# The Mitogen-activated Protein Kinase Kinase Kinase Dual Leucine Zipper-bearing Kinase (DLK) Acts as a Key Regulator of Keratinocyte Terminal Differentiation*

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In the skin, epithelial cells undergo a terminal differentiation program leading to the formation of the stratum corneum. Although it is expected that the last phases of this process must be tightly regulated since it results in cell death, the signaling pathways involved in this induction remain ill defined. We now report that a single kinase, the mitogen-activated protein kinase kinase kinase dual leucine zipper-bearing kinase (DLK), acts in the epidermis to promote the terminal differentiation of human keratinocytes. In support of this notion, we showed that DLK expression was restricted to the granular layer in situ. In addition, cultured keratinocytes infected with a recombinant adenovirus expressing DLK exhibited morphological and biochemical changes, including a suprabasal localization, altered cell shape, compacted cytoplasm, DNA fragmentation, and the up-regulation of filaggrin, that are reminiscent of a terminally differentiated phenotype. Moreover the expression of wild-type DLK in keratinocytes stimulated transglutaminase activity and the consequent formation of the cornified cell envelope, while a kinase-inactive variant of DLK did not. Together these results identify DLK as a signaling molecule implicated in the regulation of keratinocyte terminal differentiation and cornification.

The epidermis is a self-renewing stratified epithelium mainly populated by keratinocytes that undergo a complex and dynamic program of terminal differentiation throughout life (1, 2). This process begins when proliferating keratinocytes of the innermost basal layer cease to divide and initiate their migration successively through the spinous, granular, and cornified cell layers (1, 3). Upon moving upward to the suprabasal layers of epidermis, the keratinocytes progressively acquire the ability to express in a sequential manner specific gene products that are required for differentiation. For instance, the expression of keratin 1 and keratin 10 starts in the spinous layer, whereas that of filaggrin, loricrin, cystatin A, and small proline-rich protein is primarily detected in the granular layer (2, 4, 5). The transition of cells from the upper granular to the cornified layer is accompanied by the elimination of all organelles (including the nuclei) and the activation of transglutaminases, which are essential for the assembly of an insoluble barrier structure termed the cornified cell envelope. Ultimately these events culminate in the production of the outermost epidermal layers, composed of flattened, enucleated, dead cornified cells (corneocytes) that ensure the skin barrier function (2).

The ability of keratinocytes to differentiate is regulated by a number of biological signals derived from cell-cell or cell-matrix interactions that act through downstream signaling pathways (6). Among the pathways that are particularly important for the regulation of keratinocyte growth and differentiation are those involving phosphorylation cascades that lead to the activation of mitogen-activated protein kinases (MAPKs).[1] In mammals, the MAPKs are classified into at least three distinct subfamilies of related polypeptides referred to as the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 kinases (7). The activation of these proteins in response to extracellular stimuli is catalyzed by specific upstream protein kinases known as MAPKKs, which are themselves substrates for a further upstream group of protein kinases, the MAPKKKs. Once activated, MAPKs transit from their primary cytoplasmic subcellular location to the nucleus where they phosphorylate transcription factors, which in turn modulate the expression of prescribed sets of down-

[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; AdDLK, recombinant adenovirus expressing the green fluorescent protein together with a T7 epitope-tagged form of wild-type DLK; GFP, green fluorescent protein; AdGFP, recombinant adenovirus expressing the green fluorescent protein; DLK, dual leucine zipper-bearing kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; NHK, normal human keratinocyte; TG1, transglutaminase 1; TMR, tetramethylrhodamine; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; MKK, MAPK kinase; m.o.i., multiplicity of infection; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

stream effector genes (7). Although some exceptions can be found in the literature, it is generally observed that ERK signaling in keratinocytes is linked to proliferation and survival (8), whereas activation of the JNK and p38 cascades mediates prodifferentiation and proapoptotic responses (9). Evidence supporting the involvement of JNK in keratinocyte differentiation derives from the findings of Takahashi *et al.* (10), who have shown by immunohistochemistry on normal human epidermis that the active phosphorylated form of JNK is present in keratinocytes of the upper spinous and granular layers. Moreover, in studies carried out *in vitro* with cultured normal human keratinocytes, these authors demonstrated that the expression of cystatin A, a key marker of differentiation, requires the activation of JNK (11). Similarly experiments with cultured keratinocytes have also highlighted the importance of p38 MAPK signaling in the terminal differentiation of these epidermal cells. It has been proposed that a specific isoform of p38, named p38δ, plays a role in the differentiation of keratinocytes on the basis of the observation that its activation promotes involucrin gene expression (12–14).

In addition to JNK and p38, which might play complementary roles in the control of keratinocyte differentiation, numerous protein kinases acting as MAPKK and MAPKKK components of these signaling pathways have been reported to contribute to various aspects of this process. For example, overexpression experiments in cultured keratinocytes, using either activated or dominant-negative mutants, have shown that the MAPKKs MKK3, MKK6 (13), and MKK7 (15) as well as the MAPKKKs MEKK1 (12) and apoptosis signal-regulating kinase 1 (16) are required for the induction of terminal differentiation markers such as involucrin, loricrin, and transglutaminase 1. Several lines of evidence suggest that DLK as well as its human homolog (also known as human leucine-zipper protein kinase (17)) or its rat homolog (MAPK upstream kinase (18)), a serine/threonine kinase originally isolated from mouse embryonic kidney (19), is another particularly interesting MAPKKK that could also be implicated in regulating keratinocyte differentiation. This hypothesis is supported by the finding that DLK mRNA and protein are specifically expressed in the differentiated granular layer of human epidermis and in the inner root sheath of hair follicles (20), which possess histological and histochemical characteristics comparable to those of keratinizing epidermal cells (21). In addition, the transient expression of wild-type DLK by the transfection of normal human keratinocytes cultured on plastic is associated with the detection of filaggrin in these cells (20), suggesting that DLK may play an active role in the mechanisms that regulate the terminal differentiation of epidermal keratinocytes.

In an attempt to define more precisely the role of DLK in keratinocyte terminal differentiation, we extended the characterization of its expression *in situ* with normal human skin. Moreover cultured normal human keratinocytes were infected with an adenovirus vector expressing the wild-type form of DLK as a complementary model system to investigate the consequences of its expression on the late stages of terminal differentiation. Our results indicated that the expression of DLK in human skin was tightly linked to late epidermal differentiation. In addition, our results demonstrated that the expression of DLK induced phenotypic changes associated with keratinocyte terminal differentiation including the up-regulation of filaggrin, DNA fragmentation, the activation of transglutaminases, and the formation of a cornified cell envelope. Collectively these data demonstrated that DLK plays the role of an important regulator of the physiological events occurring during the final stage of the keratinocyte terminal differentiation program.

## EXPERIMENTAL PROCEDURES

*Tissue and Cell Culture*—Human newborn foreskin biopsies were embedded in OCT compound and stored at −80 °C. Normal human keratinocytes (NHKs) were isolated and cultured from a newborn foreskin as described previously (20). Briefly cells were cultured on a feeder layer of irradiated mouse 3T3 fibroblasts in Dulbecco's modified Eagle's medium with Ham's F-12 medium in a 3:1 proportion (Invitrogen) supplemented with 24.3 $\mu$g/ml adenine (Sigma), 5% fetalclone II (Hyclone, Logan, UT), 5 $\mu$g/ml insulin, 0.4 $\mu$g/ml hydrocortisone (Calbiochem), $10^{-10}$ M cholera toxin, 10 ng/ml epidermal growth factor (Austral Biologicals, San Ramon, CA), and antibiotics (100 IU/ml penicillin G and 25 $\mu$g/ml gentamicin).

*Immunological Staining*—Acetone-fixed (10 min at −20 °C) frozen sections (5 $\mu$m thick) of human tissues or formaldehyde (4%, 20 min at room temperature)-methanol (100%, 10 min at −20 °C)-fixed cultured cells were analyzed by indirect immunofluorescence as described previously (22). The primary antibodies were a rabbit antiserum directed against the N-terminal portion (223 amino acids) of recombinant mouse DLK (23) and mouse monoclonal antibodies against filaggrin, human transglutaminase 1 (Biomedical Technologies Inc., Stoughton, MA), and keratin 10 (clone RKSE60, Sanbio, Uden, Netherlands). The anti-DLK specifically recognized the mouse and human DLK proteins (20, 23). Goat anti-rabbit IgG or anti-mouse IgG coupled to fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate was used as secondary antibody (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).

*Adenoviruses and Infection of NHKs*—Recombinant adenoviruses expressing the control green fluorescent protein (AdGFP) alone or together with T7 epitope-tagged wild-type (AdDLK) and kinase-defective (AdDLK(K185R)) DLK were described previously (24). Subconfluent cultures of NHKs were infected with the recombinant adenoviruses at a multiplicity of infection (m.o.i.) of 10 or 50 in Dulbecco's modified Eagle's medium supplemented with 2% (v/v) fetal bovine serum, 0.8 mM L-arginine, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 25 $\mu$g/ml amphotericin B. After 1 h, the medium was replaced, and the cells were incubated for 3 or 5 days before being processed for further analyses.

*Flow Cytometry Analysis*—To evaluate DNA content, cells were fixed with 70% ethanol 72 and 120 h after infection. Fixed cells were then washed with 1% bovine serum albumin in phosphate-buffered saline and incubated with propidium iodide for 15 min. Labeled cells were analyzed with a flow cytometer (BD Biosciences).

*DNA Degradation Analysis*—For DNA laddering, the DNA of infected and non-infected cells was extracted with DNA ladder Suicide Track™ (Oncogene Research) according to the manufacturer's protocol. Extracted DNA was fractionated on a 1.5% agarose gel and visualized by staining with ethidium bromide (Sigma). For the TUNEL assay, cells were grown on glass coverslips, fixed in 4% paraformaldehyde, and permeabilized with 1% Triton 72 h postinfection. The cells were then incubated with a tetramethylrhodamine TUNEL reaction mixture according to the manufacturer's protocol (Roche Diagnostics) and labeled with Hoechst 33258 (Sigma). Labeled cells were visualized by fluorescence microscopy.

*Cornified Cell Envelope Assay*—Cornified cell envelope formation was detected according to the method of Cline and Rice (25). Briefly cells were harvested by trypsinization, treated with 1% sodium dodecylsulfate (Bio-Rad) in phosphate-buffered saline (pH 7.4) containing 20 mM dithiothreitol (Sigma), and boiled for 15 min at 100 °C. 5 $\mu$l of DNase (Calbiochem) were then added to the cell suspension. Detergent- and reducing agent-resistant cornified cell envelopes were visualized by light microscopy and counted in a hemacytometer.

*In Situ Transglutaminase Activity Assays*—Infected cells were incubated 48 h postinfection with 10 $\mu$M tetramethylrhodamine (TMR)-cadaverine (Molecular Probes, Eugene, OR) in culture medium. After a 24-h incubation, the cells were washed gently with phosphate-buffered saline and fixed with acetic acid-ethanol-water (1:49:50) for 20 min. The cells were then washed with ethanol to reduce the background fluorescence. The nuclei of fixed cells were labeled with Hoechst 33258 and observed with a fluorescence microscope. Alternatively cells were labeled for 2 h with 1 mM 5-(biotinamido)pentylamine. The cells were subsequently rinsed twice with phosphate-buffered saline, harvested in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 5 mM EGTA, 1 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml leupeptin, and 1 $\mu$g/ml aprotinin), and sonicated on ice. The protein concentrations in the lysates were determined by the modified Bradford protein assay (Bio-Rad). Transglutaminase activity was quantified by measuring the transglutaminase-dependent
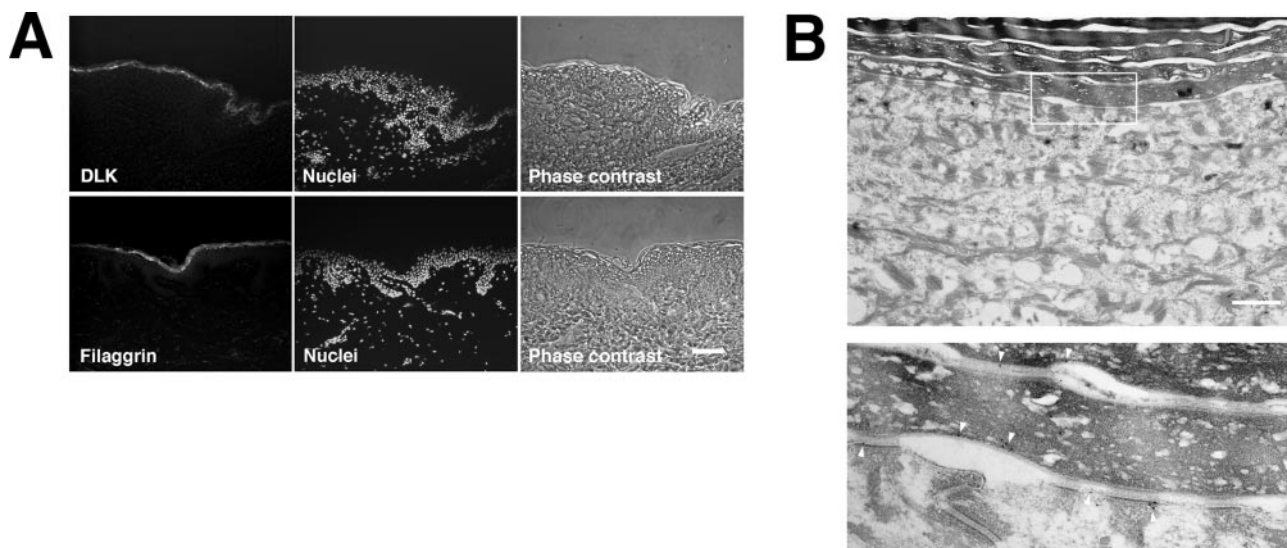
FIG. 1. **Expression of DLK in human epidermis is linked to keratinocyte terminal differentiation.** *A*, immunofluorescence staining of DLK and filaggrin in normal human skin *in situ*. The corresponding nuclear Hoechst staining and phase-contrast micrographs of the same tissue sections are also presented. Note that DLK staining is restricted to keratinocytes of the stratum granulosum. *B*, electron micrographs of normal human skin showing that the immunogold labeling of DLK is preferentially associated with plasma membranes in keratinocytes of the granular layer. The *lower panel* of *B* represents an enlargement of the boxed area in the *upper panel* of *B*. *Arrowheads*, gold particles. *Bars*, 100 $\mu$m in *A* and 1 $\mu$m in *B*.

incorporation of 5-(biotinamido)pentylamine into proteins by a microplate assay as described previously (26).

*Electron Microscopy*—Normal human newborn foreskin biopsies were fixed with 1% paraformaldehyde, 0.1% glutaraldehyde for 20 min. The fixed samples were washed with 0.1 M cacodylate buffer and postfixed with 1% $OsO_4$ for 30 min. The samples were then embedded in LRWhite and cut into thin sections. Briefly the sections were blocked with phosphate-buffered saline-bovine serum albumin and processed for immunogold labeling using a rabbit anti-DLK antibody and gold-conjugated (10-nm particles) goat anti-rabbit secondary antibody (British Biocell International, Cardiff, UK). Sections stained with uranyl acetate and lead citrate were observed with a JEOL 1200 EX transmission microscope.

*Preparation of Cell Lysates and Immunoblotting*—Cells were lysed for 60 min at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml leupeptin, and 1 $\mu$g/ml aprotinin). The lysates were clarified by centrifugation (12,000 $\times$ g for 10 min at 4 °C), and the concentration of total protein in the supernatant fraction was quantified by the modified Bradford protein assay (Bio-Rad). For immunoblotting, equal amounts of proteins were fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Roche Diagnostics) using a semidry transfer apparatus (Amersham Biosciences). The membranes were blocked in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20 containing 5% skim milk powder before addition of the primary antibody (1 h at room temperature or overnight at 4 °C). The primary antibodies were mouse monoclonal anti-T7 (Novagen, Madison, WI), anti-p16 INK4, anti-p21 WAF1, anti-p27 Kip1 (Neomarkers, Fremont, CA), anti-phospho-JNK, anti-phospho-p38, anti-phospho-p44/42 ERK, rabbit polyclonal anti-JNK total, anti-p38 total, anti-p44/42 ERK total (Cell Signaling Technology, Inc., Beverly, MA), and anti-actin (Sigma) antibodies. Immunoreactive bands were detected by enhanced chemiluminescence using secondary horseradish peroxidase-conjugated antibodies (ECL Plus Western blotting kit, Amersham Biosciences).

*Immunocomplex Kinase Assay for DLK*—Cultures of NHKs infected with the adenoviruses were harvested and homogenized in lysis buffer. The lysates were clarified by centrifugation, and the concentration of total protein in the supernatant fraction was quantified using the modified Bradford protein assay (Bio-Rad). Typically 600 $\mu$g of protein extract were incubated for 2 h at 4 °C with constant rotation using a T7 tag monoclonal antibody (Novagen, 1:2000 dilution) and protein A-Sepharose beads. After incubation, the immunocomplexes were washed three times with lysis buffer and three times with kinase buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 1 $\mu$g/ml leupeptin, and 1 $\mu$g/ml aprotinin). Immunocomplex kinase assays were performed by incubating the immune complexes in 40 $\mu$l of kinase

buffer containing 2.5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (Amersham Biosciences), 25 $\mu$M ATP, and 1 $\mu$g of myelin basic protein as a substrate. Following a 20-min incubation at 30 °C, the reaction was stopped by adding an appropriate volume of 6$\times$ SDS-PAGE sample buffer and boiling for 5 min. Phosphorylated proteins were visualized by autoradiography after fractionation by SDS-PAGE.

## RESULTS

*DLK Exhibits a Differentiation-associated Localization in Normal Human Skin Epithelium*—The cellular localization of endogenous DLK in normal human skin was investigated by immunofluorescence microscopy with a specific antiserum raised against the recombinant mouse DLK (23). DLK staining was restricted to the stratum granulosum, the upper suprabasal layers of living epidermis, where filaggrin, a terminal differentiation marker, was also found (Fig. 1*A*). Immunoelectron microscopy analysis of normal human skin confirmed this localization, showing that the immunoreactive protein was preferentially detected on the inner face of the plasma membrane in the keratinocytes of the granular layer (Fig. 1*B*). These results demonstrate that DLK expression is restricted to the most differentiated living keratinocytes in normal human skin.
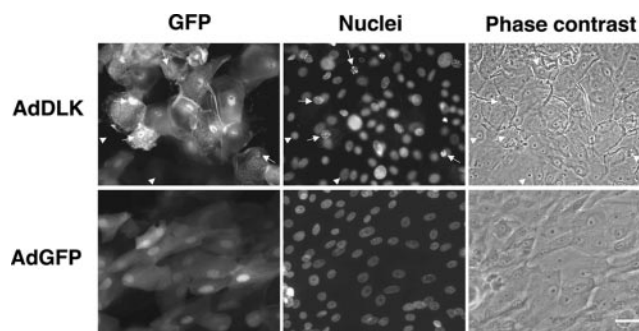
*DLK Mediates JNK Activation in Normal Human Keratinocytes*—To address the possibility that DLK plays a functional role in the late stages of terminal differentiation, we examined the effects of its forced expression using an *in vitro* model of poorly differentiated NHKs (cultured on plastic substrate). For this purpose, NHKs were infected at an m.o.i. of 10 or 50 with recombinant adenoviruses expressing the control green fluorescent protein (AdGFP) alone or together with a T7 epitope-tagged form of wild-type DLK (AdDLK). The expression of DLK in infected cells was confirmed by probing a Western blot of the lysates with the T7 antibody (Fig. 2*A*). As expected, the abundance of DLK in infected cells correlated with the amount of virally transduced cDNAs. The activity of DLK was also determined in the cell lysates by an immunocomplex kinase assay using myelin basic protein as a substrate (Fig. 2*A*). Our results using this assay indicate that the kinase activity of wild-type DLK in infected cells increased in an m.o.i.-dependent manner. In comparison, no activation of DLK was detected in non-infected or AdGFP-infected keratinocytes.

Since DLK is an essential component of the JNK MAPK
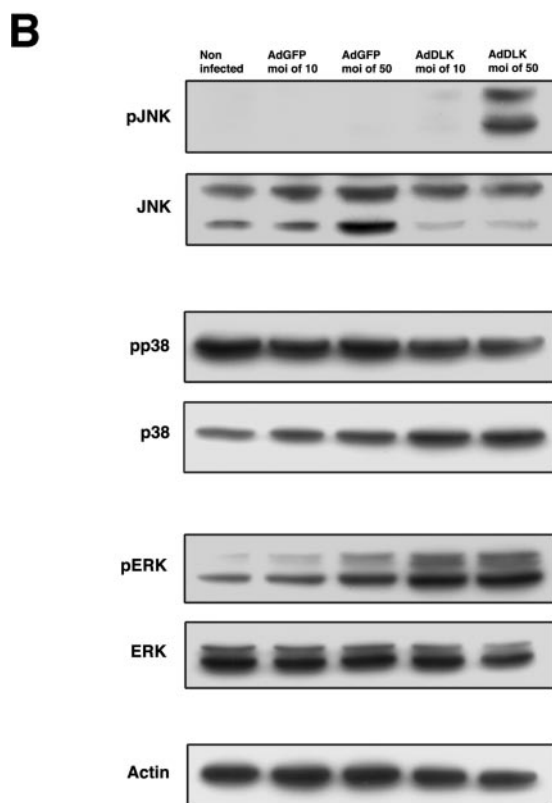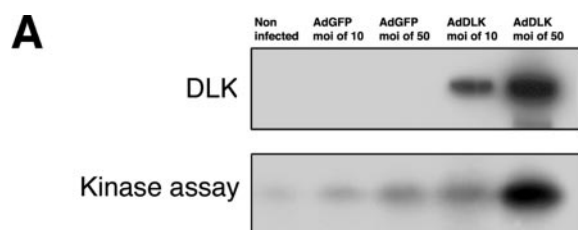
FIG. 2. **Activation of the JNK signaling pathway in normal human keratinocytes infected with an adenovirus expressing DLK.** *A*, NHKs were infected with the recombinant adenoviral vector AdDLK or the control vector AdGFP at an m.o.i. of 10 and 50. Three days after infection, the cells were lysed, and the extracts were subjected to immunoblotting with the T7 antibody and to a DLK immuno-complex kinase assay using the myelin basic protein as a substrate. *B*, NHKs were infected at an m.o.i. of 10 or 50 with AdDLK or AdGFP or mock-infected and processed 3 days postinfection for immunoblotting with antibodies that specifically recognize the phosphorylated and activated forms of JNK (*pJNK*), p38 (*pp38*), or ERK (*pERK*). The total levels of JNK, p38, and ERK in infected cells were also analyzed by Western blots using specific antibodies. γ-Actin was used as a control for protein loading.



FIG. 3. **Morphology of DLK-expressing keratinocytes.** NHKs were infected with AdDLK or AdGFP (m.o.i. = 50), and the morphology of the cells was examined 3 days later by GFP fluorescence (*left panel*) and phase-contrast microscopy (*right panel*). The nuclei were visualized by staining the DNA with Hoechst (*middle panel*). Note that AdDLK-infected NHKs are located in a suprabasal position and present a cornified-like morphology with altered nuclei in which chromatin is condensed (*arrows*). In contrast, their neighboring non-infected NHKs (not GFP fluorescent) remain attached to the plastic (basal position) and do not present these features (*arrowheads*). *Bar*, 50 μm.

pathway (27), we tested whether the activity of endogenous JNK was modulated in cells infected with the adenovirus vectors at an m.o.i. of 10 or 50. JNK activity was determined in the lysates from these cells by immunoblotting experiments with an antibody specific to the phosphorylated and activated form of JNK. Fig. 2*B* shows that DLK expression in keratinocytes infected at an m.o.i. of 50 induced sustained activation of endogenous JNK, while no such effect was observed in keratinocytes infected at an m.o.i. of 10, in mock-infected cells, or in cells infected with AdGFP. Immunoblots processed in parallel with an antibody that is insensitive to the phosphorylation

state of JNK confirmed that the increase in kinase activity was not attributable to variations in JNK protein levels (Fig. 2*B*). In conjunction with these experiments, we also investigated the consequences of DLK expression on p38 MAPK and ERK activity by immunoblotting with phosphospecific and phospho-independent antibodies. The results shown in Fig. 2*B* demonstrate that both p38 and ERK exist as constitutively phosphorylated proteins in intact NHKs cultured under our experimental conditions. When these cells were infected with AdDLK at an m.o.i. of 10 or 50, no effect on the phosphorylation level of p38 was observed, while expression of DLK was found to slightly stimulate the activity of ERK (Fig. 2*B*). Thus, the expression of DLK preferentially stimulates the activity of endogenous JNK in NHKs.

*Expression of DLK in NHKs Induces Morphological Changes Characteristic of Terminal Differentiation*—Microscopic examination of NHKs infected at an m.o.i. of 50 with the adenovirus vectors revealed that DLK expression induces morphological changes associated with terminal differentiation. Indeed 72 h after infection, cells expressing DLK were flattened and located on top of the keratinocyte colonies. In contrast to this suprabasal localization, NHKs infected with AdGFP remained in the basal layer (Fig. 3). DLK expression in NHKs also promoted the formation of cytoplasmic granules (Fig. 3). Interestingly adenovirus-mediated expression of DLK was accompanied by an increase in the number of cells with nuclei exhibiting chromatin condensation as revealed by Hoechst staining (Fig. 3). To complement these observations, we characterized in more detail the effects of DLK expression on DNA by flow cytometry, TUNEL cytochemistry staining, and DNA gel electrophoresis. As seen in Fig. 4*A*, flow cytometric analysis of propidium iodide-stained cells showed that expression of DLK reduced the percentage of NHKs in the $G_0/G_1$ phase by 12% as compared with the control. This reduction was paralleled by a marked increase (14%) in the proportion of cells in the sub-$G_1$ population, indicating that extensive DNA degradation occurred. Consistent with these flow cytometry results, AdDLK induced a significant increase in the number of TUNEL-positive cells compared with AdGFP-infected control cells (Fig. 4*B*). Finally, as predicted from these findings, the DNA laddering analysis revealed that DNA was fragmented in AdDLK- but not in AdGFP-infected cells (Fig. 4*C*). Collectively the results obtained with three different techniques suggest that DLK may contribute to the process of DNA fragmentation that is inherent to the terminal differentiation of keratinocytes.
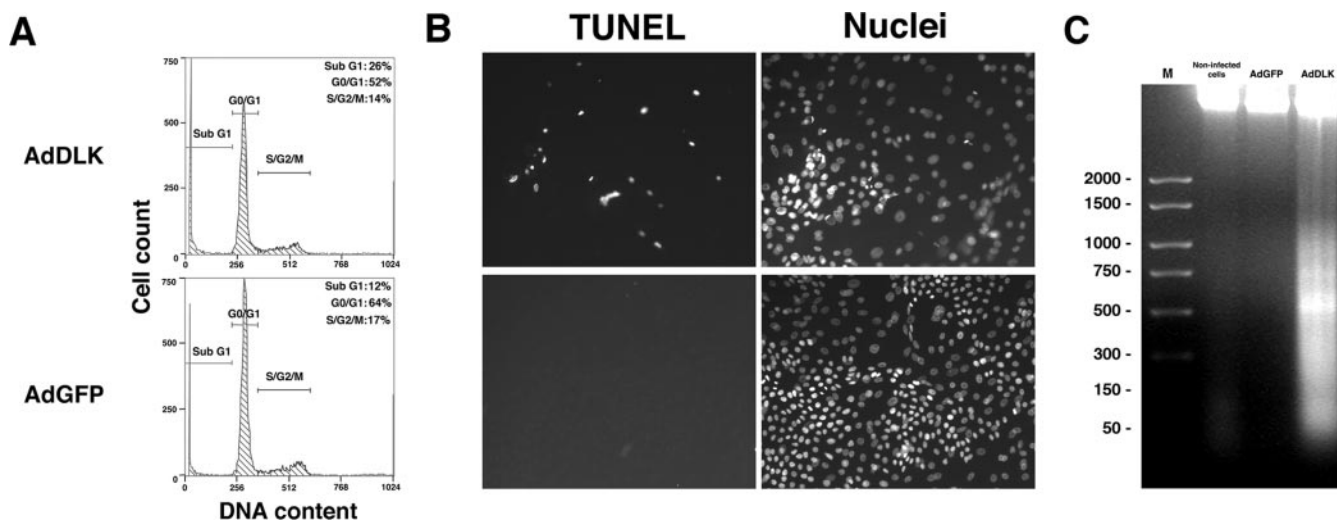
FIG. 4. **DLK expression in keratinocytes induces DNA cleavage.** *A*, NHKs were infected at an m.o.i. of 50 with AdDLK or AdGFP. 3 days after infection, the nuclei were labeled with propidium iodide, and their DNA content was determined by flow cytometry. Values are the average of three independent experiments. *B*, NHKs were infected at an m.o.i. of 50 with AdDLK or AdGFP. After 3 days, cells were fixed and stained for DNA cleavage by the TUNEL assay. The nuclei were visualized by staining the DNA with Hoechst. *C*, NHKs were infected at an m.o.i. of 50 with AdDLK or AdGFP or mock-infected and harvested 3 days postinfection to assess DNA ladder formation. DNA markers (*M*) in bp are shown on the *left*.

*Expression of DLK in NHKs Induces p21$^{cip1/waf1}$ Accumulation*—Considering that the terminal differentiation of keratinocytes is tightly linked to cell cycle withdrawal (28), we next examined the effect of DLK on the expression of the cyclin-dependent kinase inhibitors p16$^{INK4}$, p21$^{cip1/waf1}$, and p27$^{Kip1}$, which have been reported to promote growth arrest in various cell types (29). Lysates of NHKs prepared 3 days after infection with AdDLK or AdGFP were examined by immunoblot analysis with antibodies against the cyclin-dependent kinase inhibitors p16$^{INK4}$, p21$^{cip1/waf1}$, and p27$^{Kip1}$. The immunoblot data shown in Fig. 5 indicated that the expression of DLK caused a marked increase of p21$^{cip1/waf1}$ levels in NHKs, while the expression of p16$^{INK4}$ and p27$^{Kip1}$ remained constant (Fig. 5). Therefore, the expression of DLK in NHKs stimulates the preferential accumulation of the cyclin-dependent kinase inhibitor p21$^{cip1/waf1}$, which is usually induced with growth arrest and differentiation (30).

*Expression of DLK in NHKs Induces Late but Not Early Differentiation Markers*—The terminal differentiation of keratinocytes is a multistep process characterized at the biochemical level by the induction of specific proteins, which are expressed sequentially as cells migrate upward to the skin surface (4). As DLK was found to induce morphological changes associated with NHK differentiation, we next examined the effects of its expression in poorly differentiated NHKs on proteins specific to early and late stages of differentiation, namely keratin 10 and filaggrin. The presence of keratin 10 and filaggrin in NHKs after infection with AdDLK or AdGFP was assessed by immunofluorescence staining with specific antibodies (Fig. 6*A*). Adenovirus-mediated expression of DLK in NHKs did not induce the expression of the early differentiation marker keratin 10. Actually there was no increase in the number of keratin 10-expressing keratinocytes when DLK was expressed (Fig. 6*B*). In contrast, the expression of DLK in NHKs resulted in a 9-fold increase in the number of filaggrin-expressing cells relative to AdGFP-infected controls (Fig. 6*B*), suggesting that DLK causes the preferential induction of late differentiation markers upon expression in NHKs.

*Expression of DLK Enhances Transglutaminase Activity*—To extend our findings on the induction of phenotypic changes characteristic of late differentiation by DLK, we next examined its effects on transglutaminase 1 (TG1), a cross-linking enzyme whose expression and activation are essential for the formation

of the cornified cell envelope (31). To do this, we first sought to analyze the subcellular localization of TG1 and DLK in normal human skin using double immunofluorescence staining. As expected, we observed that endogenous TG1 and DLK were simultaneously expressed by keratinocytes of the granular layer in normal human skin (Fig. 7*A*). Interestingly TG1 co-localizes with DLK at the cell membrane in granular keratinocytes in normal epidermis (Fig. 7*A*).

The co-localization of TG1 with DLK in human skin prompted us to ask whether the expression of DLK in NHKs could stimulate transglutaminase activity. NHKs were therefore infected with AdGFP (controls) or AdDLK and exposed 2 days later to the transglutaminase substrate TMR-cadaverine for 24 h. The incorporation of TMR-cadaverine in infected cells was monitored by fluorescence microscopy analysis. The results presented in Fig. 7*B* demonstrated that adenovirus-mediated expression of DLK in NHKs caused a dramatic increase in TMR-cadaverine incorporation as compared with the control vector. High magnification of the cells stained with TMR-cadaverine after infection with AdDLK also showed a prominent localization of labeled cadaverine at the plasma membrane (Fig. 7*C*), the cell structure where TG1 is primarily found in granular keratinocytes (32). The measurement of *in situ* transglutaminase activity by the incorporation of 5-(biotinamido)-pentylamine into substrate proteins confirmed that DLK expression stimulated transglutaminase activity (Fig. 7*D*). Our findings led us to propose that the expression of DLK in keratinocytes of the granular layer might be important for transglutaminase activation and subsequent cornification.

*Expression of DLK Induces Keratinocyte Cornification*—Since DLK expression caused up-regulation of transglutaminase activity, which is necessary for the formation of the cell envelope and for the maturation of the stratum corneum, we undertook experiments to determine the effect of DLK on the cornification process in NHKs. Cells infected with the adenoviruses were subjected, 3 or 5 days later, to a cornified cell envelope formation assay. Results shown in Fig. 8 reveal that NHKs expressing wild-type DLK were much more likely to undergo cornification than NHKs infected with the adenovirus encoding GFP. In fact, the expression of DLK elevated the relative level of NHK cornification more than 4-fold compared with the levels observed in AdGFP-infected cells, leading us to

FIG. 5. **Expression of DLK in keratinocytes induces p21$^{cip1/waf1}$ accumulation.** NHKs were infected at an m.o.i. of 10 or 50 with AdDLK or AdGFP or mock-infected and harvested 3 days postinfection for Western blot analysis with antibodies against the cyclin-dependent kinase inhibitors p16$^{INK4}$, p21$^{cip1/waf1}$, and p27$^{Kip1}$. As a control for protein loading, immunoblots were probed in parallel with an antibody targeting γ-actin.
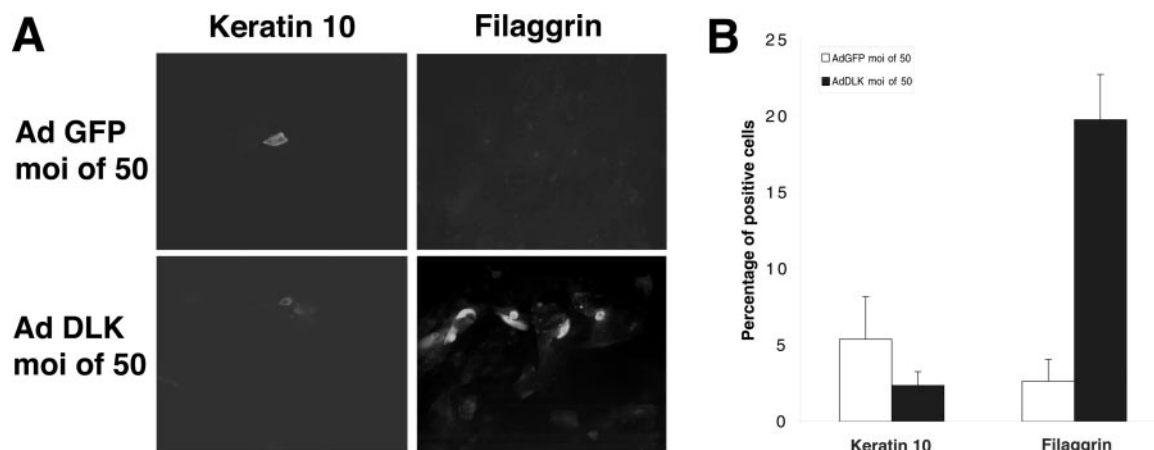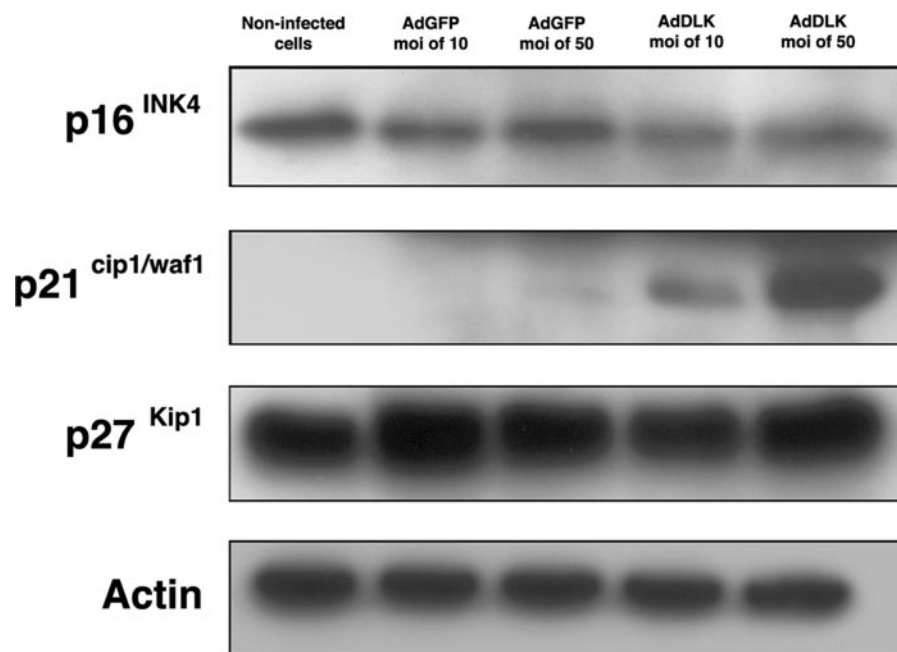


FIG. 6. **Expression of DLK in keratinocytes induces late but not early differentiation markers.** NHKs were infected with AdDLK or AdGFP at an m.o.i. of 50, fixed 3 days later, and stained with antibodies against the "early" keratinocyte differentiation marker keratin 10 or the "late" differentiation marker filaggrin. The expression of GFP, keratin 10, and filaggrin in infected cells was visualized using fluorescence microscopy (*A*). The percentage of GFP-positive cells expressing keratin 10 and filaggrin is shown in the graph (*B*). Values are the average of cell counts in 10 microscope fields from two independent experiments with the standard deviation provided. Note that the expression of DLK in NHKs induces filaggrin but not keratin 10.

conclude that DLK expression has a priming effect on NHK cornification.

*The Kinase Activity of DLK Is Required for Transglutaminase Activation and Induction of NHK Cornification*—Previous results show that the expression of wild-type DLK in NHKs is sufficient to up-regulate transglutaminase activity and induce keratinocyte cornification. To test whether these effects were dependent on DLK catalytic activity, NHKs were infected with an adenovirus expressing a T7 epitope-tagged kinase-defective form of DLK (AdDLK(K185R)) and subsequently analyzed for transglutaminase activity and cornified cell envelope formation as described above. As expected, the introduction of wild-type DLK in NHKs resulted in the activation of transglutaminases. In contrast, the catalytically inactive DLK mutant was unable to induce transglutaminase activation (data not shown), although it was expressed at levels comparable with wild-type DLK (Fig. 9*A*). These results indicate that the catalytic activity of DLK is required for transglutaminase activation. In support of this notion, the cornification analysis demonstrated that the levels of cornified envelope formation in AdDLK-infected cells

were 3–4-fold higher than those occurring in NHKs expressing the DLK mutant (Fig. 9*B*).

DISCUSSION

The terminal differentiation of epidermal cells is a complex multistep program culminating in the formation of the cornified layer. As this process ultimately leads to cell death, it is expected that cornification must be tightly regulated *in vivo* and that its regulators should be expressed in the most differentiated layers of the epidermis. Although our understanding of the mechanisms underlying cornification is still limited, the available data suggest a role for the different MAPK signaling cascades in regulating keratinocyte proliferation, survival, differentiation, and death (9). In the present study, we extended our previous work on the MAPKKK DLK (20) to investigate its potential involvement in the cascade of events leading to terminal differentiation of human epidermal cells. Our results provide strong evidence indicating that this protein kinase is a key regulator of keratinocyte terminal differentiation. This conclusion is supported by the following observations. 1) The
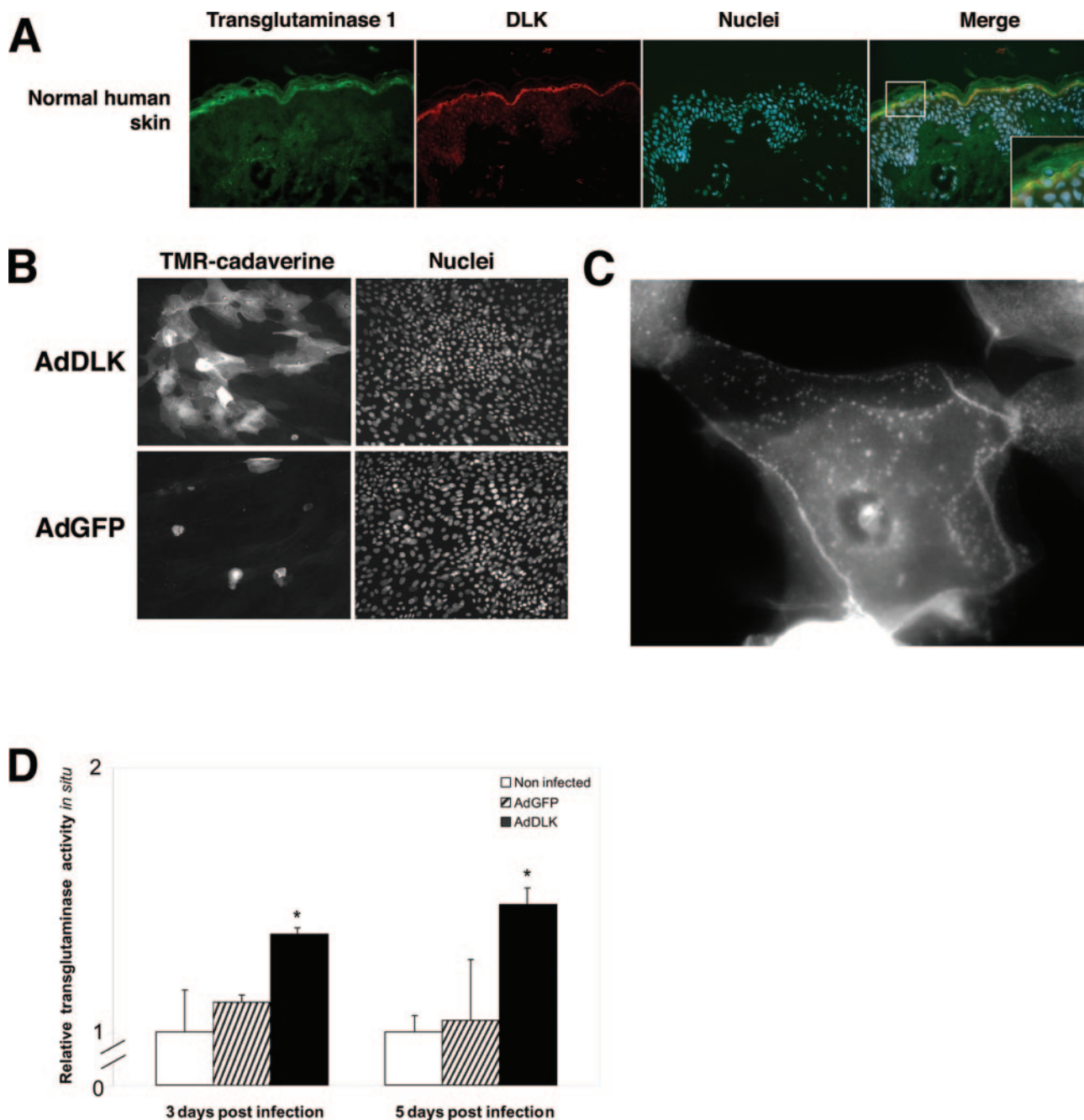
FIG. 7. **Induction of transglutaminase activity in keratinocytes expressing DLK.** *A*, double immunofluorescence staining of TG1 and DLK in normal human skin. The nuclei were visualized by staining the DNA with Hoechst. A merge of the three images shows the co-localization of TG1 and DLK in keratinocytes of the granular layer. The *orange* color indicates a co-localization of both labeling (*green* and *red*). *B*, fluorescence microscopy analysis of TMR-cadaverine incorporation in NHKs 3 days after infection (m.o.i. = 50) with AdDLK or AdGFP. The nuclei were counterstained with Hoechst. Note a stronger cadaverine labeling after DLK infection, indicating that DLK increases transglutaminase activity. *C*, high magnification of AdDLK-infected NHKs stained with TMR-cadaverine showing the preferential localization of transglutaminase activity at the cell periphery. *D*, NHKs were infected with the recombinant adenoviral vector AdDLK or the control vector AdGFP at an m.o.i. of 50. Three or five days after infection, the cells were incubated with 5-(biotinamido)pentylamine, and the incorporation of this polyamine derivative into proteins was used as a measure of *in situ* transglutaminase activity. Spectrophotometry analysis of 5-(biotinamido)pentylamine incorporation into substrate proteins was used to determine the level of transglutaminase activity. Values are the average of two independent experiments performed in triplicate with the mean ± S.D. provided. Transglutaminase activity is given in arbitrary units as -fold increase relative to NHKs infected with the control vector AdGFP. // represents a gap in the scale of the histogram. *, significantly different from the AdGFP control.

expression of DLK in normal human skin, both at the mRNA and protein levels, was confined to the stratum granulosum, the most terminally differentiated viable cell layer of the epidermis. 2) DLK expression in poorly differentiated keratinocytes caused activation of JNK, a subgroup of MAPKs involved in the control of keratinocyte differentiation. 3) The cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$ and the late differentiation marker filaggrin were induced by DLK expression. 4) The

expression of DLK induced DNA degradation, stimulated transglutaminase activity, and increased the formation of cornified cell envelopes.

Our immunohistochemical analyses on normal human epidermis showed that DLK was specifically expressed in granular keratinocytes where it was found in proximity to the epidermal cell membrane. Such a subcellular localization is a common feature of many proteins and lipids involved in corni-
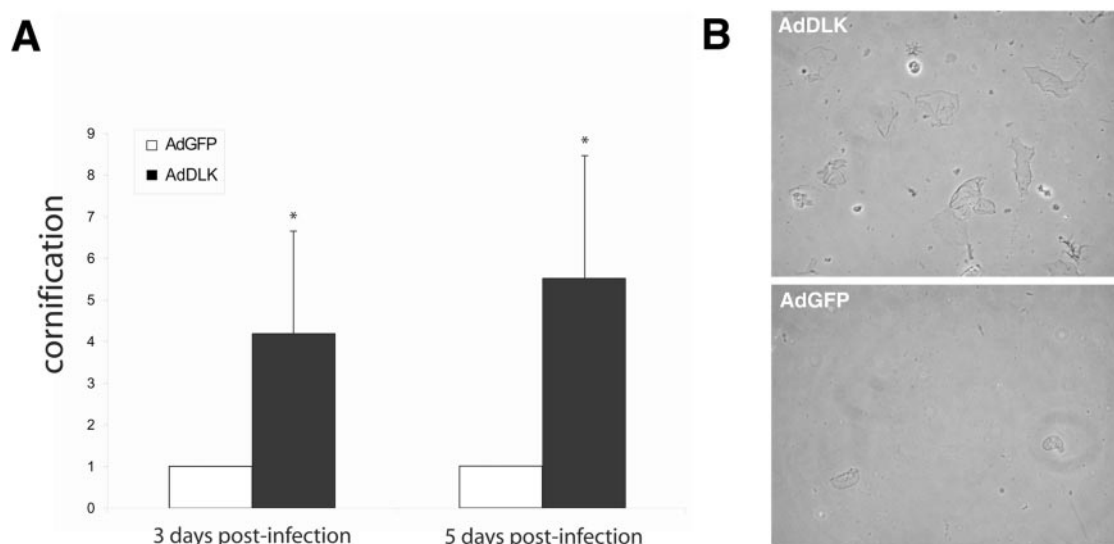
FIG. 8. **DLK expression in keratinocytes induces cornification.** *A*, NHKs were infected at an m.o.i. of 50 with AdGFP or AdDLK. At 3 or 5 days postinfection, cells were harvested and subjected to a cornified cell envelope assay. Values are the average of three independent experiments performed in triplicate with the mean ± S.D. provided. Cornification is given in arbitrary units as -fold increase relative to NHKs infected with the control vector AdGFP. *, significantly different from the AdGFP control. *B*, phase-contrast micrographs of cornified cell envelopes prepared from NHKs 3 days after infection with AdDLK or AdGFP (m.o.i. = 50).
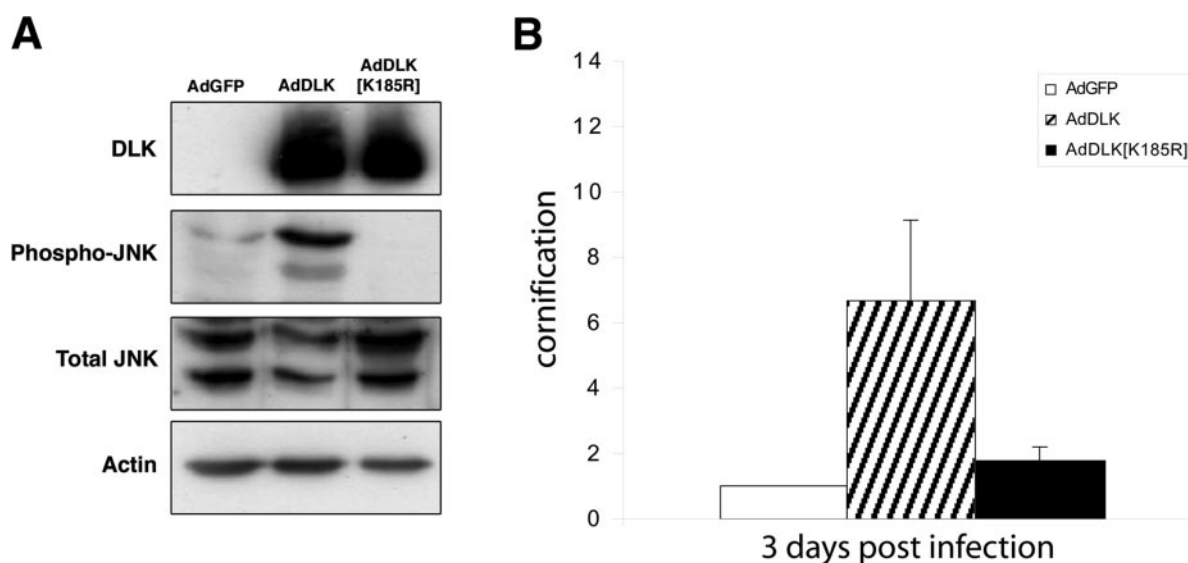


FIG. 9. **The kinase activity of DLK is required for the induction of keratinocyte cornification.** *A*, total proteins isolated from NHKs 3 days after infection were processed for immunoblotting experiments with anti-T7, anti-phospho-JNK, anti-JNK, and anti-actin antibodies. *B*, NHKs were infected at an m.o.i. of 50 with AdGFP, AdDLK, or AdDLK(K185R). At 3 days postinfection, cells were harvested and subjected to a cornified cell envelope assay. Values are the average of two independent experiments performed in triplicate with the mean ± S.D. provided. Cornification is given in arbitrary units as -fold increase relative to NHKs infected with the control vector AdGFP. Note that the increase in cornified cell envelope formation (*B*) induced by AdDLK is not observed when cells express the kinase-defective form of DLK.

fied envelope formation, including transglutaminases and ceramides (33). The modulation of the intracellular calcium concentration may be involved in the regulation of this phenomenon (34–36). Furthermore the protein kinase C$\alpha$ isoform, which is also involved in keratinocyte differentiation, is recruited to the keratinocyte membrane upon activation by calcium (37). The membrane localization of DLK in terminally differentiated keratinocytes of the granular layer is suggestive of a role in cornification since the formation of the cornified cell envelope takes place directly underneath the plasma membrane in these cells (38). Based on its association with the plasma membrane, it is possible that DLK interacts with proteins regulating the assembly of the cornified envelope as discussed below.

*In vitro* studies with cultured normal human keratinocytes have clearly demonstrated that JNK, p38, and many of their upstream activators regulate positively the expression of proteins such as involucrin, loricrin, transglutaminase 1, and cystatin A (11, 16), which are required during the final stage of the terminal differentiation program. The results presented here show that the MAPKKK DLK preferentially stimulated the JNK subgroup of MAPKs upon expression in poorly differentiated human keratinocytes. How DLK activates JNK in these cells is presently unknown. However, biochemical and genetic evidence has demonstrated that the MAPKK MKK7, which serves as a substrate for DLK (39), is essential for JNK activation (40). Immunohistochemistry experiments with normal human epidermis (11) have shown that the active phosphorylated form of JNK is present in the most differentiated cells of the granular layer like DLK and c-Jun (41, 42), the transcription factor through which JNK activation leads to the up-regulation of target genes in a variety of mammalian cells (43).

The observation that DLK, active JNK, and c-Jun are concomitantly localized in granular keratinocytes raises the intriguing possibility that DLK may act as an important regulator of the JNK pathway in human skin *in vivo*. Interestingly many studies have shown that signaling through the JNK pathway is linked to different biological functions. In some cell types, JNK has been recognized as an important regulator of programmed cell death, while in others, its activation is required for survival, proliferation, differentiation, and the suppression of apoptosis (44). Therefore, JNK appears to be a multifaceted molecule that contributes to many and sometimes even opposing functions depending on the cell lineage, the identity of its upstream regulators and downstream effectors, and the extracellular stimuli.

In keratinocytes, $p21^{cip1/waf1}$ expression is induced during early differentiation, and this induction seems to be required for the differentiation-associated growth arrest (45, 46). Studies with $p21^{cip1/waf1}$-null keratinocytes revealed an essential role for $p21^{cip1/waf1}$ in cell commitment to differentiation (47). As indicated by our findings, the expression of DLK in poorly differentiated human keratinocytes caused an accumulation of $p21^{cip1/waf1}$, whereas protein levels of the cyclin-dependent kinase inhibitors $p16^{INK4}$ and $p27^{Kip1}$ remained constant. The significance of the induction of $p21^{cip1/waf1}$ in these cells is unknown, but it may relate to its regulatory effects on the cell cycle since DLK overexpression has been reported to inhibit cell growth (48). Alternatively, as the expression of DLK in keratinocytes leads to sustained activation of the JNK cascade, a condition linked to apoptosis in many cell types (49), it is possible that $p21^{cip1/waf1}$ might influence cell survival through its recently identified antiapoptotic properties (50). In support of this hypothesis, Huang *et al.* (55) recently demonstrated that $p21^{cip1/waf1}$ is antagonistic to the cell death response induced by rapamycin, an inhibitor of the mammalian target of rapamycin, through a mechanism involving apoptosis signal-regulating kinase 1, a MAPKKK that like DLK acts as a regulator of the JNK pathway.

In addition to $p21^{cip1/waf1}$, we also found by immunostaining that the expression of DLK in poorly differentiated cultured keratinocytes led to the up-regulation of filaggrin protein levels. This finding is of particular interest since DLK and filaggrin are both localized primarily in terminally differentiated cells of normal human epidermis. Together these results support the possibility that DLK signaling and the induction of filaggrin expression could be functionally linked. In light of the roles of filaggrin in the cytoskeletal remodeling that occurs during the terminal differentiation of epidermal keratinocytes (51), it seems likely that its expression must be stringently controlled to maintain skin barrier functions.

Morphological analyses showed that keratinocytes expressing DLK have the features of terminally differentiated cells with a dense cytoplasm, a flattened appearance on top of the colonies, and a large proportion of them containing degraded DNA as established by three independent methods (Hoechst and propidium iodide staining, DNA laddering, and TUNEL assay). The induction of DNA degradation, one of the latest events in keratinocyte terminal differentiation, suggests that DLK is sufficient to promote the irreversible process leading to epidermal cell death. Additionally we found that the expression of wild-type DLK in normal human keratinocytes increased transglutaminase activity at the cell membrane, as assessed by the TMR-cadaverine incorporation assay, and the subsequent formation of cornified envelopes. These effects were entirely dependent on the kinase activity of DLK since a catalytically inactive DLK mutant consistently failed to activate transglutaminases *in situ* and to stimulate cornified envelope forma-

tion. The incorporation of TMR-cadaverine at the cell membrane suggests that DLK could be a specific activator of TG1, an isozyme of the transglutaminase family that exists as a membrane-bound form in keratinocytes (52). This hypothesis is supported by our findings that DLK and TG1 co-localized at the cell membrane in granular keratinocytes of normal human epidermis. Of the different transglutaminases known to be expressed in the epidermis (53), only TG1 appears to be required for the formation of cornified cell envelopes *in vivo*. Human epidermal keratinocytes lacking TG1 are unable to form cornified envelopes either spontaneously or upon induction with calcium (31). Similarly studies with mice deficient for TG1 have clearly demonstrated an essential role for this enzyme in the formation of the cell envelope and the maturation of the cornified layer (54). Thus, on the basis of these data, we propose that DLK, through its ability to phosphorylate and activate downstream effectors, functions as a positive regulator of TG1 activity, which in turn contributes to the formation of the cornified cell envelope.

In summary, our findings indicated that the MAPKKK DLK was expressed in the most terminally differentiated viable keratinocytes of human epidermis *in situ*. Upon expression in poorly differentiated cultured keratinocytes, DLK was sufficient to induce a set of morphological and biochemical changes that are reminiscent of the physiological events that occur during the final stages of the terminal differentiation program. Together these data support the hypothesis that DLK is a very important regulator of keratinocyte terminal differentiation, especially in the initiation of the cornification process. Moreover these findings are extremely relevant for epidermal integrity since several skin pathologies involve a defect in differentiation or cornification.

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