was detectable within 1-5 minutes after incubation with staurosporine (Fig. 4A,B). Both α and β GSK-3 isoforms were dephosphorylated at serine residues by staurosporine treatment (Fig. 4A,B). Tyrosine phosphorylation of GSK-3α/β on residues 279/216 is a prerequisite for its activity (Hughes et al., 1993). Tyrosine phosphorylation of GSK-3 was not markedly altered by staurosporine (Fig. 4C). Thus, staurosporine induced changes in GSK-3 phosphorylation that were consistent with its activation.

Extended lamellipodia formation requires the activity of GSK-3

The direct involvement of GSK-3α/β in E-lam formation was tested by treating cells with staurosporine in the presence of the GSK-3 inhibitors $LiCl_2$ and SB-415286 (Klein and Melton, 1996; Coghlan et al., 2000). Both of these GSK-3 inhibitors caused a specific and concentration-dependent reduction of the incidence of E-lams without a change in general cell morphology. This experiment indicated that GSK-3 activity is specifically involved with the extension of E-lams but does not affect lamellar spreading (Fig. 5A-C). In agreement with this function, immunostaining for GSK-3 was restricted to the extended part of the E-lam in staurosporine-treated cells and was not present in lamellae (Fig. 6B,C). In EGF-treated cells, the localization of GSK-3 was more diffuse, but it was still more abundant away from cellular perimeters (data not shown).

Intracellular free Ca^{2+} is essential for E-lam formation

GSK-3 is activated in response to an increase in intracellular Ca^{2+} (Hartigan and Johnson, 1999), and staurosporine can

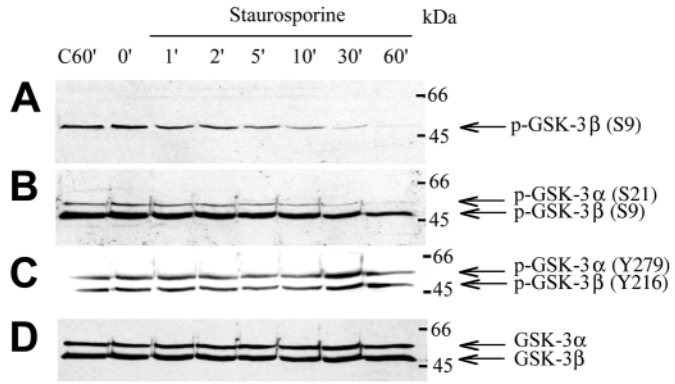

Fig. 4. Changes in GSK-3 phosphorylation induced by staurosporine. The cells were allowed to spread on fibronectin for 120 minutes and then treated with 50 nM staurosporine for either 0-60 minutes or left untreated for 60 minutes (C60'). The cell lysates were separated by SDS-PAGE and immunoblotted with antibodies recognizing GSK-3α/β phosphorylated on serine 21/9 (A,B), GSK-3α/β phosphorylated on tyrosine 279/216 (C) or total GSK-3 (D). The bands visible in A represent GSK-3β. GSK-3α phosphorylation followed a similar trend but was much weaker. P-GSK-3α is visible in B, which represents a longer exposure of A.

induce a long-lasting increase of intracellular free Ca^{2+} in primary human keratinocytes (Jones and Sharpe, 1994). We found that in HaCaT cells, 50 nM staurosporine induced a slow and steady increase in cytoplasmic free Ca^{2+} that was detectable within 30 seconds after adding the staurosporine and reached a plateau in 300 seconds (in 6 out of 15 cells measured; 40%). A typical response is shown in Fig. 7A. None of the cells treated with vehicle only showed any Ca^{2+} increase (data not shown). To test the importance of this Ca^{2+} influx for E-lam formation, we treated spread HaCaT cells with either BAPTA/AM (100 nM-10 μM) or EGTA (2 mM) to chelate intracellular or extracellular Ca^{2+}, respectively, together with staurosporine. Chelation of extracellular Ca^{2+} by EGTA slightly increased cell spreading but did not affect E-lam formation (Fig. 7B). The effect of BAPTA/AM on cell morphology, however, was dependent on staurosporine (Fig. 7B): the spread morphology of control cells was unaffected by BAPTA/AM. The cells exposed to staurosporine, however, underwent concentration-dependent collapse in response to BAPTA/AM, and E-lam formation was completely abolished at 10 μM BAPTA/AM. Staurosporine thus sensitized the cells to chelation of intracellular Ca^{2+}. The Ca^{2+} concentration-dependent disappearance of E-lams indicates that intracellular Ca^{2+} may play a crucial role in staurosporine-induced cell spreading and E-lam formation.

Treatment with staurosporine induces relocalization of tyrosine phosphorylated phospholipase C-γ1 to E-lams and localized actin assembly

As phospholipase C-γ (PLC-γ) is a key mediator in the pathway that releases Ca^{2+} from intracellular stores (Berridge, 1993), we tested the involvement of PLC-γ in E-lam formation. Treatment of keratinocytes with the specific PLC-γ inhibitor U-71322 induced a concentration-dependent

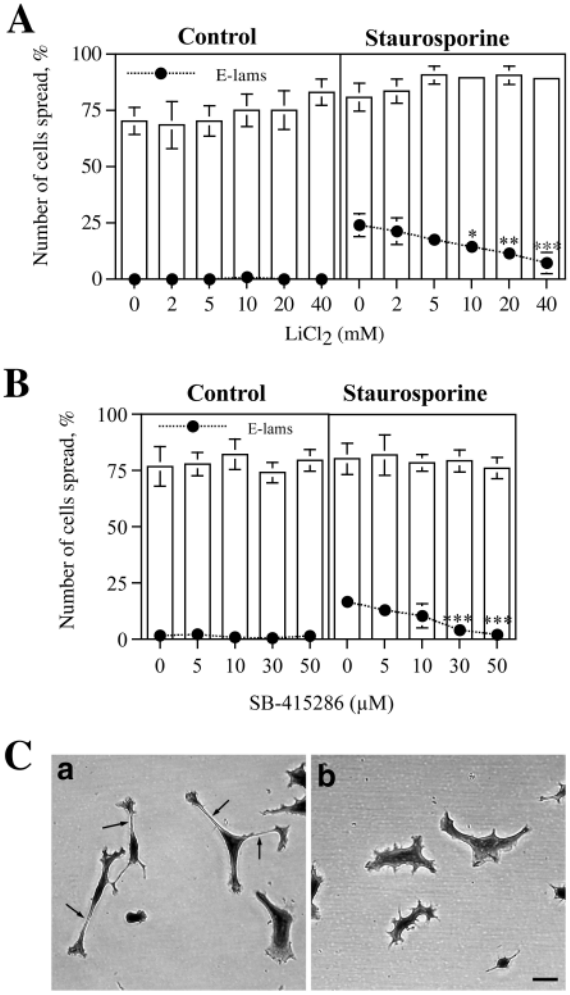

Fig. 5. The role of GSK-3 in E-lam formation. To investigate the role of GSK-3 in E-lam formation, the cells were treated with $LiCl_2$ (2-40 mM; A) or SB-415286 (1-50 μM; B) in presence or in absence of staurosporine. The percentage of cells that were spread or representing E-lams was calculated. Results (mean±s.d., n=8) of one representative experiment are shown. (*P<0.05; **P<0.01; ***P<0.001.) (C) The morphology of cells treated with 50 nM staurosporine only (a) or in presence of 30 μM SB-415286 (b). E-lams are marked with arrows. Scale bar: 10 μm.

loss of cell spreading both in control and staurosporine-treated cells, as well as inhibition of E-lam formation (Fig. 8A). Thus PLC-γ activity was involved in the spreading behavior of keratinocytes, but a direct role for PLC-γ in E-lam formation could not be ascertained from inhibitor studies. As the activity of PLC-γ1 depends on phosphorylation on Tyr-783 (Kim et al., 1991), indirect evidence for the importance of PLC-γ1 in E-lam formation was obtained by immunocytochemistry. These experiments demonstrated localization of active, tyrosine phosphorylated PLC-γ1 at the tips of E-lams and co-localization with actin filaments (Fig. 8B). Consistent with the importance of PLC-γ in staurosporine-induced morphological change, disturbance of cellular Ca^{2+} gradients by thapsigargin (a sarcoplasmic-endoplasmic reticulum ATPase (SERCA) inhibitor; ≥2 nM)

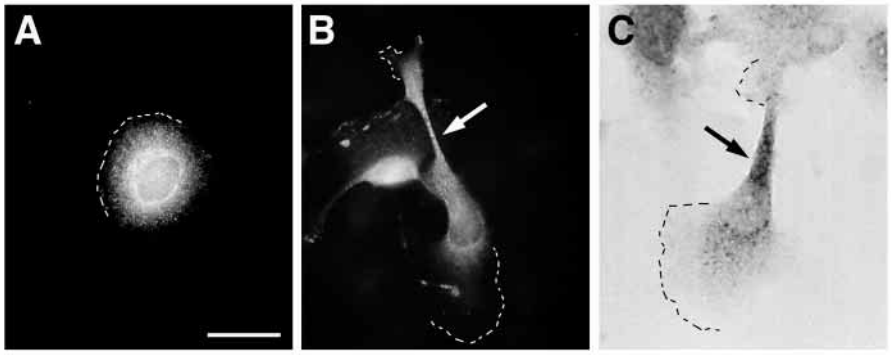

Fig. 6. The localization of GSK-3 in E-lams. Immunolocalization of GSK-3 in control (A) and staurosporine-treated (B,C) cells. GSK-3 accumulated in the extended part of the E-lam is marked with arrows. In A and B GSK-3 was visualized by immunofluorescence, whereas in C, GSK-3 was detected by biotin-avidin-peroxidase complex using VIP as a chromogen. To help visualize the cell shape, partial outlines of cells are marked with dashed lines. Scale bar: 10 μm.

blocked cell spreading (inhibition by 63%, n=8, P<0.001) and E-lam formation in staurosporine-treated cells. Thapsigargin also reduced the spreading of control cells but less potently (inhibition by 25%, n=8, P<0.001).

Agents that interfere with cellular Ca^{2+} signaling modulate GSK-3α/β serine phosphorylation

Because both the activity of GSK-3 and the intracellular Ca^{2+} signaling participated in E-lam formation, we tested whether these two pathways were connected. Notably, the activity of GSK-3 has been reported to be upregulated by increased intracellular Ca^{2+} (Hartigan and Johnson, 1999). Therefore, we tested whether disturbance of cellular Ca^{2+} metabolism by PLC-γ inhibitor U-71322 (4 μM), SERCA inhibitor thapsigargin (100 nM) or intracellular Ca^{2+} chelator BAPTA-AM (10 μM) that all blocked E-lam formation affected staurosporine-induced serine dephosphorylation of GSK-3. None of the agents had any significant effect on tyrosine phosphorylation of GSK-3 (data not shown). Thapsigargin prevented staurosporine-induced GSK-3 serine dephosphorylation (Fig. 9A). U-71322 did not completely prevent but significantly delayed GSK-3 dephosphorylation: in cells treated with staurosporine only, the dephosphorylation of GSK-3 was evident after a 5-minute incubation (Fig. 9G), whereas in cells treated with a combination of U-71322 and staurosporine, the reduction in serine phosphorylation was only evident after 30 minutes incubation (Fig. 9C). In contrast, BAPTA-AM that buffers cytoplasmic Ca^{2+} greatly accelerated serine dephosphorylation of GSK-3 (Fig. 9E). Thus, agents that affect cellular Ca^{2+} signaling seem to modulate the degree of serine phosphorylation of GSK-3 in keratinocytes, but the GSK-3 dephosphorylation does not always lead to E-lam formation.

Staurosporine-induced cell migration is dependent on GSK-3 activity

To determine if E-lam formation induced by staurosporine is indeed associated with the migratory phenotype in keratinocytes, cells were allowed to migrate in the presence of staurosporine in scratch-wounded cultures. Staurosporine potently induced cell migration resulting in wound closure within 24 hours (Fig. 10B). Staurosporine also induced the migration of primary mouse keratinocytes, although these cells were more sensitive to staurosporine and a smaller concentration was required (data not shown). To investigate whether keratinocyte migration was dependent on GSK-3 activity, staurosporine- and EGF-treated HaCaT cells were allowed to migrate in scratch wound assays in the presence of GSK-3 inhibitors. Consistent with the importance of GSK-3 in E-Lam formation in keratinocytes, staurosporine-induced

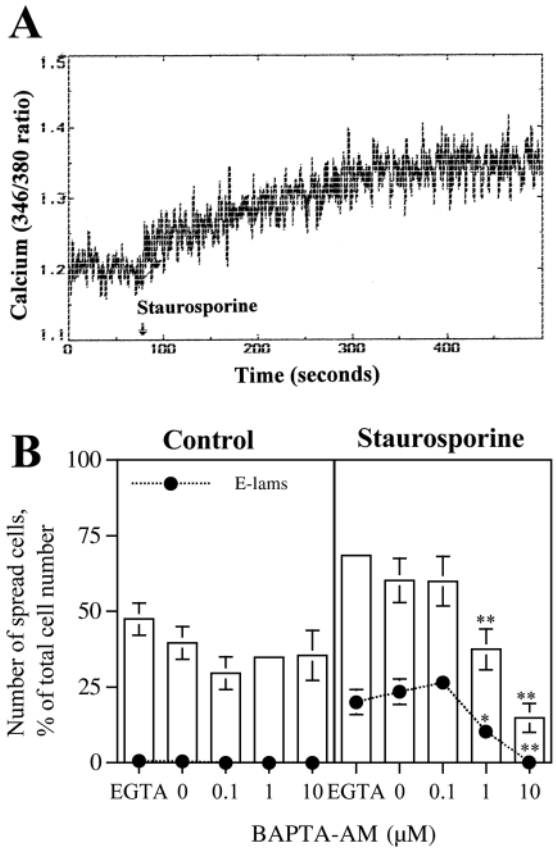

Fig. 7. The role of intracellular free Ca^{2+} in E-lam formation. (A) The spread cells were loaded with fura-2, and the ratio of 346/380 nm excitation indicating the level of intracellular free Ca^{2+} was measured by fluorimetry. Stable baseline Ca^{2+} was obtained for 50 seconds before the addition of staurosporine. A typical response is shown. (B) To study the role of intracellular free Ca^{2+}, the spread cells were treated with Ca^{2+} chelators 2 mM EGTA or BAPTA-AM (0-10 μM) in presence or in absence of staurosporine and analyzed for cell spreading and E-lam formation. The results show the mean±s.d. of a typical experiment (n=8). (*P<0.05; **P<0.01; ***P<0.001.)

migration was completely blocked by 30 μM SB-415286 and by 30 mM $LiCl_2$ (Fig. 10E,H). EGF-induced cell migration was also blocked when the wounded cell cultures were incubated with GSK-3 inhibitors before the addition of EGF to the cells (Fig. 10F,I), indicating that GSK-3 activity also contributes to EGF-induced cell migration. If the GSK-3 inhibitors were added to the cells simultaneously with EGF, the cell migration was still strongly reduced although not totally prevented: SB-415286 and $LiCl_2$ inhibited the number of cell within wounds by about 35% and by 45%, respectively, compared to cells treated with EGF only (data not shown).

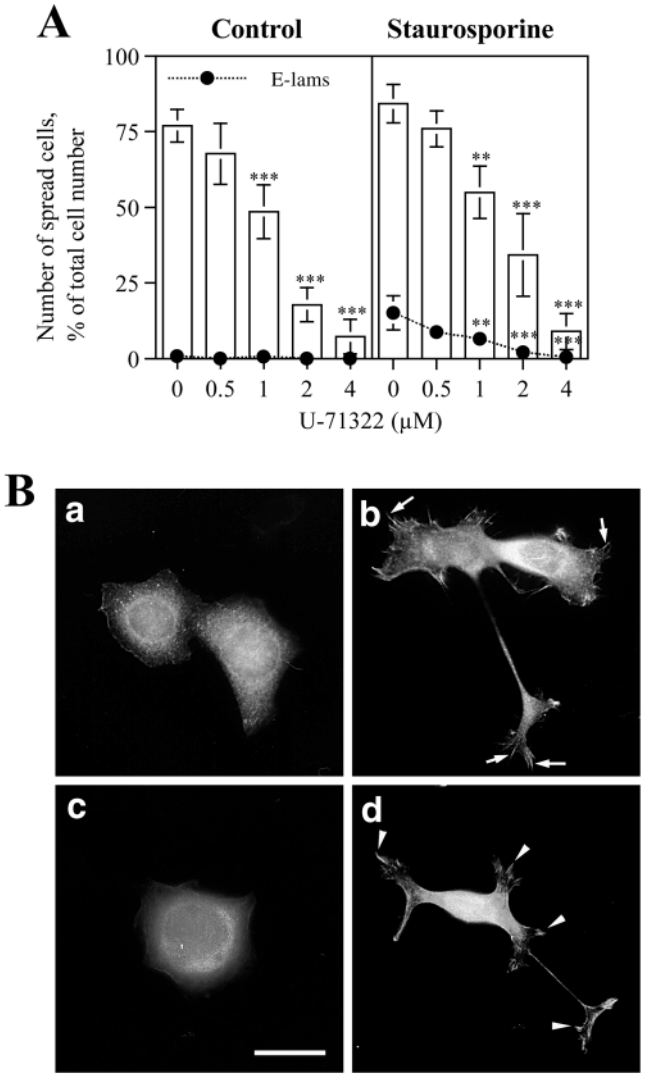

Fig. 8. The role of PLC-γ1 in E-lam formation. (A) To study the role of PLC-γ in E-lam formation, the spread cells were treated with PLC inhibitor U-71322 (0-4 μM) in the presence or in absence of staurosporine and analyzed for cell spreading and E-lam formation. The results show the mean±s.d. of one typical experiment (n=8). (*P<0.05; **P<0.01; ***P<0.001.) (B) Immunolocalization of tyrosine phosphorylated (active) PLC-γ1 (a,b) and actin (c,d) in control (a,c) and staurosporine-treated (b,d) cells. PLC-γ1 and actin accumulated in E-lams are marked with arrows and arrowheads, respectively. Scale bar: 10 μm.

Discussion

In the present study, we demonstrated that GSK-3 regulates the formation of long lamellipodia in human keratinocytes and their migration into in vitro wounds. This is a novel function for GSK-3 and makes it potentially a key molecule for tissue repair. GSK-3 is known to regulate many signaling as well as structural molecules providing multiple targets for GSK-3 action (Grimes and Jope, 2001). There are two highly homologous isoforms of mammalian GSK-3, GSK-3α and GSK-3β that are encoded by separate genes (Woodgett, 1990). The two GSK-3 isoforms show different tissue expression patterns and their expression is differentially regulated, suggesting that they may be functionally distinct (Lau et al., 1999), although in vitro they exhibit similar biochemical and substrate specificities (Woodgett, 1991). The only known difference between GSK-3α and GSK-3β to date is the specific ability of GSK-3β to activate the transcription factor NF-κB (Hoeflich et al., 2000).

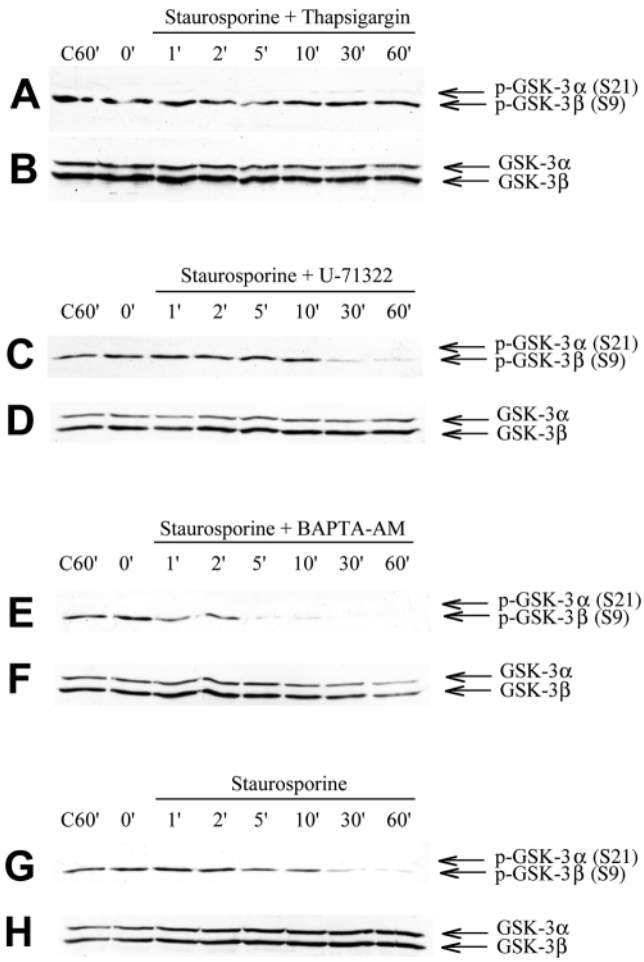

Fig. 9. The effect of Ca^{2+} modulators on GSK-3 phosphorylation. The cells were allowed to spread on fibronectin for 120 minutes and then treated with 50 nM of staurosporine alone (G,H) or together with thapsigargin (100 nM; A,B), PLC-γ1 inhibitor U-71322 (4 μM; C,D) or BAPTA-AM (10 μM; E,F) for either 0-60 minutes or left untreated for 60 minutes (C60'). The cell lysates were separated by SDS-PAGE and immunoblotted with antibodies recognizing GSK-3α/β phosphorylated on serine 21/9 (A,C,E,G) or total GSK-3 (B,D,F,H).

GSK-3 has an important role in determination of cell fate during development (Kim and Kimmel, 2000). GSK-3 complexes also serve tumor-suppressive functions in cells (Kim and Kimmel, 2000). An excessive and dysregulated activity of GSK-3 is, however, linked to many pathological conditions (Cohen and Frame, 2001). The majority of studies about the function of GSK-3 are attributed to GSK-3β because of its high expression in brain tissues and its role in Alzheimer's disease (Lau et al., 1999; Grimes and Jope, 2001). Accordingly, most of the data concerning the function of GSK-3 comes from neuronal cells, and there is no information about its function in keratinocytes during wound repair. We show that both GSK-3 isoforms are expressed by cultured HaCaT keratinocytes and that GSK-3 catalytic activity is involved in the regulation of E-lam formation.

GSK-3α/β catalytic activity is positively regulated by tyrosine phosphorylation (Y^{279}/Y^{216}) and is inhibited by phosphorylation on Ser-21/9 (Hughes et al., 1993; Grimes and Jope, 2001). GSK-3 is a serine/threonine kinase, and staurosporine can inhibit GSK-3 activity in vitro (Leclerc et al., 2001). However, this inhibition has not been shown to occur in living cells. In contrast, Bhat et al., (Bhat et al., 2000) reported an increase in GSK-3 tyrosine phosphorylation and activity in neuronal cells treated with staurosporine. In the present study, staurosporine did not, however, markedly regulate tyrosine phosphorylation of GSK-3. The protein-tyrosine kinase inhibitor genistein can induce GSK-3 dephosphorylation and inactivation, and this inhibition can be reversed by the protein-tyrosine phosphatase inhibitor sodium orthovanadate (Yu and Yang, 1994; Yu et al., 1997). In agreement with these previous results, we found that treatment of HaCaT cells with genistein and herbimycin A inhibited E-lam formation whereas sodium orthovanadate enhanced E-lam formation. In addition to GSK-3, tyrosine phosphorylation regulates the activities of PLC-γ1 and Rac1 (Kim et al., 1991; Sastry et al., 2002), molecules that are required for E-lam formation. In the present study, we also demonstrated that treatment of keratinocytes with staurosporine potently reduced serine phosphorylation of GSK-3, an alteration that has been linked to its inactivation. Staurosporine has been reported to inhibit cAMP-dependent protein kinase, PKB/Akt and several isoforms of PKC, all of which can potentially phoshorylate GSK-3 on Ser-21/9 (Goode et al., 1992; Cross et al., 1995; Fang et al., 2000; Li et al., 2000). These findings suggest one potential mechanism for GSK-3 activation by staurosporine. Based on the relative degree of serine dephosphorylation in HaCaT cells, GSK-3α shows greater relative activity than GSK-3β. We have not, however, been able to discriminate the functions of GSK-3α and GSK-3β as both $LiCl_2$ and the specific inhibitor, SB-415286, inhibit both isoforms to the same extent (Klein and Melton, 1996; Coghlan et al., 2000).

Staurosporine is commonly used to induce apoptosis; the putative functions of GSK-3 are often discussed in this context. In concentrations significantly higher than that used in this study, staurosporine (at 100-500 nM) induces nuclear translocation of GSK-3β in neuronal cells, a phenomenon linked to the onset of apoptosis (Bhat et al., 2000; Bijur and Jope, 2001). The narrow window of staurosporine concentrations that induce E-lam formation indicates a high level of regulation of cell signaling by staurosporine. At optimal concentrations of staurosporine, GSK-3 localizes to the nascent E-lam whereas at higher concentrations GSK-3 may associate with the nucleus. Conceivably, one of the factors that distinguishes normally regulated wound healing from malignant epithelial invasion is the susceptibility of the migrating cells to undergo programmed cell death. The mechanisms that regulate cell migration and apoptosis may be linked, the end result being determined by the degree of activation and/or cellular location of regulatory molecules such as GSK-3.

A pool of GSK-3 is active in resting cells, and it can be further activated by transient Ca^{2+} influxes (Hughes et al., 1993; Hartigan and Johnson, 1999). Our findings suggest that an increase in intracellular Ca^{2+} concentration was involved in staurosporine-induced changes in cell morphology and probably participated in GSK-3 activation. Staurosporine induced an early but relatively slow increase in the total level of intracellular free Ca^{2+} in HaCaT cells. Similar results demonstrating a slow Ca^{2+} increase were obtained previously with primary human keratinocytes exposed to staurosporine (Jones and Sharpe, 1994). The increase in cytoplasmic Ca^{2+} influx is likely caused by the release of Ca^{2+} from

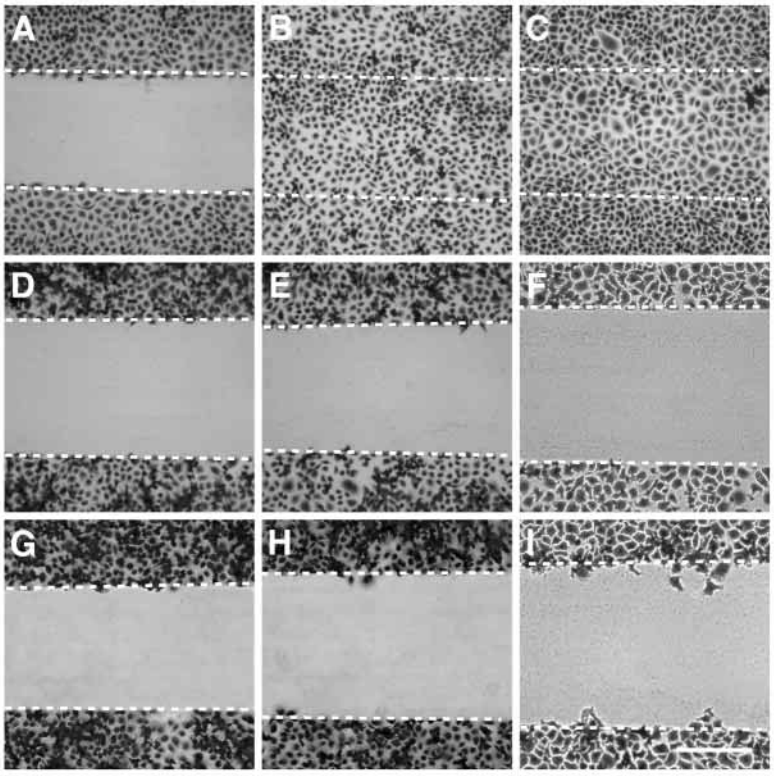

Fig. 10. Effect of GSK-3 inhibitors on cell migration. Confluent cell layers were scratch-wounded and then treated with staurosporine (50 nM; B,E,H), EGF (10 ng/ml; C,F,I) or left untreated (A,D,G) together with GSK-3 inhibitors SB-415286 (30 μM; D-F) and $LiCl_2$ (30 mM; G-I) for 24 hours. In EGF-treated samples, the wounded cultures were preincubated for two hours before adding EGF to the samples. The cells were then fixed, stained with crystal violet and photographed. Scale bar: 100 μm.

intracellular storage compartments (Jones and Sharpe, 1994) and is consistent with our finding that chelation of extracellular Ca^{2+} with EGTA did not inhibit E-lam formation whereas the intracellular Ca^{2+} chelator BAPTA-AM did. BAPTA-AM induced a selective rounding of the staurosporine-treated keratinocytes and an inhibition of E-lam formation but did not affect the untreated control keratinocytes.

The increase of intracellular Ca^{2+} by staurosporine was probably mediated by PLC-γ1, a PLC-γ isoform that is expressed by HaCaT cells (Haase et al., 1997). Notably, PLC-γ1 is activated by tyrosine phosphorylation (Kim et al., 1991). In the present study, we demonstrated that tyrosine phosphorylated PLC-γ1 colocalized with actin filaments at the tips of lamellipodia, in agreement with previously published data (Yu et al., 1998). Conceivably, PLC-γ1 activity may also be spatially restricted in this keratinocyte model system. The importance of PLC-γ1 activity in E-lam formation was also suggested by the blockade of E-lam extension following a disturbance of cellular Ca^{2+} gradients by thapsigargin. The treatment of cells with PLC-γ1 inhibitor or thapsigargin also delayed or prevented the staurosporine-induced dephosphorylation of GSK-3. It appears, therefore, that the localized and controlled release of Ca^{2+} by the activity of PLC-γ1 is essential for formation of long lamellipodia and may also contribute to dephosphorylation of GSK-3 by an unknown mechanism in keratinocytes. Surprisingly, treatment of HaCaT cells with intracellular Ca^{2+} chelator BAPTA-AM, which inhibited E-lam formation, accelerated rather than inhibited GSK-3 serine dephosphorylation. The effects of BAPTA-AM on cellular Ca^{2+} are, however, complex, and by buffering intracellular Ca^{2+}, BAPTA-AM may actually increase the amount of Ca^{2+} in cells in a high-calcium medium such as DMEM (Hofer et al., 1998). Ca^{2+} is a ubiquitous intracellular messenger that regulates a number of cellular processes, and the amplitude, speed and spatiotemporal patterning of Ca^{2+} signals affect the way cells interpret them (Berridge et al., 2000). Therefore, these results suggest that intracellular Ca^{2+} also regulates other aspects in E-lam formation than just GSK-3 activity, as witnessed by the coincident enhanced serine dephosphorylation of GSK-3 and inhibition of E-lam formation by BAPTA-AM. In addition keeping in mind the broad effect of staurosporine on cellular signaling events, while GSK-3 activity is required for E-lam formation, GSK-3 activation is unlikely the sole cause of E-lam formation, but it also requires other coordinated changes in cellular signaling.

As demonstrated in the present and previous studies, long cellular extensions are found in keratinocytes migrating into the wound provisional matrix (Odland and Ross, 1968; Larjava et al., 1996; Woodley, 1996). Long lamellipodia seen in vitro may represent simple forms of lamellipodia seen inside three-dimensional matrix in vivo. EGF is a natural wound cytokine that may regulate the formation of long lamellipodia in vivo. Stimulation of cells with EGF activates multiple downstream signaling pathways in a complex and cell-type-specific manner that is not currently completely understood (Bogdan and Klämbt, 2001; Yarden and Sliwkowski, 2001; Danielsen and Maihle, 2002). It is commonly believed that EGFR activation leads to the activation of PKB/Akt followed by inactivation rather than activation of GSK-3 (Burgering and Coffer, 1995). EGFR activation, however, also leads to activation of Fyn, a member of Src family tyrosine kinases (Mariotti et al., 2001). Interestingly, Fyn has been shown to be involved in GSK-3 activation (Lesort et al., 1999). GSK-3β is rapidly and transiently activated followed by its inhibition by extracellular stimuli including insulin (Lesort et al., 1999). It is speculated that even this rapid and transient GSK-3β activation plays a significant role in modification of cytoskeletal structures in neuronal development by modulating cellular plasticity and by inducing neuronal outgrowth (Lesort et al., 1999). In fibroblasts, GSK-3α appears to be transiently deactivated downstream of EGFR activation (Shaw and Cohen, 1999), whereas in human epidermoid carcinoma A431 cells, EGF caused cellular translocation of active GSK-3α without affecting its overall activity (Yang et al., 1989). Induction of long lamellipodia (E-lams) by EGF in keratinocytes in vitro appears to be a slow process probably involving several second messenger signals that may eventually lead to GSK-3 activation. Similarly, keratinocyte migration induced by EGF may involve cyclic transient activation and inactivation of GSK-3 as well as modulation of its cellular localization.

Based on the results and the data discussed above, we propose that E-lam formation involves the activation and membrane translocation of PLC-γ1. PLC-γ1-induced elevation of intracellular Ca^{2+} may contribute to the activation of GSK-3, which mediates E-lam formation by modulating the organization of the cytoskeleton. We suggest, therefore, that GSK-3 is potentially a key molecule in re-epithelization during wound repair.

This study was supported by grants from The Canadian Institutes of Health Research and The Finnish Cultural Foundation.

References

Aoki, M. and Tavassoli, M. (1981). OTO method for preservation of actin filaments in electron microscopy. *J. Histochem. Cytochem.* **29**, 682-683.

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315-325.

Berridge, M. J., Lipp, P. and Bootman, M. D. (2000). The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell. Biol.* **1**, 11-21.

Bhat, R. V., Shanley, J., Correll, M. P., Fieles, W. E., Keith, R. A., Scott, C. W. and Lee, C. M. (2000). Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3beta in cellular and animal models of neuronal degeneration. *Proc. Natl. Acad. Sci. USA* **97**, 11074-11079.

Bijur, G. N. and Jope, R. S. (2001). Proapoptotic stimuli induce nuclear accumulation of glycogen synthase kinase-3 beta. *J. Biol. Chem.* **276**, 37436-37442.

Bogdan, S. and Klämbt, C. (2001). Epidermal growth factor signaling. *Curr. Biol.* **10**, R292-R295.

Boukamp, P., Petrusevska, R. T., Breitkreut, D., Hornung, J., Markham, A. and Fusenig, N. E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte line. *J. Cell Biol.* **106**, 761-771.

Burgering, B. M. and Coffer, P. J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602.

Coghlan, M. P., Culbert, A. A., Cross, D. A., Corcoran, S. L., Yates, J. W., Pearce, N. J., Rausch, O. L., Murphy, G. J., Carter, P. S., Roxbee Cox, L. et al. (2000). Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem. Biol.* **7**, 793-803.

Cohen, P. and Frame, S. (2001). The renaissance of GSK3. *Nat. Rev. Mol. Cell. Biol.* **2**, 769-776.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin is mediated by protein kinase B. *Nature* **378**, 785-789.

Danielsen, A. J. and Maihle, N. J. (2002). The EGF/ErbB receptor family and apoptosis. *Growth Factors* **20**, 1-15.

Dlugosz, A. A. and Yuspa, S. H. (1991). Staurosporine induces protein kinase

C agonist effects and maturation of normal and neoplastic mouse keratinocytes in vitro. *Cancer Res.* **51**, 4677-4684.

Fang, X., Yu, S. X., Lu, Y., Bast, R. C. Jr, Woodgett, J. R. and Mills, G. B. (2000). Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc. Natl. Acad. Sci. USA* **97**, 11960-11965.

Feng, J., Ito, M., Kureishi, Y., Ichikawa, K., Amano, M., Isaka, N., Okawa, K., Iwamatsu, A., Kaibuchi, K., Hartshorne, D. J. et al. (1999). Rho-associated kinase of chicken gizzard smooth muscle. *J. Biol. Chem.* **274**, 3744-3752.

Fenteany, G., Janmey, P. A. and Stossel, T. P. (2000). Signaling pathways and cell mechanics involved in wound closure by epithelial cell sheets. *Curr. Biol*. **10**, 831-838.

Firth, J. D., Putnins, E. E., Larjava, H. and Uitto, V. J. (1997). Bacterial phospholipase C upregulates matrix metalloproteinase expression by cultured epithelial cells. *Infect. Immun*. **65**, 4931-4936.

Fulga, T. A. and Rørth, P. (2002). Invasive cell migration is initiated by guided growth of long cellular extensions. *Nat. Cell Biol*. **4**, 715-719.

Goode, N., Hughes, K., Woodgett, J. R. and Parker, P. J. (1992). Differential regulation of glycogen synthase kinase-3 beta by protein kinase C isotypes. *J. Biol. Chem*. **267**, 16878-16882.

Grimes, C. A. and Jope, R. S. (2001). The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. *Prog. Neurobiol*. **65**, 391-426.

Grinnell, F. (1992). Wound repair, keratinocyte activation and integrin modulation. *J. Cell Sci.* **101**, 1-5.

Haase, I., Liesegang, C., Henz, B. M. and Rosenbach, T. (1997). Retinoic acid attenuates phospholipase C-mediated signaling in HaCaT keratinocytes. *Arch. Dermatol. Res.* **289**, 533-539.

Häkkinen, L., Koivisto, L. and Larjava, H. (2002). An improved method for culture of epidermal keratinocytes from newborn mouse skin. *Methods Cell Sci*. **23**, 189-196.

Hartigan, J. A. and Johnson, G. V. (1999). Transient increases in intracellular calcium result in prolonged site-selective increases in Tau phosphorylation through a glycogen synthase kinase 3beta-dependent pathway. *J. Biol. Chem*. **274**, 21395-21401.

Herbert, J. M., Seban, E. and Maffrand, J. P. (1990). Characterization of specific binding sites for [3H]-staurosporine on various protein kinases. *Biochem. Biophys. Res. Commun.* **171**, 189-195.

Hill, M. M., Andjelkovic, M., Brazil, D. P., Ferrari, S., Fabbro, D. and Hemmings, B. A. (2001). Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase. *J. Biol. Chem*. **276**, 25643-25646.

Hoeflich, K. P., Luo, J., Rubie, E. A., Tsao, M. S., Jin, O. and Woodgett, J. R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* **406**, 86-90.

Hofer, A. M., Landolfi, B., Debellis, L., Pozzan, T. and Curci, S. (1998). Free [Ca2+] dynamics measured in agonist-sensitive stores of single living intact cells: a new look at the refilling process. *EMBO J*. **17**, 1986-1995.

Hughes, K., Nikolakaki, E., Plyte, S. E., Totty, N. F. and Woodgett, J. R. (1993). Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. *EMBO J.* **12**, 803-808.

Hughes, P. E. and Pfaff, M. (1998). Integrin affinity modulation. *Trends Cell Biol.* **8**, 359-364.

Jones, K. T. and Sharpe, G. R. (1994). Staurosporine, a non-specific PKC inhibitor, induces keratinocyte differentiation and raises intracellular calcium, but Ro31-8220, a specific inhibitor, does not. *J. Cell. Physiol.* **159**, 324-330.

Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J. and Rhee, S. G. (1991). PDGF stimulation of inositol phospholipid hydrolysis requires PLC-gamma 1 phosphorylation on tyrosine residues 783 and 1254. *Cell* **65**, 435-441.

Kim, L. and Kimmel, A. R. (2000). GSK3, a master switch regulating cell-fate specification and tumorigenesis. *Curr. Opin. Genet. Dev*. **10**, 508-514.

Klein, P. S. and Melton, D. A. (1996). A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. USA* **93**, 8455-8459.

Koivisto, L., Larjava, K., Häkkinen, L., Uitto, V.-J., Heino, J. and Larjava, H. (1999). Different integrins mediate cell spreading, haptotaxis and lateral migration of HaCaT keratinocytes on fibronectin. *Cell Adh. Commun.* **7**, 245-257.

Larjava, H., Peltonen, J., Akiyama, S. K., Yamada, S. S., Gralnick, H. R., Uitto, J. and Yamada, K. M. (1990). Novel function for β1 integrins in keratinocyte cell-cell interactions. *J. Cell Biol.* **110**, 803-815.

Larjava, H., Haapasalmi, K., Salo, T., Wiebe, C. and Uitto. V. J. (1996). Keratinocyte integrins in wound healing and chronic inflammation of the human periodontium. *Oral Diseases* **2**, 77-86.

Larjava, H., Koivisto, L. and Häkkinen, L. (2002). Keratinocyte interaction with fibronectin during wound healing. In *Cell Invasion* (ed. J. Heino and V.-M. Kähäri), pp. 42-64. Landes Biosciences, Eurecah.com, Georgetown, TX.

Lau, K. F., Miller, C. C., Anderton, B. H. and Shaw, P. C. (1999). Expression analysis of glycogen synthase kinase-3 in human tissues. *J. Pept. Res*. **54**, 85-91.

Leclerc, S., Garnier, M., Hoessel, R., Marko, D., Bibb, J. A., Snyder, G. L., Greengard, P., Biernat, J., Wu, Y. Z., Mandelkow, E. M. et al. (2001). Indirubins inhibit glycogen synthase kinase-3 beta and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? *J. Biol. Chem.* **276**, 251-260.

Lesort, M., Jope, R. S. and Johnson, G. V. (1999). Insulin transiently increases tau phosphorylation: involvement of glycogen synthase kinase-3beta and Fyn tyrosine kinase. *J. Neurochem*. **72**, 576-584.

Levizki, A. and Gazit, A. (1995). Tyrosine kinase inhibition: An approach to drug development. *Science* **267**, 1782-1788.

Li, M., Wang, X., Meintzer, M. K., Laessig, T., Birnbaum, M. J. and Heidenreich, K. A. (2000). Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3beta. *Mol. Cell. Biol*. **20**, 9356-9363.

Mariotti, A., Kedeshian, P. A., Dans, M., Curatola, A. M., Gagnoux-Palacios, L. and Giancotti, F. G. (2001). EGF-R signaling through Fyn kinase disrupts the function of integrin alpha6beta4 at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *J. Cell Biol*. **155**, 447-458.

Masure, S., Haefner, B., Wesselink, J. J., Hoefnagel, E., Mortier, E., Verhasselt, P., Tuytelaars, A., Gordon, R. and Richardson, A. (1999). Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3. *Eur. J. Biochem.* **265**, 353-360.

Matsui, M. S., Chew, S. L. and DeLeo, V. A. (1992). Protein kinase C in normal human epidermal keratinocytes during proliferation and calcium-induced differentiation. *J. Invest. Dermatol.* **99**, 565-571.

Matsui, M. S., Illarda, I., Wang, N. and DeLeo, V. A. (1993). Protein kinase C agonist and antagonist effects in normal human keratinocytes. *Exp. Dermatol.* **2**, 247-256.

Mikhailov, A. and Gundersen, G. G. (1998). Relationship between microtubule dynamics and lamellipodium formation revealed by direct imaging of microtubules in cells treated with nocodazole and taxol. *Cell Motil. Cytoskeleton* **41**, 325-340.

Nobes, C. D. and Hall, A. (1995). Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem. Soc. Trans.* **23**, 456-459.

Odland, G. and Ross, R. (1968). Human wound repair. I. Epidermal regeneration. *J. Cell Biol*. **39**, 135-151.

Osherov, N. and Levizki, A. (1997). Tyrphostin AG 494 bolcks Cdk2 activation. *FEBS Lett*. **410**, 187-190.

Rasouly, D., Rahamim, E., Ringel, I., Ginzburg, I., Muarakata, C., Matsuda, Y. and Lazarovici, P. (1994). Neurites induced by staurosporine in PC12 cells are resistant to colchicine and express high levels of tau proteins. *Mol. Pharmacol.* **45**, 29-35.

Rasouly, D., Shavit, D., Zuniga, R., Elejalde, R. B., Unswotrh, B. R., Yayon, A., Lazarovici, P. and Lelkes, P. I. (1996). Staurosporine induces neurite outgrowth in neuronal hybrids (PC12EN) lacking NGF receptors. *J. Cell. Biochem.* **62**, 356-371.

Sako, T., Tauber, A. I., Jeng, A. Y., Yuspa, S. H. and Blumberg, P. M. (1988). Contrasting actions of staurosporine, a protein kinase inhibitor, on human neutrophils and primary mouse epidermal cells. *Cancer Res.* **48**, 4646-4650.

Sastry, S. K., Lyons, P. D., Schaller, M. D. and Burridge, K. (2002). PTP-PEST controls motility through regulation of Rac1. *J. Cell Sci.* **115**, 4305-4316.

Shaw, M. and Cohen, P. (1999). Role of protein kinase B and the MAP kinase cascade in mediating the EGF-dependent inhibition of glycogen synthase kinase 3 in Swiss 3T3 cells. *FEBS Lett*. **461**, 120-124.

Small, V. J., Rotter, K. and Kaverina, I. (1999). Functional design in the actin cytoskeleton. *Curr. Opin. Cell Biol.* **11**, 54-60.

Stanwell, C., Denning, M. F., Rutberg, S. E., Cheng, C., Yuspa, S. H. and Dlugosz, A. A. (1996). Staurosporine induces a sequential program of mouse keratinocyte terminal differentation through activation of PKC isoenzymes. *J. Invest. Dermatol.* **106**, 482-489.

Stenn, K. S. and Malhorta, R. (1992). Epithelialization. In *Wound Healing:*

Biochemical and Clinical Aspects (eds. I. K. Cohe, R. F. Diegelmann and W. J. Lindblad), pp. 115-127. WB Saunders Co., Philadelphia.

Sudbeck, B. D., Parks, W. C., Welgus, H. G. and Pentland, A. P. (1994). Collagen-stimulated induction of keratinocyte collagenase is mediated via tyrosine kinase and protein kinase C activities. *J. Biol. Chem.* **269**, 30022-30029.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/ Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**, 397-402.

Wagner, U., Utton, M., Gallo, J. M. and Miller, C. C. (1996). Cellular phosphorylation of tau by GSK-3 beta influences tau binding to microtubules and microtubule organisation. *J. Cell Sci*. **109**, 1537-1543.

Woodgett, J. R. (1990). Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J*. **9**, 2431-2438.

Woodgett, J. R. (1991). cDNA cloning and properties of glycogen synthase kinase-3. *Methods. Enzymol*. **200**, 564-577.

Woodley, D. T. (1996). Reepithelization. In *The Molecular and Cellular Biology of Wound Repair* (ed. R. A. F. Clark), pp. 339-354. NY: Plenum Press.

Yamazaki, Y., Sanokawa, R., Fujita, Y., Zhou, D., Kawasaki, K., Tanaka, H., Komatsu, T., Nagasawa, T. and Oka, S. (1999). Cytoplasmic elongation and rupture in megakaryoblastic leukemia cells via activation of adhesion and motility by staurosporine on fibronectin-bound substratum. *J. Cell. Physiol.* **179**, 179-192.

Yang, S. D., Chou, C. K., Huang, M., Song, J. S. and Chen, H. C. (1989). Epidermal growth factor induces activation of protein kinase FA and ATP.Mg-dependent protein phosphatase in A431 cells. *J. Biol. Chem.* **264**, 5407-5411.

Yarden, Y. and Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell. Biol.* **2**, 127-137.

Yu, H., Fukami, K., Itoh, T. and Takenawa, T. (1998). Phosphorylation of phospholipase Cgamma1 on tyrosine residue 783 by platelet-derived growth factor regulates reorganization of the cytoskeleton. *Exp. Cell Res.* **243**, 113-122.

Yu, J. S. and Yang, S. D. (1994). Tyrosine dephosphorylation and concurrent inactivation of protein kinase FA/GSK-3 alpha by genistein in A431 cells. *J. Cell. Biochem*. **56**, 131-141.

Yu, J. S., Chen, H. C. and Yang, S. D. (1997). Reversible tyrosine phosphorylation/dephosphorylation of proline-directed protein kinase FA/glycogen synthase kinase-3alpha in A431 cells. *J. Cell. Physiol*. **171**, 95-103.

Zeng, Q., Lagunoff, D., Masaracchia, R., Goeckeler, Z., Cote, G. and Wysolmerski, R. (2000). Endothelial cell retraction is induced by PAK2 monophosphorylation of myosin II. *J. Cell Sci.* **113**, 471-482.