Zyxin Redistributes Without Upregulation in Migrating Human Keratinocytes During Wound Healing

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Cell migration, growth, and survival is modulated by focal adhesions linking extracellular matrix proteins, cell adhesion molecules, and the cytoskeleton. Zyxin is a focal adhesion phosphoprotein that shares homology with *Listeria* ActA protein in promoting actin filament assembly; it also has specialized protein–protein interface domains implicating an important role in cell growth and differentiation. We investigated the distribution of zyxin in normal and migrating human keratinocytes in wounds *in vitro* and *in situ* using confocal laser microscopy. Zyxin expression in high-density nonmigrating keratinocytes *versus* low-density migrating keratinocytes was determined by western immunoblotting and time lapse image analysis. In normal epidermis, zyxin exhibited a punctate staining pattern throughout the cytoplasm and was excluded from the intercellular spaces. In wounds, the punctate staining also localized in the edge of the migrating keratinocyte sheets; however, intercellular spaces were absent. Likewise, *in vitro* keratinocytes showed punctate staining throughout the cytoplasm. Migrating cultured keratinocytes next to wounds, however, had large focal contacts in the cell periphery where actin bundles converged at focal adhesions. Western immunoblots and confocal experiments with protein synthesis inhibition by cycloheximide confirmed that this difference in distribution of zyxin in migrating *versus* nonmigrating keratinocytes is due to the redistribution and not upregulation of zyxin. The abundance of zyxin and its relative change in distribution from normal to migrating keratinocytes in wounds is consistent with its role in cytoskeletal organization of actin bundles. ***Key words: actin bundles/cytoskeleton/focal contact/wound. J Invest Dermatol 113:651–657, 1999***

Keratinocytes forming the cutaneous epidermis adhere to extracellular matrix components and each other through specialized membrane complexes located at focal adhesions. These focal contacts are transmembrane junctions where the heterodimeric integrins are the prevailing adhesive receptors. Indeed, the linkage of the actin cytoskeleton to the integrins appears to be critical for relaying information about the extracellular environment to the cell interior (Ruoslahti, 1997). *In vivo*, integrins are expressed in basal keratinocytes but are downregulated in differentiating upper cell layers (Klein *et al*, 1990; Poumay *et al*, 1994). Moreover, several reports have described the modulation of integrin expression in keratinocytes migrating during wound healing and have demonstrated that integrins play an important part in keratinocyte proliferation and adhesion (Cavani *et al*, 1993; Larjava *et al*, 1993; Haapasalmi *et al*, 1996). To regulate cell behavior, such as cell locomotion and cell proliferation, however, integrins must function in association with numerous other molecules in the focal adhesion as structural and signaling partners (Burridge and Chrzanowska-Wodnicka, 1996). A focal adhesion molecule called zyxin exhibits specialized zinc-binding sequences that indicate an important role in modular protein binding interfaces (Sadler *et al*, 1992). Zyxin appears to relay information from the focal contact to the nucleus (Nix and Beckerle, 1997) and is important in actin organization (Golsteyn *et al*, 1997; Beckerle, 1998).

Zyxin is a phosphoprotein with an apparent molecular weight of 84 kDa in human cells (Macalma *et al*, 1996). In fibroblasts, zyxin and integrins colocalize at sites of cellular substratum attachment (focal adhesions) (Crawford *et al*, 1992; Sadler *et al*, 1992). Zyxin distributes along actin-containing stress fibers and circumferential actin bundles of epithelium; highest concentrations are present at the ends of stress fibers or sites of focal cell adhesion (Crawford and Beckerle, 1991). Indeed, recent data suggest that zyxin acts as a nucleating protein for actin as it shares structural and functional features with a *Listeria monocytogenes* actin-nucleating protein (Golsteyn *et al*, 1997; Beckerle, 1998). To date, there are no investigations on zyxin expression in normal human skin keratinocytes or migrating keratinocytes in wounds. We hypothesized that zyxin, as other focal adhesion molecules, would redistribute during keratinocyte migration and would be upregulated in proliferating cells in wounds as well. Therefore, we analyzed zyxin distribution in nonmigrating keratinocytes in confluent cultures and normal skin and in migrating keratinocytes in wounds *in vitro* and *in situ*. Furthermore, we performed western immunoblots on migrating *versus* nonmigrating keratinocytes *in vitro* and confocal microscopy on cycloheximide-treated cells to determine if zyxin expression is upregulated during migration. In this report, we demonstrate a unique focal punctate distribution of zyxin in human cultured keratinocytes and in histologic sections of human

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Abbreviations: K-STIM, keratinocyte stimulation media; LIM, Lin-11, Isl-1, Mec-3.

epidermis. In addition, our findings indicate that focal contact formation in migrating keratinocytes accompanies redistribution and not upregulation of zyxin.

MATERIALS AND METHODS

Cell culture and *in vitro* wound procedure Human foreskin keratinocytes were isolated as described previously with modifications (Gilchrest, 1979). Cells were cultured in a low calcium medium (K-STIM, Becton Dickinson, Bedford, MA) without serum and used at second passage. For image analysis and immunofluorescent studies, cells were seeded on 35 mm Petri dishes (Falcon Plastics, Becton Dickinson, Bedford, MA) or on eight-chamber slides (Permanox Tissue Tek, Nunc, Naperville, IL), coated with 10 μg per ml fibronectin. Sliding a plastic sterile pipette tip over the substrate with confluent keratinocytes induced mechanically the *in vitro* wound. Immunoblotting assays (see below) were performed on sparsely plated keratinocytes ($<10^3$ cells) and confluent keratinocytes ($>10^6$ cells) per 60 mm Petri dish. In addition, intact epidermal sheets were isolated and used for immunoblot analysis.

Video time-lapse and image analysis Keratinocyte migration was analyzed by time-lapse computerized microscopy as described previously with modifications (Byers *et al*, 1991). Briefly, cell cultures were placed on the stage of a Nikon Microphot inverted microscope equipped with a Plexiglas housing, Nikon incubator and CO_2/air flow mixer to obtain constant pH and temperature (37°C). Digitized images were taken every 30 min for 24 h after the wound using the phase contrast 10 × objective lens, a Cohu High performance CCD camera (San Diego, CA), a power Macintosh 7100/66 with a LG-3 scientific frame grabber card (Scion, Frederick, MD) and IPlab spectrum software (Scanalytics, Fairfax, VA).

Double fluorescent localization of zyxin and actin filaments Human keratinocytes were cultured until confluence on 10 μg per ml fibronectin-coated eight-chamber slides (Nunc). Immunofluorescent studies were done at 0, 6, 12, and 24 h, after *in vitro* wounding. Confluent cultures were pretreated for 24 h in 0, 10, 20, and 30 μg per ml cycloheximide to study the effect of protein synthesis inhibition on keratinocyte migration and zyxin distribution. Cultures were then wounded, maintained in cycloheximide and immunofluorescent studies performed after 24 h. A rabbit polyclonal anti-zyxin antibody against human platelet zyxin (B38) was a generous gift of M.C. Beckerle (Macalma *et al*, 1996). The chambers were washed three times with phosphate-buffered saline (PBS) and fixed for 15 min with 5% formaldehyde, 2 mM glycol ether diamine tetraacetic acid and 2 mM $MgCl_2$, washed with PBS and permeabilized for 2 min with 0.2% Triton X-100. After washing with PBS, 5% goat serum in PBS was added for 30 min to block nonspecific binding sites, followed by 1 h incubation with the rabbit polyclonal antihuman zyxin antibody at a 1:1200 dilution in PBS with 0.5% bovine serum albumin. After rinses with PBS, goat anti-rabbit fluorescein isothiocyanate (Sigma, St. Louis, MO) was added at a 1:80 dilution in PBS and 0.5% bovine serum albumin and incubated for 1 h, washed again with PBS and incubated with rhodamine-phalloidin to label actin filaments (Molecular Probes, Eugene, OR) at a 1:40 dilution in PBS and 0.5% bovine serum albumin. Control preparations included the goat anti-rabbit fluorescein isothiocyanate and the rhodamine-phalloidin without the anti-zyxin primary antibody. After washing with PBS, the cells on the chamber slides were mounted using the slowfade kit (Molecular Probes) followed by coverslipping. All manipulations were performed at room temperature and incubation times were done at 37°C. The preparations were examined with a Leica confocal laser scanning microscope using the 25 × (n.a. = 0.75) water immersion lens. The images were stored on an optical disc, transferred to the Macintosh computer and processed using the IPlab spectrum software described above.

Immunodetection of zyxin Western blotting was performed by modification of the procedure developed by Towbin *et al* (1979). Briefly, low-density migrating keratinocyte cultures (in four 60 mm dishes less than 20% confluent) and confluent keratinocyte cultures (60 mm dish) were rinsed three times with cold PBS and lysed in lysis buffer containing 20 mM Tris, 2 mM ethylenediamine tetraacetic acid, 0.5 mM glycol ether diamine tetraacetic acid, 1% Triton X-100, 5 mM dithiothreitol, 10 μg aprotinin per ml, 50 nM okadaic acid, and 1 mM phenylmethylsulfonyl fluoride. Migrating (low-density) and nonmigrating (high-density) keratinocyte lysates were centrifuged, pellets were discarded and protein assay of the supernatant was performed. Eighty micrograms of protein were electrophoresed using a 10% sodium dodecyl sulfate–polyacrylamide gel. Wet transfer of proteins on to nitrocellulose paper by electrophoresis was performed in 25 mM Tris, 192 mM glycine, and 20% methanol. After transfer, the upper two-thirds of the nitrocellulose membrane were blocked in 5% nonfat dry milk in PBS for 1 h at room temperature with shaking. The lower part of the membrane was stained with amido black solution (0.5% amido black, 45% methanol, 9% acetic acid) for 1 min and was then destained with 45% methanol, 9% acetic acid, and 46% water for 1 h at room temperature with agitation. The rabbit polyclonal anti-zyxin antibody was diluted 1:5000 in 1% nonfat dry milk in PBS and incubated with the transferred protein–nitrocellulose membrane at 4°C overnight. After incubation, the membrane was washed in PBS once for 10 min and then three times in PBS containing 0.05% Tween 20 followed by incubation with a secondary goat anti-rabbit antibody (Pierce, Rockford, IL) in 1% milk for 1 h. Nitrocellulose membrane was washed again and then incubated for 1 min with the enzyme-linked chemiluminescence Western Blotting Detection Reagent (Amersham, Buckinghamshire, U.K.). The membrane was then exposed to preflashed Kodak X-Omat film and developed after a few seconds of exposure. Densitometry was performed using computerized scanning of the film using a Macintosh scanner and the IPlab spectrum software (Scanalytics). Keratinocytes from three different donors were assayed in three different experiments in the migrating low-density *versus* nonmigrating high-density comparison studies. Sensitivity of changes in the zyxin immunoblot assay was determined by increasing protein concentration and performing densitometry gel scans to quantify the protein loading.

Immunofluorescence labeling of human tissue sections Re-excisions of 7, 10, and 14 d cutaneous wounds made by previous shave excisions were used for the immunolocalization of zyxin at the wound and in normal skin up to 1 cm away from the wound. Re-excision specimens were selected in cases where cutaneous melanoma had been excised in the original shave excisional biopsies but had required re-excision for greater margins. A pathologist (HRB) examined re-excision specimens and no residual tumor was present. Likewise, the original excisional biopsies were re-examined and confirmed that the margins revealed normal skin without inflammation present. Therefore, the migrating keratinocytes within the wound bed in the re-excision specimens met with normal wounded dermis and not tumor stroma. Tissue used in the study was excess human tissue and no longer needed for diagnostic purposes. The tissue was fixed routinely in 10% formalin and paraffin embedded. Tissue sections were cut 3 μm thick, placed on plus-coated slides (Fischer, Pittsburgh, PA) and held overnight. The sections were deparaffinized by first placing the slides for 10 min in a 50°C oven, then 5 min cooling, followed by 1 min in xylene immersion. The sections were rehydrated in alcohol and washed with PBS. No proteolytic digestion was performed. The sections were then incubated with anti-zyxin antibodies following the same procedure described previously for cultured keratinocytes. After the last washing with PBS, sections were mounted using the slowfade kit (Molecular Probes). Student's t test compared mean fluorescence intensity (obtained by Scanalytics image analysis software described above) of the cytoplasm of 30–40 cells in the basal layer and the first two spinous cell layers with upper spinous cell layers.

RESULTS

Zyxin is concentrated at focal adhesion plaques during keratinocyte migration *in vitro* To determine the optimal time to perform immunofluorescent studies in migrating keratinocytes, we used time-lapse image analysis of the *in vitro* wound bed. **Figure 1** shows different time points of the keratinocyte migration during the *in vitro* wound healing: keratinocytes colonized approximately 25% of the wound surface after 6 h and 50% after 12 h. One day after the *in vitro* wound, more than 80% of the surface was healed. We noted donor variability and intradonor variability; on occasion 10% healing was observed at 6 h and 100% healing was noted at 24 h. Healing at 12 h varied from 25% to 75%; therefore, we performed experiments at 12 h when a significant number of keratinocytes was migrating into the wound. Time-lapse analysis confirmed that keratinocytes covering the wound was primarily due to migration and secondarily to cell division. Immunofluorescence detection of zyxin and actin distribution in confluent *versus* migrating cells was examined. Migrating keratinocytes fixed at the edge of wounds (**Fig 2*A***, ***F***) revealed more developed actin-containing stress fibers than those seen in areas of confluent keratinocytes (**Fig 2*A***, ***E***). These findings confirmed previous studies (Kubler and Watt, 1993). The actin filaments labeled with rhodamine-phalloidin in keratinocytes were also incubated with the fluorescein-labeled goat anti-rabbit antibody

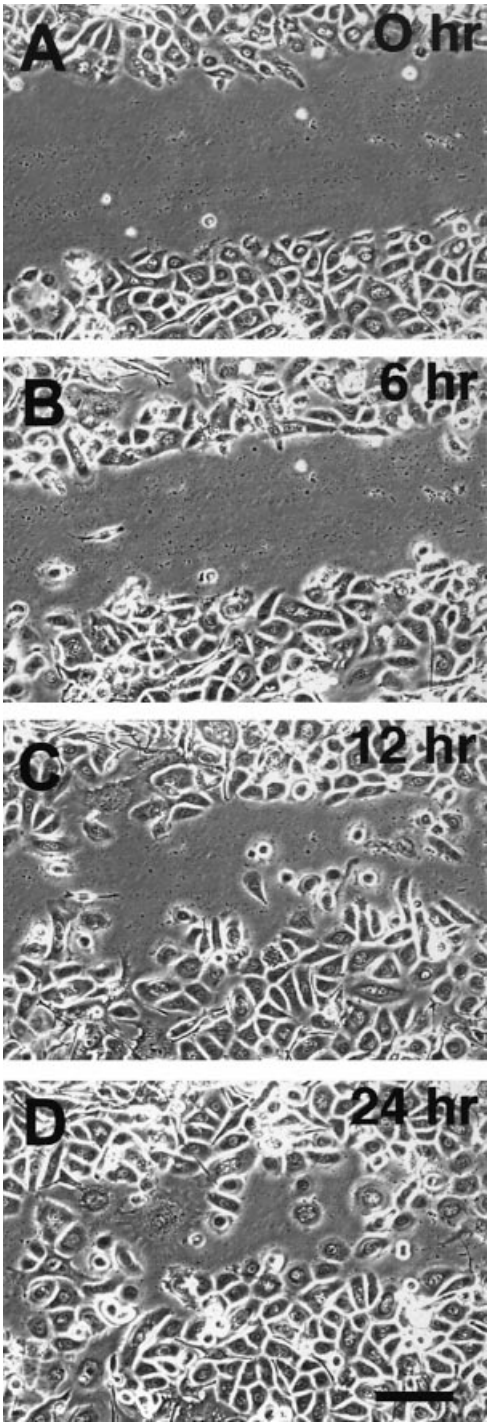

Figure 1. Migrating human keratinocytes cover *in vitro* wound after 24 h. Phase-contrast time-lapse images, at 0 h (*A*), 6 h (*B*), 12 h (*C*), and 24 h (*D*). By 24 h, the wound is 80% covered by migrating keratinocytes. *Scale bar.* 100 μm.

without the zyxin primary antibody (control). These preparations exhibited no fluorescent transfer or bleed-through of the rhodamine fluorescence (**Fig 2*C***) with the fluorescein-labeled secondary antibody control (**Fig 2*D***), indicating complete separation of the fluorescent signals. Zyxin distribution in areas of confluent keratinocytes was punctate in the central cytoplasm (**Fig 2*B***, ***G***). It showed increased staining at the periphery (**Fig 2*B***, ***G***), however, similar to the actin staining pattern (**Fig 2*A***, ***E***, ***I***). In contrast, in migrating keratinocytes, zyxin staining was intense at focal sites of the peripheral cytoplasm corresponding to ends of actin stress fibers at focal adhesion plaques (**Fig 2*B***, ***H***, ***J***). In order to determine whether the formation of zyxin staining at focal adhesion plaques is a redistribution of zyxin or dependent on upregulation, we pretreated cultures for 24 h with increasing concentrations of the protein synthesis inhibitor cycloheximide. The cultures were then wounded, maintained in cycloheximide then fixed and stained after 24 h. Zyxin redistributed to focal contacts in migrating cells despite concentrations of up to 30 μg per ml cycloheximide for 48 h (**Fig 3**).

Zyxin upregulation is not detected during keratinocyte migration *in vitro* To test further whether the increased staining of zyxin at the focal adhesion plaques during *in vitro* keratinocyte migration is linked to redistribution of the protein as opposed to upregulation, we performed immunoblot analysis for zyxin using lysates of human cutaneous keratinocytes plated at low or high density. Computerized time-lapse analysis on keratinocytes plated at low density confirmed that keratinocytes migrated at speeds equivalent to those observed into the wound. Likewise, keratinocytes in confluent cultures showed low migration rates similar to those cells away from the wound. Typically, four to five plates of low-density plated cells were harvested for each confluent plate. We found no difference in zyxin expression in the migrating *versus* nonmigrating keratinocytes from three different donors as detectable by the immunoblot technique (**Fig 4*A***) and densitometry gel scanning (**Fig 4*B***). In order to define the sensitivity of our immunoblot assay to detect differences in zyxin protein levels, we performed immunoblot analysis on increasing protein concentrations of cultured human keratinocyte lysates (**Fig 4*C***). Densitometric scanned gel plots of zyxin immunoblots revealed that changes of 10% are detectable by western blotting but changes less than 10% are uncertain (**Fig 4*D***).

Zyxin is expressed in epidermis of normal human skin Several studies have shown that integrin distribution in normal human skin is confined primarily to the basal and lateral membranes of the basal layer (Klein *et al*, 1990; Larjava *et al*, 1990; Hertle *et al*, 1991; Poumay *et al*, 1994). Using the polyclonal anti-zyxin antibody for immunofluorescence labeling, we found that zyxin is expressed in all cellular layers of the normal cutaneous epidermis in a distinct punctate cytoplasmic staining pattern with increased staining in keratinocytes in the basal and first two spinous cell layers (**Fig 5*A***) compared with the upper spinous cell layers. Image analysis revealed that mean fluorescence intensity for zyxin in the lower cell layers was 166 ± 13 and in the upper layers 104 ± 09 ($p < 0.001$). In addition, the intercellular spaces in the first few keratinocyte layers were delineated clearly in zyxin and control preparations (**Fig 5*A***, ***B***), as is seen typically in fixed, paraffin-embedded tissue.

To confirm that zyxin is present in the epidermis of normal human skin and to exclude the possibility of cross-reactivity to other proteins in the epidermis, normal human foreskin epidermis was isolated and a lysate prepared for immunoblot analysis. Fifty microgram protein loading with extended enzyme-linked chemiluminescence exposure revealed a single prominent band at the appropriate relative mobility of zyxin (**Fig 6**). No other bands were detected indicating specificity of the antibody for zyxin in lysates of human epidermis.

Punctate redistribution of zyxin during epidermal wound healing *in situ* It has been shown that integrin expression is upregulated during wound healing (Cavani *et al*, 1993; Juhasz *et al*, 1993; Haapasalmi *et al*, 1996). Conversely, cytoplasmic bridges at desmosomal-associated proteins are downregulated (Kurpakus *et al*, 1991; Poumay *et al*, 1994). In this study, the distribution of zyxin was studied in a skin wound when keratinocyte sheets showed marked migration into the wound bed (**Fig 5*C***). The actin labeling pattern (**Fig 5*D***) revealed peripheral cytoplasmic staining as reported previously (Kaiser *et al*, 1993), except that in paraffin-embedded tissue the intercellular spaces are broadened in areas away from the wound due to contraction artifact. At the edge of the migrating epithelial sheet, zyxin distribution showed a punctate staining pattern throughout the cytoplasm and at the leading edge and basal

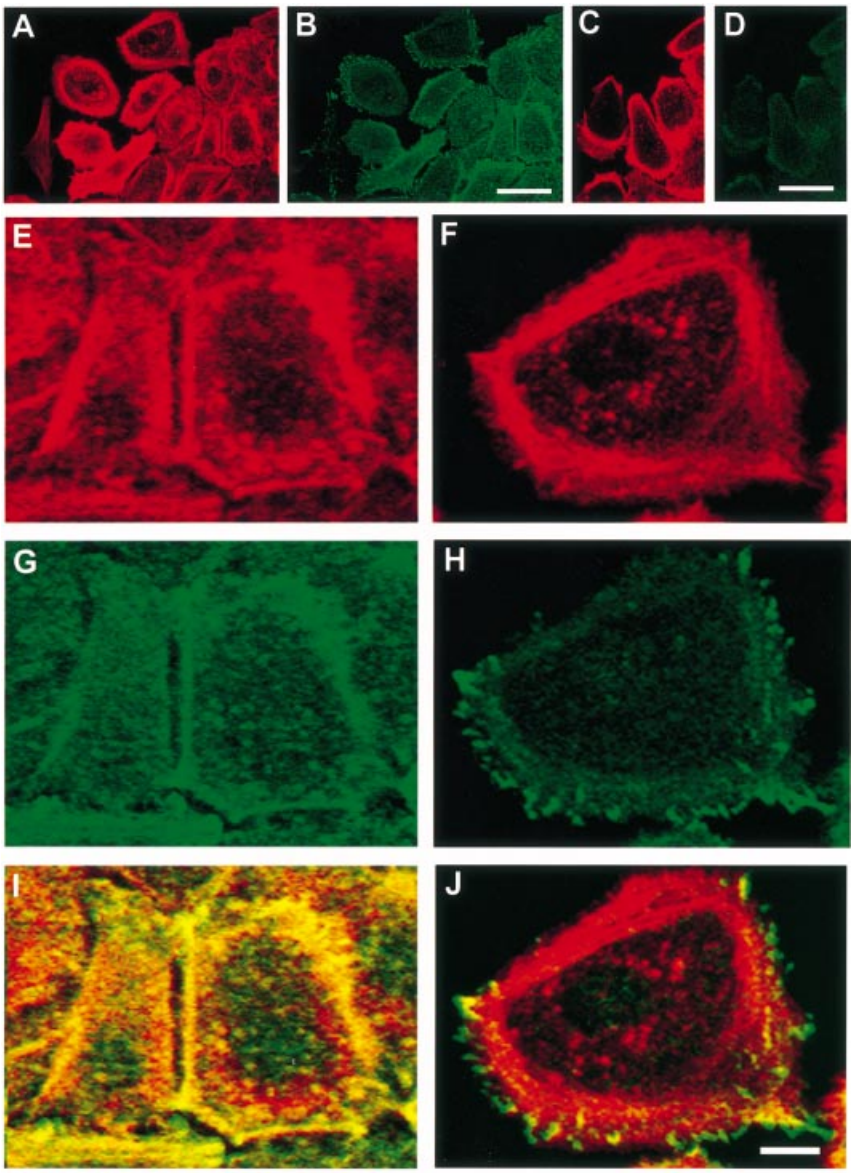

Figure 2. Zyxin redistributes to focal contacts in human keratinocytes migrating into the *in vitro* wound. Double fluorescent confocal microscopic images of actin filaments (*A*) and zyxin (*B*) distribution in human keratinocytes next to the *in vitro* wound at low magnification. Migrating keratinocytes show developed actin stress fibers (*A*, *F*) and focal peripheral zyxin staining (*B*, *H*). Color overlay analysis (*J*) demonstrates colocalization of zyxin and ends of actin stress fibers or focal adhesions (yellow color). Non-migrating keratinocytes show peripheral actin staining (*E*), punctate zyxin staining centrally with marked peripheral localization (*G*), and marked colocalization with actin peripherally (*I*). Control preparation stained for actin (*C*) and the secondary fluorescein labeled antibody alone (*D*) revealed faint nonspecific fluorescein signal and absent "bleed-through". *Scale bar*: 20 μm (A–D), 5 μm (E–J).

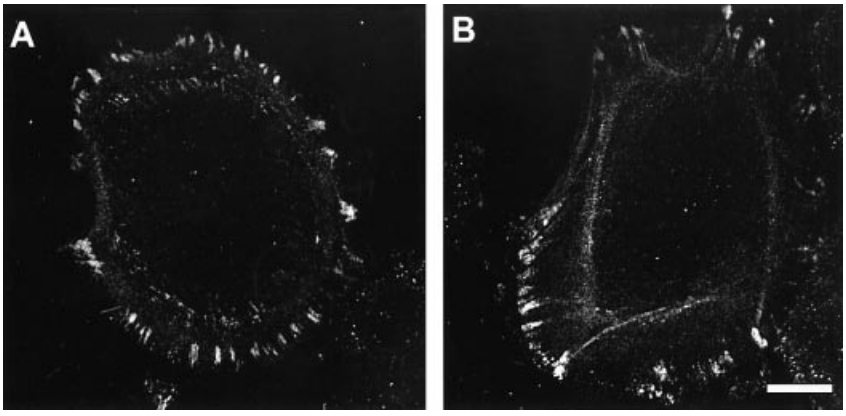

Figure 3. Zyxin redistributes to focal contacts in migrating keratinocytes in the presence of the protein synthesis inhibitor cycloheximide. Migrating human keratinocytes show peripheral zyxin staining at focal contacts in the presence of 10 μg cycloheximide per ml (*A*) or 20 μg cycloheximide per ml (*B*). *Scale bar*: 5 μm.

aspect of migrating cells (**Fig 5*E***, ***F***, *arrows*). Another significant change compared with the paraffin-embedded normal skin pattern was the loss of the distinct intercellular spaces among the leading edge keratinocytes. Flattened keratinocytes further from the wound exhibited broadened intercellular spaces seen in nonwounded paraffin-embedded skin, with exclusion of punctate zyxin (**Fig 5*E***, *arrowheads*).

DISCUSSION

Keratinocyte expression of adhesion molecules appears critical for normal growth and differentiation of the cutaneous epidermis and for the healing of cutaneous wounds. Some of these molecules are localized at focal contacts, structures organized as cytoskeletal and transmembrane connections between the cytoskeleton and the

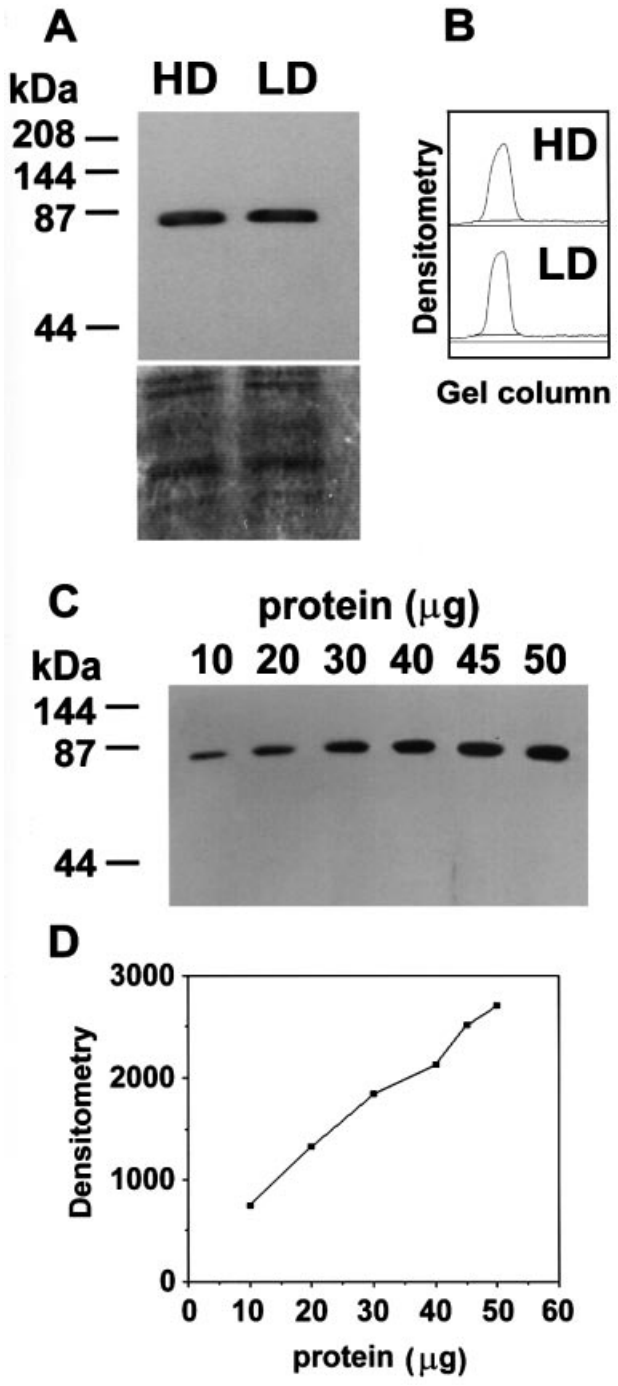

Figure 4. Zyxin upregulation is not detectable in migrating keratinocytes. (*A*) Western immunodetection of zyxin expression in high-density (HD) nonmigrating keratinocytes *versus* low-density (LD) migrating keratinocytes. *Top panel*: a single band at relative mobility of 84 kDa corresponds to zyxin. *Bottom panel*: Amido-black-stained bottom portion of nitrocellulose membrane shows protein loading of gel above. (*B*) Densitometry gel scan of zyxin immunoblots in (*A*); no significant difference was detectable between HD (nonmigrating) and LD (migrating) keratinocytes. (*C*) Immunoblot assay to determine sensitivity for detectable changes in zyxin protein levels. Zyxin immunoblot of increasing protein loading from lysates of confluent keratinocyte cultures. (*D*) Densitometry gel scan plot of zyxin immunoblot in (*C*) showing linear relationship of protein loading and densitometry gel scan values.

extracellular matrix; the integrin family of adhesion molecules plays an important part in focal contacts. Integrin distribution in normal human skin, keratinocyte cultures, and during wound healing, has been studied extensively (Watt and Jones, 1993). Integrins interact with a number of cytoskeletal proteins after binding extracellular matrix proteins (Burridge and Chrzanowska-Wodnicka, 1996). The phosphoprotein zyxin plays a part in signal transduction at those specialized contacts of the cell membrane (Nix and Beckerle, 1997). To our knowledge, the distribution of zyxin in keratinocytes has not been investigated. We demonstrate in this work that zyxin is expressed in cutaneous keratinocytes, both in cultures and *in situ*. We demonstrate that zyxin in cultured migrating keratinocytes is concentrated at adhesion plaques and focally along the actin filament bundles; findings that are similar to studies on migrating fibroblasts (Crawford and Beckerle, 1991). Evidence suggests that zyxin regulates the spatial control of actin assembly within cells (Macalma *et al*, 1996; Beckerle, 1998). Indeed, recent studies confirmed that zyxin plays a nucleating part in the organization of actin filaments (Golsteyn *et al*, 1997).

The healing of skin wounds requires stimulation of keratinocyte adhesion, migration, and associated reorganization of actin filaments. In this study, we used the *in vitro* model of wounding to contrast zyxin expression in confluent keratinocytes *versus* keratinocytes migrating into the wound. The double-labeling of zyxin and actin filaments demonstrated that keratinocytes at the edge of the wound exhibit a change in zyxin distribution: in the migrating cells, zyxin concentrates at the focal points in the cell periphery corresponding to actin initiation sites and focal adhesions. We have not detected significant upregulation within the limits of the immunoblot technique, that is, no fold-increase nor upregulation greater than 10%. Thus we did not detect a significant difference in zyxin levels between the confluent *versus* the sparsely plated migrating keratinocytes. These findings, in conjunction with the protein synthesis blocking experiments, indicate a redistribution of zyxin and not upregulation during migration into a wound. Likewise, they suggest that other molecules regulate actin assembly during migration.

The mechanism of zyxin redistribution is most consistent with release from cell–cell contacts and contact with cell matrix. The expression of integrins such as $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 5$, or $\alpha v\beta 6$, is induced in keratinocytes during wound healing *in situ* (Cavani *et al*, 1993; Juhasz *et al*, 1993; Larjava *et al*, 1993; Watt and Jones, 1993; Haapasalmi *et al*, 1996). As the epidermal cell barrier is disrupted, these integrins bind the newly exposed fibronectin, vitronectin, and other extracellular matrix molecules, signaling an increase in cell migration. Clustering of integrins at sites of matrix contact induces formation of adhesion plaques that are nucleation sites for actin filament formation (Burridge and Chrzanowska-Wodnicka, 1996). Our *in vitro* and *in situ* findings show a peripheral shift in punctate cytoplasmic staining for zyxin that is consistent with evidence that zyxin helps direct the spatial control of actin filament nucleation (Golsteyn *et al*, 1997; Beckerle, 1998).

Cellular control of proliferation, migration and differentiation is dependent on cell–cell and cell–matrix signaling pathways that involve integrins that interact with the cytoskeleton (Burridge and Chrzanowska-Wodnicka, 1996; Ruoslahti, 1997). Zyxin presents the molecular features of an intracellular signal transducer exhibiting a proline-rich sequence and three LIM domains (Schmeichel and Beckerle, 1994). LIM domains are conserved regions found in a number of developmental transcription factors (Lin-11, Isl-1, Mec-3) that are known to regulate cell proliferation and differentiation (Sadler *et al*, 1992; Macalma *et al*, 1996). Although zyxin is detected primarily in the cytoplasm, zyxin displays a nuclear export signal and is capable of regulated shuttling between the nucleus and the cytoplasm (Nix and Beckerle, 1997). Furthermore, zyxin exhibits structural and functional characteristics of the *Listeria monocytogenes* ActA protein; both zyxin and ActA nucleate actin polymerization, thus forming actin filament bundles (Golsteyn *et al*, 1997). These findings place zyxin in a regulatory role of cytoskeletal organization and may thus modulate cytoarchitecture, differentiation, and tissue homeostasis. The abundance of focal zyxin staining in human skin keratinocytes and its redistribution in migrating keratinocytes in wounds is consistent with a role in focal cell

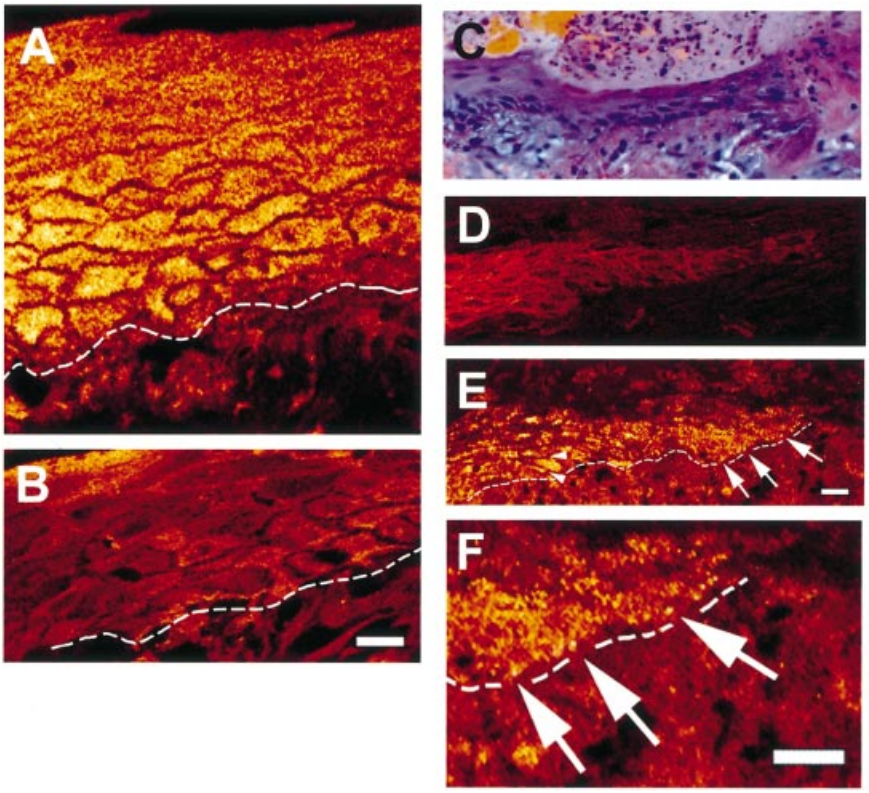

Figure 5. Zyxin is localized in a punctate staining pattern throughout the keratinocyte cytoplasm in normal human epidermis and exhibits focal peripheral staining in wounds. (*A*) Confocal immunofluorescent microscopy reveals punctate zyxin staining throughout the keratinocyte cytoplasm in normal epidermis. Note increased staining in lower cell layers compared with upper layers. Sections incubated with the fluorescein-labeled goat anti-rabbit antibody alone exhibit faint nonspecific staining (*B*). (*C*) Hematoxylin and eosin stained section of wound site. (*D*) Migrating epidermal sheet shows peripheral actin localization (rhodamine-phalloidin staining). (*E*) The keratinocytes show zyxin dispersed throughout the cytoplasm with focal peripheral increased staining at the leading edge (*arrows*). In contrast, keratinocytes away from the leading edge show a punctate staining pattern (*arrowheads*), similar to normal skin zyxin distribution. (*F*) Magnification of leading edge of migrating keratinocytes in (*E*), showing focal peripheral increased staining adjacent to dermis. The dashed line indicates the dermoepidermal junction. *Scale bar*: 20 μm.

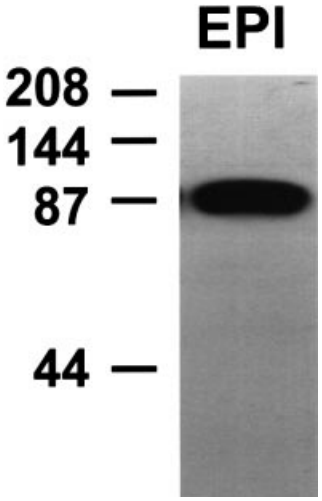

Figure 6. Zyxin is present in human epidermis. Immunoblot analysis of lysate from whole isolated human epidermis. Single band at relative mobility of 84 kDa corresponds to zyxin.

contact formation and intracytoplasmic cytoskeletal organization of actin bundles in these cells.

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