The Mixed Lineage Kinase Leucine-Zipper Protein Kinase Exhibits a Differentiation-Associated Localization in Normal Human Skin and Induces Keratinocyte Differentiation upon Overexpression

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Leucine-zipper protein kinase/dual leucine zipper bearing kinase/mitogen-activated protein kinase-upstream kinase is a recently described protein serine/threonine kinase which belongs to the mixed lineage kinase family. The overall pattern of expression of the leucine-zipper protein kinase/dual leucine zipper bearing kinase/mitogen-activated protein kinase-upstream kinase gene in embryonic and adult mouse tissues suggested that this kinase could be involved in the regulation of epithelial cell proliferation and differentiation. In order to get more insights into the potential role of leucine-zipper protein kinase in these cellular processes, we characterized its expression in normal human skin, both at the mRNA and protein levels. *In situ* hybridization, western blotting, and indirect immunofluorescence studies were conducted to localize leucine-zipper protein kinase on various human skin tissues. This is one of the first reports that leucine-zipper protein kinase has a very precise pattern of expression in human skin epithelia, as both mRNA and protein are restricted to the granular layer of the epidermis and inner root sheath of hair follicles. Detection of leucine-zipper protein kinase protein on skin from various body sites, donors of different ages as well as on reconstructed human skin always reveals that leucine-zipper protein kinase is present only in the very differentiated keratinocytes of epidermis and hair follicles. To determine directly whether leucine-zipper protein kinase exhibits any effect on cell growth and differentiation, keratinocytes were transfected with an expression vector harboring the leucine-zipper protein kinase cDNA. The presence of this construct in keratinocytes results in growth arrest together with a concomitant increase in filaggrin expression. Collectively, our results indicate that leucine-zipper protein kinase plays an active part in cellular processes related to terminal differentiation of epidermal keratinocytes. ***Key words: epidermis/granular layer/hair follicle/reconstructed skin. J Invest Dermatol 115:860–867, 2000***

A wide variety of circulating polypeptides and hormones play crucial roles in the regulation of growth, differentiation, and apoptosis in multicellular organisms. Many of these extracellular signaling molecules are transduced from the plasma membrane to the nucleus by a cascade of protein kinases that involves members of the mitogen-activated protein kinase (MAPK) family (Kyriakis and Avruch, 1996; Ip and Davis, 1998; Tomic-Canic *et al*, 1998). In mammals, MAPK are classified into at least three distinct subfamilies of related polypeptides, commonly known as the extracellular signal-regulated kinases (ERK), the Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK), and the p38 kinases. Although some cross-talk between them does appear to exist, each MAPK subgroup is preferentially activated by different types of extracellular stimuli. The ERK are activated in response to mitogens (Robinson and Cobb, 1997), whereas the JNK/SAPK and p38 kinases respond predominantly to inflammatory cytokines and environmental stresses such as ultraviolet light, osmotic shock γ-irradiation, and ceramides (Kyriakis and Avruch, 1996; Ip and Davis, 1998).

An interesting feature of each MAPK subgroup is that their activation by external stimuli requires phosphorylation of both a threonine and a tyrosine residue, and this is catalyzed by specific MAPK kinases (MAPKK/MEK). These kinases in turn are themselves activated by upstream MAPK kinase kinases (MAPKKK/MEKK), whose activation appears to be regulated by members of the Ras superfamily of GTPases. Numerous protein kinases acting as MAPKKK/MEKK components of the MAPK

Manuscript received June 4, 1999; revised March 31, 2000; accepted for publication April 26, 2000.

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Abbreviations: DLK, dual leucine zipper bearing kinase; GFP, green fluorescent protein; IRS, inner root sheath; MAPK, mitogen-activated protein kinase; MLK, mixed lineage kinase; MUK, MAPK-upstream kinase; ZPK, leucine-zipper protein kinase.

cascade have been identified and characterized so far. These include, for example, Raf, MAPK/ERK kinase kinases (MEKK), germinal center kinase (GCK), transforming growth factor β-activated kinase (TAK), tumor progression locus-2 (Tpl-2), and the mixed lineage kinases (MLK) (Fanger *et al*, 1997). The MLK form a group of five distinct serine/threonine kinases that have in common structural characteristics unique among the protein kinase family, namely a catalytic domain hybrid between those found in serine/threonine and tyrosine kinases, and two leucine/isoleucine zipper motifs. In addition to these hallmark features, most members of the MLK family share the ability to activate preferentially the JNK/SAPK pathway when overexpressed in cultured cells (Tibbles *et al*, 1996; Hirai *et al*, 1997). One of the MLK family member is ZPK/DLK/MUK, DLK and MUK being, respectively, the murine and rat homolog of human ZPK (leucine-Zipper Protein Kinase). As MLK have been only recently identified, little is known about their mechanisms of activation and their physiologic roles (Dorow *et al*, 1993; Bergeron *et al*, 1997; Fanger *et al*, 1997).

The aim of this study was to investigate the relationship existing between ZPK expression and differentiation. We analyzed its pattern of distribution in normal human skin, a multilayered structure in which cell proliferation and cell differentiation take place in spatially restricted compartments (Holbrook and Wolff, 1987; Watt, 1989; Fuchs, 1990; Dale *et al*, 1994). Our results of ZPK localization reveal a very precise pattern of expression in human skin epithelia as both mRNA and protein are specifically restricted to the granular layer of epidermis and inner root sheath (IRS) of hair follicles. We also report that ZPK expression is detected in reconstructed human skin that have been cultured at the air–liquid interface but not in keratinocytes cultured in the monolayer. In these monolayer cultures, the overexpression of ZPK leads to a proliferation arrest in all ZPK-expressing cells and to an increase in filaggrin expression in the majority of them. Collectively, our data support an active role for this kinase in the terminal differentiation of epidermal keratinocytes.

MATERIALS AND METHODS

Tissues and cell culture The biopsies included: newborn (24 h) and children (2 and 4 y) foreskin, child finger (8 y), adult scalp (53 y) and trunk (16 and 25 y) skin. Specimens of skin samples and reconstructed skin were embedded in OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen and stored at −70°C until use. Normal human keratinocytes (newborn foreskin, child finger) were isolated and cultured on a feeder layer of irradiated mouse 3T3 fibroblasts (Green *et al*, 1979; Germain *et al*, 1993). Swiss 3T3 cells and adult human dermal fibroblasts were cultured in Dulbecco's modified Eagle medium (DME) containing 10% fetal bovine serum and antibiotics as described (Berthod *et al*, 1997). Cultured cells were grown on glass coverslips, ethanol-fixed for 10 min at −20°C and processed for immunocytochemistry or grown on Petri dishes for protein extraction.

Immunohistochemical staining Acetone-fixed frozen sections (4 μm thick) of human tissues or ethanol-fixed cultured cells were analyzed by indirect immunofluorescence as described (Michel *et al*, 1996). The primary antibodies used include: a rabbit anti-serum directed against the N-terminal portion (223 amino acids) of recombinant mouse ZPK (Douziech *et al*, 1999), and mouse monoclonal antibodies against involucrin (Sigma, Oakville, Canada), filaggrin, and transglutaminase (BTI, Stoughton, MA) proteins. The goat secondary antibodies used were: tetramethylrhodamine isothiocyanate conjugated anti-rabbit and anti-mouse (Chemicon, Temecula, CA). Negative controls consisted of omission of the primary antibody during the labeling reaction. Cell nuclei were labeled with Hoechst reagent 33258 (Sigma).

Protein extraction and immunoblotting Swiss 3T3 and dermal human fibroblasts were cultured until confluence or 3 wk after confluence, respectively. Cells in 100 mm Petri dishes were lyzed on ice in 1 ml of lysis buffer [phosphate-buffered saline containing 1% Triton and protease inhibitor cocktail (Roche Diagnostics, Laval, Canada)]. Human epidermis was obtained from breast skin incubated in thermolysin solution (500 μg per ml in 10 mM HEPES and 1 mM $CaCl_2$) overnight at 4°C (Germain *et al*, 1995). Epidermis was separated from dermis with forceps, rinsed in phosphate-buffered saline and cut in 1 ml of lysis buffer. All these extracts were incubated 30 min in lysis buffer at 4°C under agitation, and centrifuged 15 min at 12,000 × *g*. The concentration of total proteins in the supernatants was measured using the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL). Proteins (35 μg) from cultured S3T3, cultured fibroblasts, or fresh epidermis were separated under reducing conditions on a 7.5% sodium dodecyl sulfate–polyacrylamide mini-gel according to Laemmli (1970). Proteins were transferred on to Hybond-C extra nitrocellulose membrane (Amersham Pharmacia Biotech, Québec, Canada) by electroblotting. Nitrocellulose membrane was blocked overnight at 4°C with 5% nonfat dry milk in a 10 mM Tris solution at pH 8.0 containing 0.9% NaCl and 0.05% Tween 20 (TBST), and then incubated with ZPK anti-serum (1/2000 in TBST, 1% milk) for 1 h (Douziech *et al*, 1999). After washing, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG (Sigma, 1:4000) and detection done using the ECL system (Amersham) and Scientific Imaging Film (Kodak, Rochester, NY). In addition, adsorption of the rabbit anti-serum has been done by incubation with recombinant glutathione S-transferase-ZPK coupled to glutathione Sepharose beads (100 μg), 2 h at 4°C. After centrifugation, the supernatant was used for western blotting.

Production of reconstructed skin *in vitro* Reconstructed skin was produced as described (Michel *et al*, 1999). Briefly, human fibroblasts were cultured for 35 d in medium supplemented with 50 μg per ml of ascorbate (Sigma). The resulting sheets of cells and extracellular matrix were superimposed and formed the dermal equivalent on which human newborn foreskin keratinocytes were seeded. These reconstructed skin were cultured for 8 d in submerged conditions and then raised at the air–liquid interface for 21 d in order to achieve terminal differentiation (Pruniéras *et al*, 1983).

***In situ* RNA hybridization** *In situ* hybridization experiments were performed essentially as described previously (Blouin *et al*, 1996; Nadeau *et al*, 1997) using digoxigenin-labeled sense and anti-sense riboprobes generated from linearized pBluescript KS^+ plasmid (Stratagene, CA) containing a 192 bp fragment of the murine ZPK cDNA (Blouin *et al*, 1996). Immunologic detection of the hybridized probes was performed with alkaline phosphatase-conjugated anti-digoxigenin antibody and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate color substrates (Roche Diagnostics).

ZPK overexpression in cultured keratinocytes

Cell proliferation assay Human foreskin keratinocytes (8000 per cm^2) were seeded with irradiated 3T3 fibroblasts (20,000 per cm^2) in 60 mm Petri dishes and cotransfected after 2 d (40% confluence) with 0.5 μg of pEGFP-N1 plasmid (Clontech, Palo Alto, CA) and 4.5 μg of a pcDNA3 (Invitrogen, Carlsbad, CA) expression vector containing the entire coding region of mouse ZPK cDNA (Douziech *et al*, 1999). Keratinocytes transfected with 0.5 μg of pEGFP-N1 plasmid alone were used as controls. Transfection of the expression plasmids was done using Superfect Reagent (Qiagen, Missisauga, Ontario, Canada) according to the manufacturer's protocol. After a 4 h incubation with the transfection mixture, the medium was replaced with 5 ml of fresh medium. Twenty-four hours later, cells were trypsinized and plated on glass coverslips (20,000 cells per cm^2). Cells were fixed with 4% formol–100% methanol 24 h, 48 h, 72 h, 96 h, or 120 h after transfection (four coverslips at each time point). Cells were immunostained by incubating with a rabbit anti-ZPK anti-serum (Douziech *et al*, 1999). followed by tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit secondary antibody (Chemicon). Negative controls and nuclei labeling were performed as described above. To evaluate if keratinocytes expressing ZPK had proliferated, the percentage of colonies containing one or more labeled cells was counted as a function of time after transfection. The experiment was performed twice.

Filaggrin expression Cells (human foreskin keratinocytes) were transfected with 5 μg of either the mouse ZPK pcDNA3 expression construct (Douziech *et al*, 1999) or the pEGFP-N1 plasmid as described above. Transfected cells were processed as mentioned before. Double indirect immunostaining was performed on ZPK transfected cells and nontransfected cells using, first, the rabbit anti-ZPK anti-serum with tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit secondary antibody (Chemicon) and, second, a mouse anti-human filaggrin antibody (BTI, Stoughton, MA) with a fluorescein-conjugated goat anti-mouse secondary antibody (Chemicon). Immunostaining of pEGFP-N1 transfected control cells was performed using the mouse anti-human filaggrin antibody (BTI) and a tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse secondary antibody (Chemicon). Cells were observed and counted by immunofluorescence microscopy. Cell nuclei were labeled with Hoechst reagent 33258 (Sigma).

RESULTS

Localization of ZPK mRNA in human skin To examine the localization of ZPK mRNA in human skin, we performed *in situ* hybridization with either anti-sense or sense digoxigenin-labeled ZPK probes on human newborn foreskin sections. As seen in **Fig 1(*b*)**, ZPK mRNA was exclusively detected in the epidermal portion of the skin. Higher magnification (**Fig 1*c***) of the same section demonstrated that ZPK mRNA expression occurs specifically in keratinocytes of the granular layers of human epidermis. Control hybridization with the ZPK sense probe gave no signal (**Fig 1*a***). Similar localization in the granular layer of epidermis was observed for human adult trunk skin (data not shown).

ZPK protein is detected in extracts of human epidermis To monitor ZPK protein expression in human skin tissues, western blot analysis were carried out using a rabbit anti-serum raised against the N-terminal portion of recombinant mouse ZPK (Douziech *et al*, 1999). In human epidermis extracts (**Fig 2,** *lane 3*), the anti-serum recognized a protein with an apparent M_r of 104,000 that corresponds approximately to the molecular mass (94 kDa) predicted by its cDNA sequence (Reddy and Pleasure, 1994). Therefore, this anti-serum recognizes the human (**Fig 2,** *lanes 2* and *3*) as well as murine (DLK) (S3T3 extracts, **Fig 2,** *lane 1*) form of ZPK. ZPK protein was also detected when long-term cultured human fibroblast extracts were analyzed (**Fig 2,** *lane 2*). The detection of an additional band at 56 kDa for all the extracts is intriguing and to test further the specificity of the antibody, we adsorbed the rabbit anti-serum to recombinant glutathione S-transferase-ZPK protein coupled to glutathione Sepharose beads. Western blotting with the adsorbed anti-serum did not lead to the detection of any band (**Fig 2,** *lane 4*). Reblotting of the same membrane with the nonadsorbed anti-serum revealed the identical pattern of bands (as **Fig 2**, *lanes 1–3*) and led us to conclude that the lower 56 kDa band represents a cleavage product of the ZPK protein. These results demonstrate that the anti-serum was highly specific for human ZPK, and therefore suitable for immunohistochemical localization of the ZPK protein in human skin.

ZPK protein localizes to keratinocytes of the granular layer of human epidermis and IRS of hair follicles Skin from various body sites and different donor ages was examined to localize ZPK protein expression in normal human skin. Staining of newborn foreskin, children finger skin, adult trunk and scalp skin frozen sections with anti-ZPK demonstrates that the protein was strongly expressed by the keratinocytes of the granular layer (**Fig 3*a***), the last nucleated layer of epidermis (**Fig 3*b***). When hair follicles were present, the IRS was strongly labeled (**Fig 3*d***). Some positive cells were also seen in sweat glands and at the openings of sebaceous glands on scalp skin sections (not shown). We compared ZPK expression pattern with those of well known differentiated-related proteins such as involucrin (**Fig 3*j***), filaggrin, and transglutaminase (not shown). ZPK localized to the same suprabasal compartments, namely the granular layer of epidermis and the IRS of human hair follicles (**Fig 3**).

ZPK expression in cultured keratinocytes and reconstructed skin In order to better define the distribution of ZPK in human keratinocytes, we have analyzed its expression pattern in cultured cells. For human keratinocytes, ZPK was not detected when these

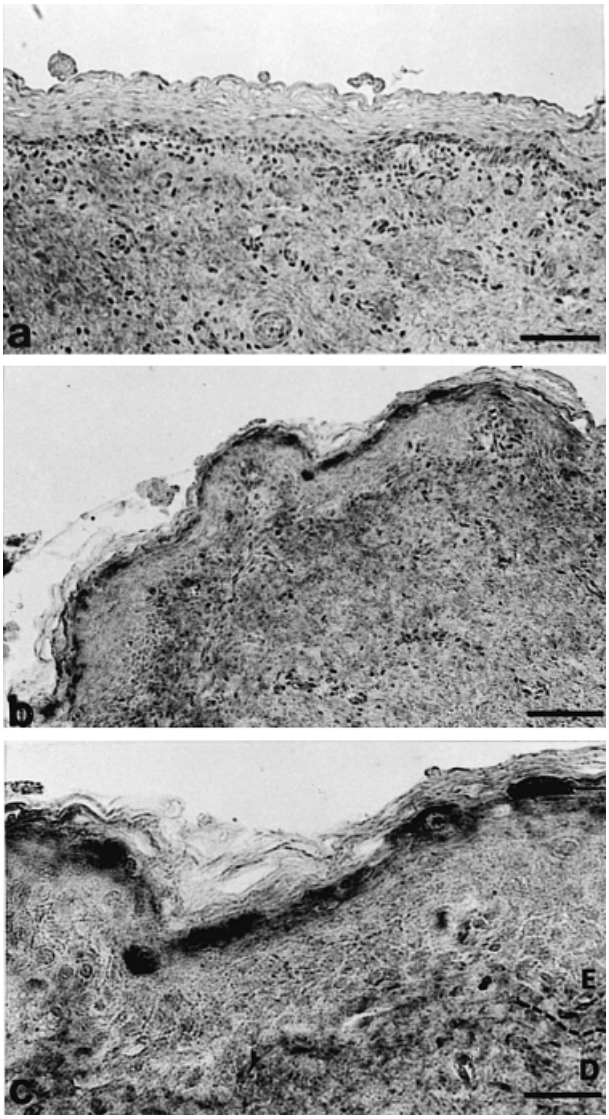

Figure 1. ZPK mRNA is only detected in the differentiated granular layer of human epidermis. *In situ* hybridization of human newborn foreskin sections with ZPK (*a*) control sense probe and (*b*) anti-sense probe. (*c*) Higher magnification of (*b*) showing the presence of *ZPK* mRNA in the granular layer of epidermis. E, epidermis; D, dermis; dotted line, dermo–epidermal junction. *Scale bars*: (*a*, *b*) 85 μm; (*c*) 30 μm.

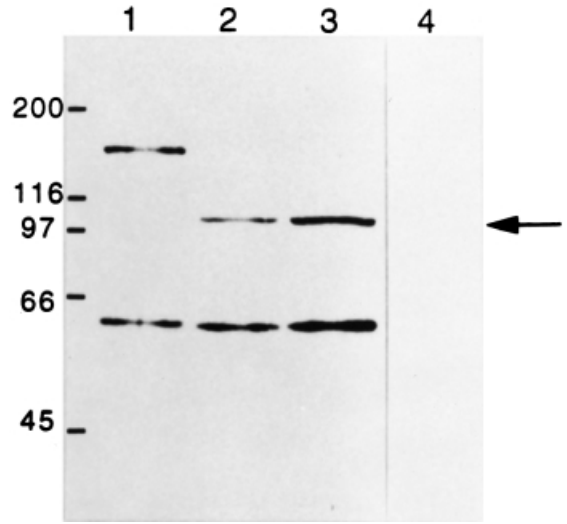

Figure 2. Detection of human ZPK by western blot analysis using a rabbit anti-serum against recombinant mouse ZPK. Protein extracts (35 μg) from (1) cultured mouse S3T3 fibroblasts, (2) cultured human dermal fibroblasts, and (3, 4) fresh human epidermis were separated on a 7.5% sodium dodecyl sulfate–acrylamide gel under reducing conditions; they were immunoblotted using (1–3) an anti-serum directed against the N-terminal portion of recombinant mouse ZPK and (4) the anti-serum previously adsorbed to recombinant ZPK coupled to Sepharose beads.

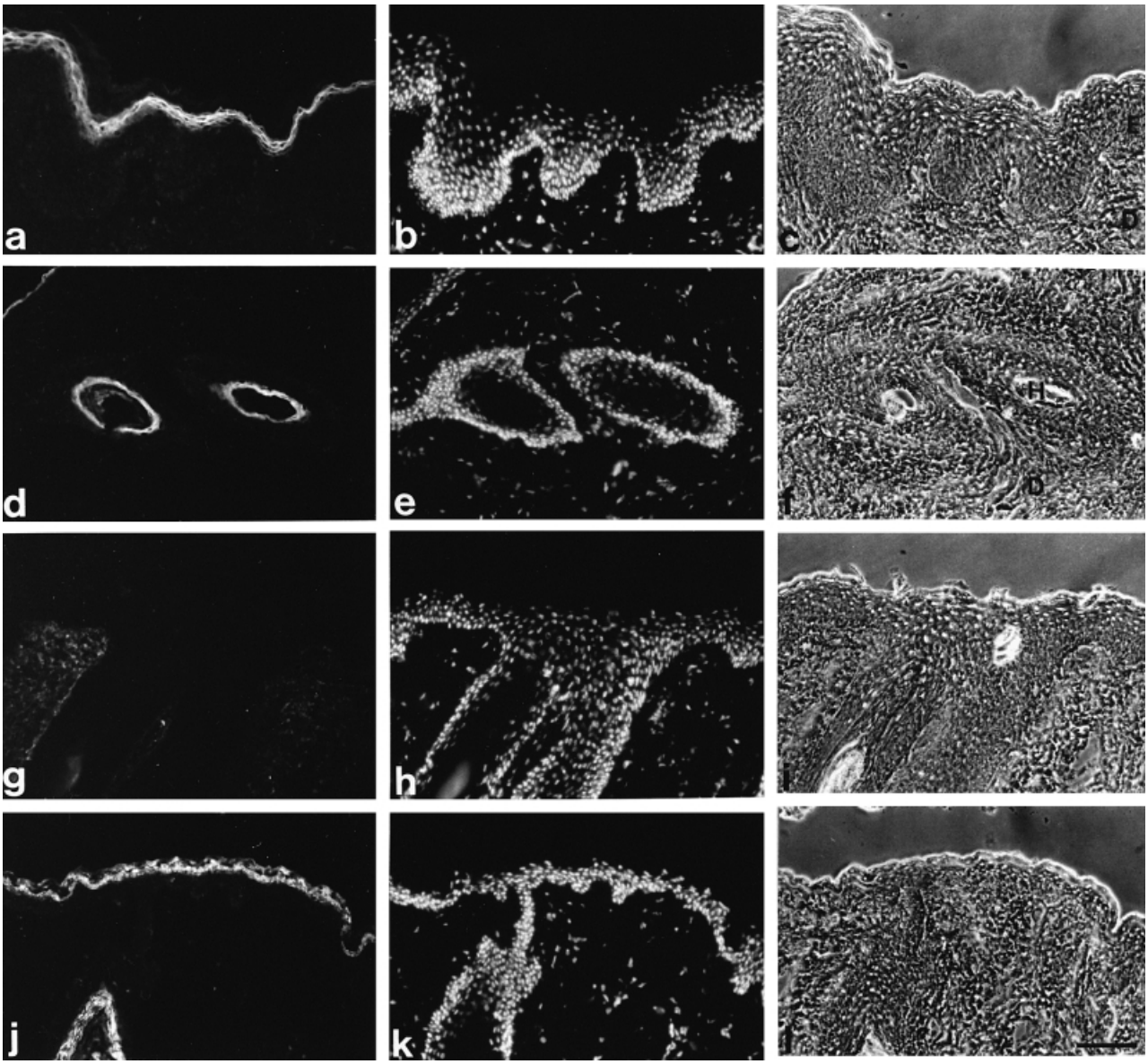

Figure 3. ZPK protein is strongly expressed in the differentiated granular layer of human epidermis and the IRS of hair follicles. Indirect immunofluorescence labelings (*a*, *d*, *g*, *j*) of human adult scalp skin using (*a*,*d*) an anti-serum against ZPK protein showing reactivity within (*a*) epidermis and (*d*) hair follicles. (*g*) Negative control by omission of the primary antibody. (*j*) Labeling for involucrin, a protein expressed in the granular layer. (*b*, *e*, *h*, *k*) Nuclear Hoescht staining and (*c*, *f*, *i*, *l*) phase-contrast micrographs corresponding to (*a*), (*d*), (*g*), (*j*), respectively. E, epidermis; D, dermis; H, hair follicle. *Scale bar*: 90 μm.

cells were cultured as monolayers with a feeder layer of irradiated 3T3 cells (not shown). In contrast, ZPK was detected in cultured human adult fibroblasts and NIH 3T3 cells (data not shown and **Fig 2**, *lanes 1* and *2*). This might indicate a less restricted control of ZPK expression *in vitro* as no labeling of fibroblasts was seen in tissue sections (**Fig 3*a***). To test if ZPK expression could be found again in keratinocytes *in vitro* after the induction of terminal differentiation, we engineered reconstructed skin consisting of foreskin keratinocytes seeded on the top of a reconstructed dermis made up of human fibroblasts secreting their own matrix proteins (Michel *et al*, 1999). After 21 d of culture at the air–liquid interface, the reconstructed skin reached a high level of differentiation, as can be seen by the expression of filaggrin protein (**Fig 4*d***). In this reconstructed skin, ZPK expression was restored and localized to the upper suprabasal layers of epidermis. Even if ZPK expression was not as sharply defined in reconstructed skin (**Fig 4*a***) compared with normal skin (**Fig 3*a***), it appears that gene expression of ZPK is tightly controlled and differentiation related in human keratinocytes under our culture conditions (**Fig 4**).

ZPK overexpression in cultured keratinocytes induces growth arrest and differentiation We took advantage of the absence of ZPK in keratinocytes cultured as a monolayer to evaluate the effect of its overexpression on keratinocyte proliferation and differentiation. The effect of ZPK expression on keratinocyte proliferation was determined by counting the number of ZPK-expressing cells present in each keratinocyte colony at different times after transfection with expression vectors for green fluorescent protein (GFP) and ZPK (**Fig 5**). Cells expressing ZPK after transfection were always single in keratinocyte colonies, indicating that they did not proliferate at all (**Fig 6*a–d***). In contrast, when GFP was expressed alone, the number of GFP expressing cells increased with time (**Fig 5**). Two to 12 cells per colony were present in more than 60% of colonies containing GFP expressing cells 5 d after transfection indicating that GFP-transfected keratinocytes had proliferated (**Fig 6*e–h***). Therefore, ZPK expression in keratinocytes induced a growth arrest. To evaluate if ZPK was involved in keratinocyte differentiation, we examined the percentage of keratinocytes expressing filaggrin

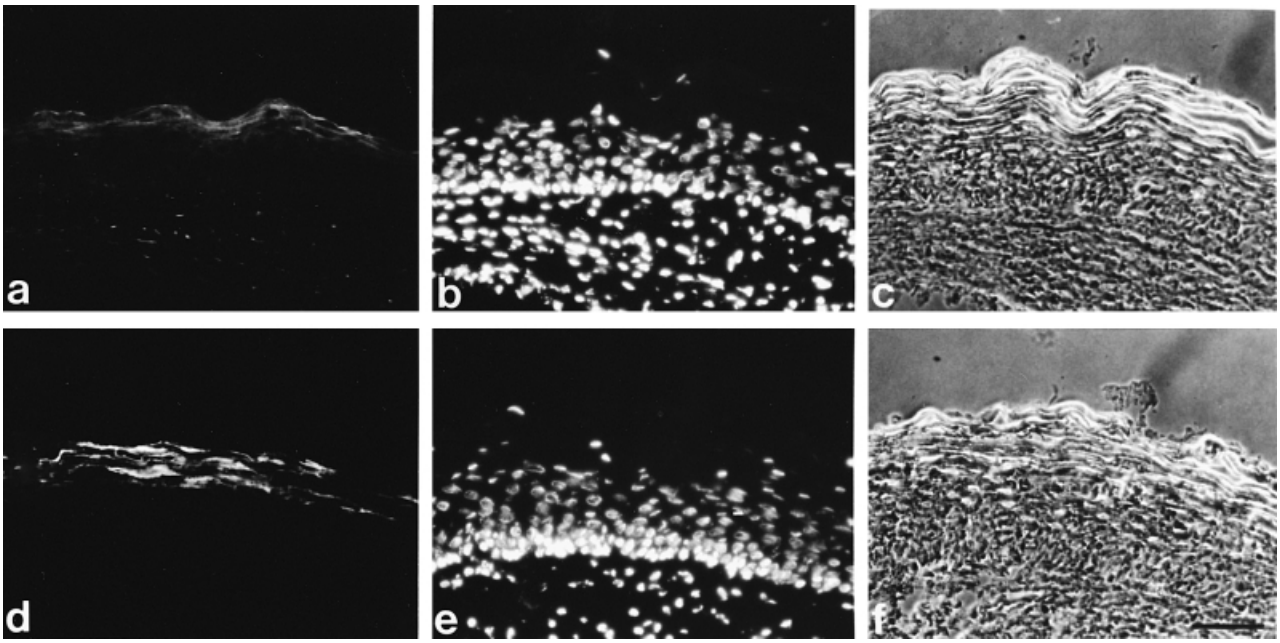

Figure 4. *In vitro* detection of ZPK in suprabasal layers of reconstructed human skin cultured at the air–liquid interface. Indirect immunofluorescence labelings of reconstructed human skin sections using (*a*) ZPK anti-serum and (*d*) an anti-filaggrin antibody. (*b*, *e*) Nuclear Hoescht stainings and (*c*, *f*) are corresponding phase-contrast micrographs of (*a*, *d*), respectively. *Scale bar*: 80 μm.

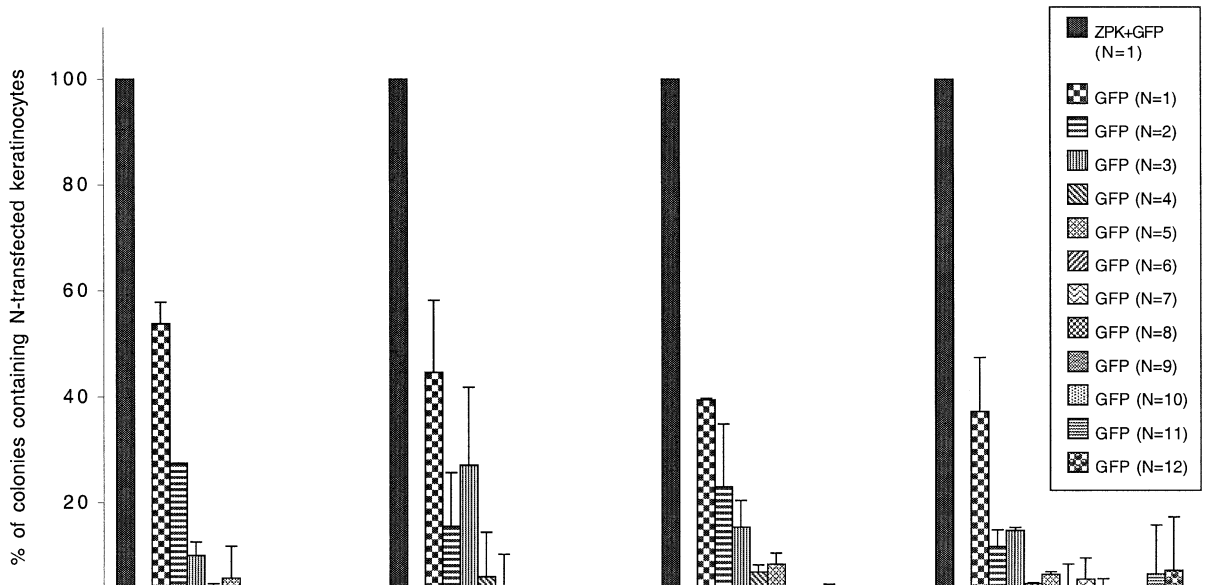

Figure 5. ZPK expression in keratinocytes induces growth arrest. Cells were cotransfected with expression vectors for ZPK and GFP (black column), or GFP alone (controls, white columns) as described in *Materials and Methods*. The histogram represents the percentage of colonies containing N-labeled keratinocytes (N = 1–12) on the total number of colonies containing labeled keratinocytes. Only one column is shown for ZPK-expressing keratinocytes as no colony containing more than one keratinocyte was observed. Values represent the mean ± SD of samples from two independent experiments.

among the cells transfected with expression vectors for ZPK or GFP (control). The number of filaggrin expressing keratinocytes increased with time after transfection of the ZPK construct to reach 53% after 4 d (**Fig 7**). Cells expressing ZPK and filaggrin were large and located on top of the colonies (**Fig 6*i–l***). In contrast, most of the GFP expressing keratinocytes were present in the basal layer of the colony. Moreover, only 4% of keratinocytes expressed filaggrin in these control cells (GFP). Therefore, we conclude that ZPK expression is sufficient to promote keratinocyte differentiation.

DISCUSSION

This study reports on the distribution of the recently identified MLK ZPK in human skin. We took advantage of human epidermis to determine the histologic localization of ZPK. This tissue comprises numerous cell layers (more than 15) in which a strict balance between the proliferation and differentiation processes ensure homeostasis of the epidermal compartment. Keratinocyte proliferation is restricted to cells of the basal layer. The

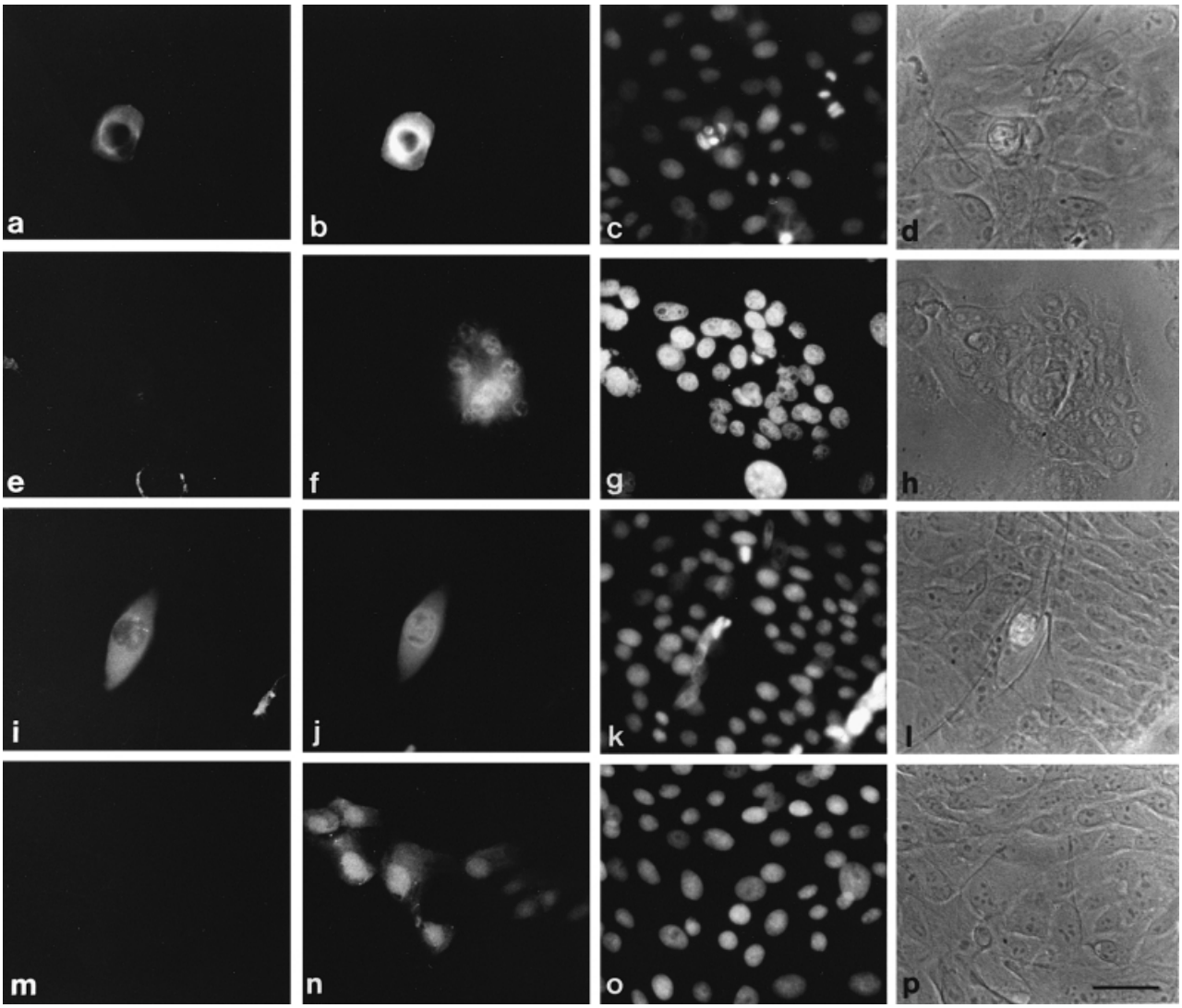

Figure 6. ZPK expression induces keratinocyte differentiation and suprabasal localization. Cells were transfected with expression vectors for either ZPK and GFP (*a–d*), ZPK (*i–l*), or GFP (*e–h*, *m–p*) and labeled by indirect immunofluorescence using (*a*, *e*, *i*) ZPK anti-serum and (*j*, *m*) an anti-filaggrin antibody as described in *Materials and Methods*. (*b*,*f*,*n*) GFP fluorescence. (*c*, *g*, *k*, *o*) Nuclear Hoescht staining and (*d*, *h*, *l*, *p*) are corresponding phase-contrast micrographs of (*a*, *e*, *i*, *m*), respectively. *Scale bar*: 50 μm.

differentiation process occurs as the keratinocytes migrate upwards successively through the spinous, granular, and cornified cell layers (Holbrook and Wolff, 1987; Watt, 1989; Fuchs, 1990; Dale *et al*, 1994). Our results show that ZPK mRNA as well as protein is restricted to the granular layer of the epidermis, the highest nucleated cell layer. In the hair follicle, it is also expressed in a terminally differentiated layer: the IRS.

Although ZPK mRNA expression has previously been detected by northern blots in human tissues such as brain, kidney, skeletal muscle, and lung (Reddy and Pleasure, 1994), this is the first precise localization of ZPK at the mRNA and protein levels in human skin epithelia. Comparatively to ZPK for which little is known, its murine homolog DLK is better characterized. Analysis of fetal and adult mouse tissues demonstrated that DLK mRNA is expressed in postmitotic cells of brain, and of various organs relying on epitheliomesenchymal interactions during development, including intestine and kidney (Blouin *et al*, 1996; Nadeau *et al*, 1997). The results reported here for ZPK mRNA are consistent with the detection of DLK mRNA in mouse skin tissues. DLK mRNA is expressed from embryonic day 15.5 and onwards, and is restricted to the differentiated granular layer of murine epidermis and IRS of whisker follicles (Nadeau *et al*, 1997). The overall expression pattern of DLK mRNA in a variety of specialized cells led to the hypothesis that this kinase might be an important mediator in signaling pathways devoted to the regulation of cell growth and differentiation.

Using a specific rabbit anti-serum (Douziech *et al*, 1999), we extended the findings to the precise immunolocalization of ZPK protein in human skin from various anatomic sites and donor ages as well as for reconstructed human skin. The restricted distribution of both ZPK mRNA and protein in such differentiated cells of the granular layer of epidermis and IRS of hair follicles strongly suggest that this kinase plays an active role in the late stages of terminal differentiation. A few other examples of such mRNA synthesis in the granular layer have been reported, namely for the cytochrome P450 enzyme CYP2B19 in mouse skin (Keeney *et al*, 1998), filaggrin (Dale *et al*, 1994; Nirunsuksiri *et al*, 1995), and the transcription factor c-jun (Briata *et al*, 1993; Welter and Eckert, 1995) in normal human skin. The latter is of particular interest in the context of this study as c-jun is one of the final target of the JNK signal transduction cascade and DLK/MUK has been shown to be an upstream activator of this pathway (Fan *et al*, 1996; Hirai *et al*, 1996). c-jun is a member of the AP-1 family of transcription factors known to play a central role in the regulation of the expression of keratinocyte genes such as involucrin, loricrin, profilaggrin, and transglutaminase 1 (Jang *et al*, 1996; for review see

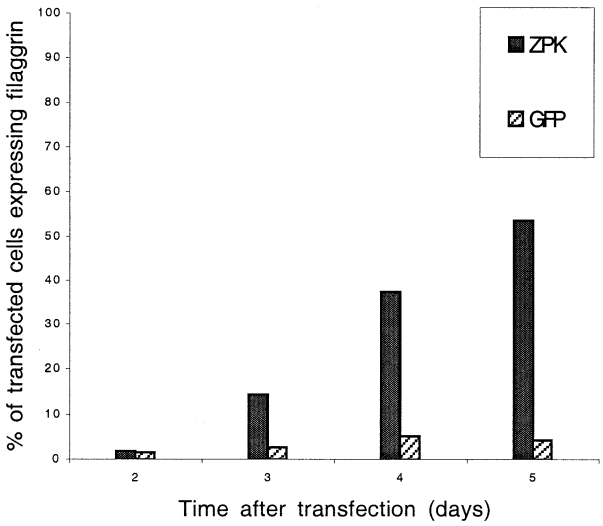

Figure 7. ZPK expression induces keratinocyte differentiation. Cells were transfected with expression vectors for ZPK or GFP as described in *Materials and Methods*. The histogram represents the percentage of keratinocytes transfected with ZPK (black column), or GFP (controls, white column) that coexpresses filaggrin.

Eckert *et al*, 1997). As a member of the MLK family of protein kinases and considering this concomitant distribution of ZPK and c-jun in the granular layer, ZPK is likely to be involved in the JNK signal transduction network as well.

The results obtained with the transfection experiments demonstrate that ZPK expression is sufficient to stop growth and induce keratinocyte differentiation. Consequently, it is not surprising that its expression is restricted to the granular layer of the epidermis. Indeed, the keratinocyte terminal differentiation process must be very tightly controlled as it is an irreversible pathway that culminates in the formation of anucleated cell layers, which insure the functional barrier of the skin. If ZPK is a key regulatory element signaling the entrance in terminal differentiation, its expression must be controlled to avoid problems of skin homeostasis that would result from an excessive number of cells entering the late stages of terminal differentiation.

In conclusion, we have established the precise localization of ZPK mRNA and protein *in situ* in normal human skin and *in vitro* in reconstructed skin. These informations are particularly valuable for the characterization of this kinase and perhaps even of other MLK family members. Of particular interest in this study are the results of the transfection-based experiments, which demonstrate a causative role for ZPK in the induction of terminal differentiation. From its localization in skin epithelia and its ability to induce differentiation, it is tempting to speculate that ZPK could exert a putative role in mechanisms regulating apoptosis, degradation of nuclei, or lipid organization. Recent reports on the intracellular localization of ZPK on the cytoplasmic face of the Golgi apparatus in NIH3T3 cells raise also the possibility that this protein kinase is involved in the processing of intracellular lipid and protein transport (Douziech *et al*, 1999). Although there is no experimental evidence at present, the presence of ZPK in the granular layer where components of the cornified envelope are assembling with lipids is in agreement with such an hypothesis. Upcoming studies addressing the targets of ZPK kinase activity will surely help us to elucidate the precise part played by this protein that induces terminal differentiation of epidermal keratinocytes.

We are grateful to Drs Gabriel Gosselin (Jeffery Hale), Alphonse Roy, Félix-André Têtu (Hôpital du Saint-Sacrement, Québec) and Louise Caouette-Laberge (Hôpital Sainte-Justine, Montréal) for providing skin specimens. We also thank Eric Grandbois and Claude Marin for technical and photographic assistance, respectively.

This work was supported by Medical Research Council of Canada to L.G. and National Science and Engineering Research Council of Canada to R.B. L.G. is recipient of a scolarship from the Fonds de la Recherche en Santé du Québec, J.F. is recipient of a studentship from the Medical Research Council of Canada.

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