Regulation of transcription factor activity by extracellular signals in epidermal keratinocytes.

L. S. Rao, W. S. Long, T. Kaneko and M. Blumenberg

SUMMARY

Environmental signals, such as growth factors and cytokines, modulate the activity of nuclear transcription factors, thus regulating gene expression. This regulation is of particular importance in skin where a large variety of signals, especially in activated keratinocytes associated with disease states or wound healing, stimulate new gene expression in keratinocytes. In this study, we used electrophoretic mobility-shift assays (EMSA) to define the changes in DNA binding activity of transcription factors in such activated keratinocytes. Normal human epidermal keratinocytes were grown in a serum-free medium and treated with 10% fetal calf serum, EGF, TNFα or Oncostatin M (OsM) for different periods of time. Whole-cell extracts, without purification of the nuclei, were prepared and combined with a series of ^{32}-P-labeled synthetic oligonucleotide probes containing consensus binding sequences for the transcription factors AP1, NFκB, C/EBPβ, and Sp1. Serum activates all four transcription factors in keratinocytes; TNFα activates AP1, NFκB and C/EBPβ, but not Sp1, whereas EGF activates AP1 and NFκB, but not C/EBPβ or Sp1. On the other hand, OsM specifically activates Sp1. The activation of transcription factors is transient, usually reaching a peak 20 to 60 minutes after stimulation and returning to the basal level after two hours. We conclude that different signaling pathways are activated in response to various extracellular signals. This orchestrates the activity of transcription factors on the promoters of regulated genes in epidermis thus determining the levels of gene expression.

KEY WORDS

transcription factors, activity regulation, extracellular signals, epidermal keratinocytes

Introduction

Epidermis, our first line of defense from environmental injury, consists predominantly of keratinocytes, which form a multilayered protective sheet. In healthy epidermis, basal keratinocytes proliferate, while the supra basal ones cease to divide and terminally differentiate forming an impermeable protective layer called stratum corneum (1-3). This passive protective role of a mechanical barrier is expanded into a more active protective role when the skin is injured and the keratinocytes become activated. Keratinocytes respond to injury by releasing and reacting to a large number of protein signaling molecules, growth factors and cytokines, which are both paracrine signals that alert the internal immunologic defenses and autocrine signals that activate keratinocytes (4-7).

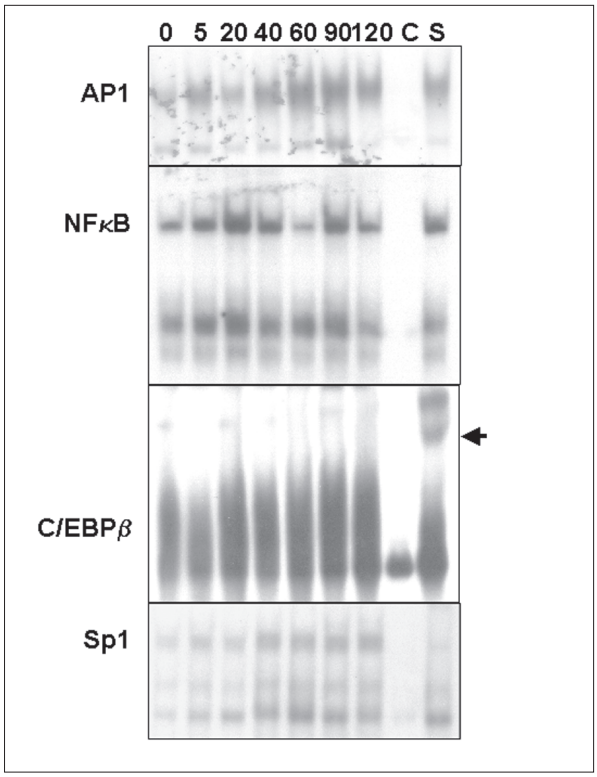

Figure 1. The effect of serum addition on AP1, NFκB, C/EBPβ and Sp1.
Consensus elements for AP1, NFκB, C/EBPβ and Sp1 were used in EMSA as radioactive probes with protein extracts of cells treated with 10% fetal calf serum for 5 minutes to two hours. Extracts from untreated cells are marked with O. These bands were shown to be specific by a combination of specific competitors and antibodies. Competition with the cognate DNA eliminates the specific binding in lanes marked C. The nonspecific bands are usually due to an artifact present in whole cell extracts, but absent from nuclear extracts (data not shown). Antibodies to specific transcription factors are present in lanes marked S; the arrow marks the specific retarded bands. Note that the antibodies can either cause appearance of a new band (C/EBPb and Sp1), or a disappearance of a band, on occasion failing to have an effect. The pictures of all electrophoretic mobility shift assays are representative of two or more experiments performed with different batches of keratinocytes.

Activated keratinocytes are migratory and hyperproliferative due, in part, to the induction of expression of cytoskeletal, cell surface and secreted proteins that are not found in the healthy, differentiating epidermis (8-11). Examples of activation-induced proteins are keratins K6 and K16, ICAM-1 and fibronectin (12-15). The induction of their expression occurs primarily at the level of transcription initiation, but the regulatory mechanisms by which extracellular signals affect transcription in keratinocytes have not been extensively studied. The signals that affect transcription factors and cause activating, hyperproliferative and inflammatory responses in keratinocytes are growth factors and cytokines produced by several skin cell types, including keratinocytes. Signals from the extracellular environment are received by the cell surface receptors that initiate enzymatic cascades, usually of protein phosphorylation, which lead to the activation of transcription factors (16,17). Activated transcription factors then regulate gene expression by several mechanisms that include binding to specific DNA sequences and interaction with other transcription factors or nuclear proteins.

Perhaps the most widely studied transcription factors are those belonging to the AP1 and NFκB families. AP1 is a nuclear transcription complex composed of dimers encoded by the *fos* and *jun* families of proto-oncogenes (18). Whereas the Fos proteins only heterodimerize with members of the Jun family, the Jun proteins may either homo- or heterodimerize with both Fos and other Jun proteins. AP1 activity can be induced by growth factors such as serum, EGF, and TGFα, cytokines such as IL-1 and TNFα, as well as tumor promoters, such as TPA and UV light (18-20). While such inducers cause *de novo* synthesis of Fos, Jun is activated by mitogen-activated protein kinases, particularly ERKs and JNKs (19,21,22). In the epidermis, AP1 regulates cell growth, differentiation and transformation (23-26).

The NFκB family includes proteins p65, p50 and c/rel, which both homo- and heterodimerize amongst themselves (27). Activation of these proteins is not dependent upon new protein synthesis. Rather, they are stored in the cytoplasm bound to the inhibitory protein, IκB. The inflammatory processes that induce IκB phosphorylation and degradation results in the nuclear translocation and activation of the NFκB complex. Signaling by EGF, TNFα and IL-1 result in activation of NFκB (28,29). In epidermis NFκB, particularly p65, regulates transcription of activation-specific markers, including ICAM1 and keratin K6 [12]. Conversely, genetic ablation of IκB leads to constitutive activation of NFκB, which causes severe and widespread skin inflammation (30). NFκB proteins interact with AP1 and other transcription factors, such as C/EBPβ (31,32).

Transcription factor C/EBPβ (also known as NF-IL6 or LAP) belongs to a family of several C/EBP proteins and has been originally characterized as the transcription factor responsible for IL-1 mediated induction of the IL-6 gene (33). Its activity is enhanced by the phosphorylation induced by extracellular signals (34,35). The

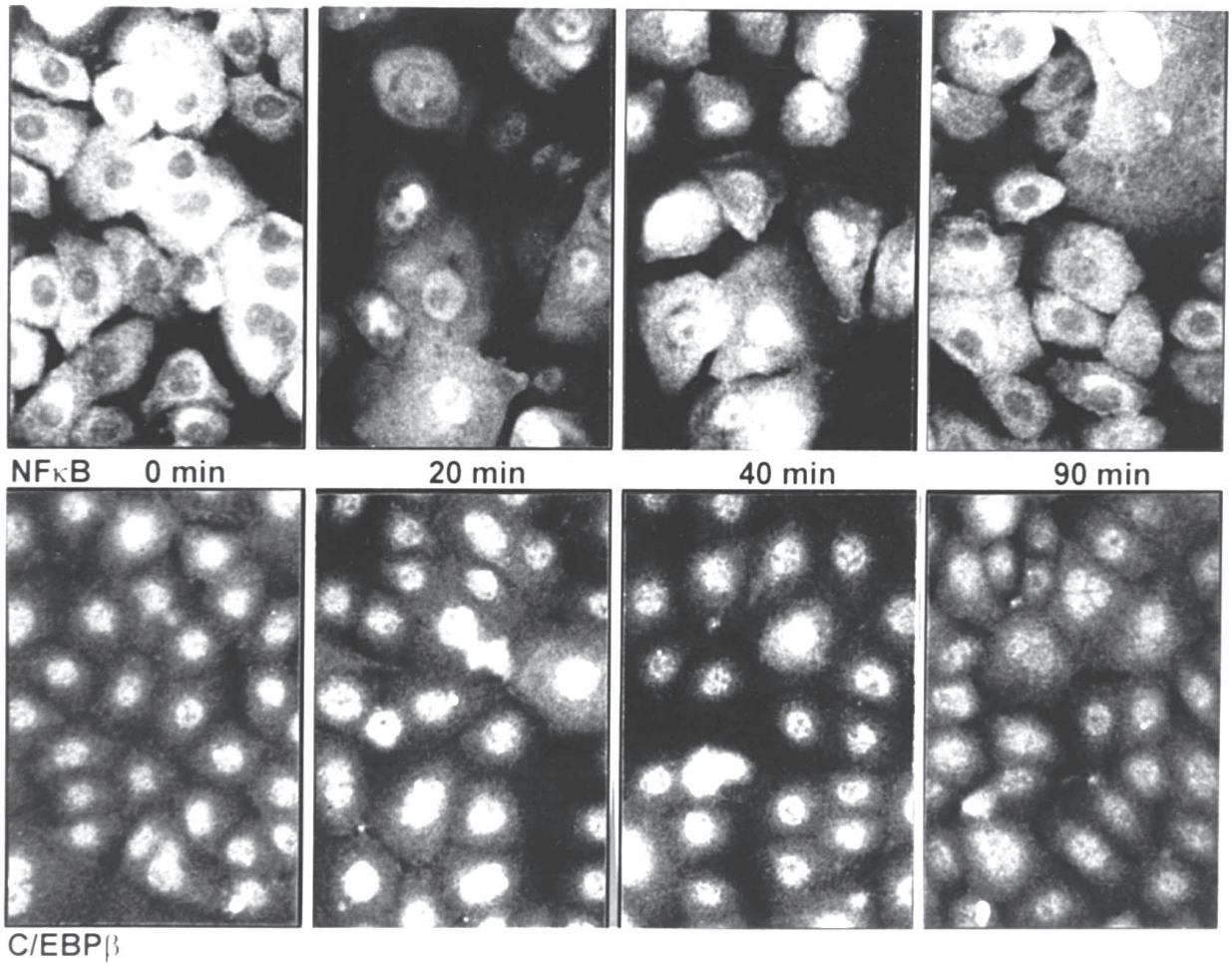

Figure 2. TNFα causes nuclear translocation of the NFκB transcription factor. Keratinocytes grown on coverslips were starved without growth factors overnight, then treated with TNFα, and at time points fixed and stained with a p65-specific antibody. Note that at the later time points the NFκB proteins appear to leave the nucleus and reenter the cytoplasm.

function of C/EBPβ in epidermis is not clear. C/EBPβ interacts with many other transcription factors, such as the glucocorticoid receptor, Myc and RB protein (36-39) and, importantly for the studies presented here, with AP1 and NFκB (20,31,40-42). In these interactions, C/EBPβ modulates the expression of a wide variety of genes in many cell types.

One of the first transcription factors to be purified and cloned, Sp1, also belongs to a gene family (43,44). The mechanisms that modulate Sp1 function are not known, although members of the family seem to antagonize one another. Sp1 interacts with other transcription factors, such as NF1 and Ets, which often have adjacent or overlapping binding sites in DNA (45-47). Importantly, Sp1 can multimerize while bound to DNA, thus looping out sequences between Sp1 sites and bringing additional factors into proximity of each other (48). This process seems important for expression of keratin genes (49). Sp1 proteins have been implicated in regulation of epidermal differentiation and Sp1 sites are present and functional in genes expressed in the epidermis (50).

To determine the molecular changes that convey the extracellular signals to the transcriptional machinery in healthy and hyperproliferative conditions in human epidermal keratinocytes, we grew these cells in culture and treated them with a selection of signaling agents. Specifically, we used serum because it is a rich source of many growth factors and cytokines, TNFα, because it initiates cutaneous inflammation, EGF because it is a strong mitogen for keratinocytes (6), and OsM, because it is an immunomodulator. We followed the course of activation, or deactivation, of AP1, NFκB, C/EBPβ and Sp1 transcription factors in response to these extracellular signals using electrophoretic mobility shift assays (EMSA).

We found that different signaling agents activate different and specific sets of transcription factors. For ex-

ample, serum activated all transcription factors tested. TNFα activated AP1, NFκB and C/EBPβ, while EGF, the proliferative agent, activated AP1 and NFκB. Interestingly, Sp1 was not activated by the proinflammatory and mitogenic signals, but was activated by OsM. Thus, the extracellular signals orchestrate the activation of transcription factors in human epidermal keratinocytes and thus coordinate the levels of gene expression.

Materials and methods

Cell Culture and Preparation of Cell Extracts

Human epidermal keratinocytes, a generous gift from Dr M. Simon, were derived from newborn foreskins obtained at circumcisions. The epidermis that remained after most of the dermis was surgically removed, incubated for 4 x 30 minutes at 37°C with constant stirring in a 1:1 solution of 0.1 % trypsin containing 5 mM glucose and phosphate-buffered saline pH 7.4 (PBS) with 50 mM EDTA. At the end of each incubation, cells obtained by centrifuging (800 x g, 5 min) were plated onto a 1/3 confluent layer of γ-irradiated 3T3 cells and grown in a modified culture medium made up of three parts of DMEM and one part of Ham's F-12 medium, supplemented with 5 % fetal bovine serum, adenine (18 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), hydrocortisone (0.4 μg/ml), insulin (5 μg/ml), EGF (10 ng/ml) and cholera toxin (1.2 pM). A week later, the keratinocytes were removed using the trypsin-EDTA solution described above. After the keratinocyte cultures were expanded one more time on g-irradiated 3T3 cells in medium containing serum, the 3T3 cells were removed by incubation with PBS containing 5 mM EDTA for 10 minutes. A 10 % DMSO (v/v) keratinocyte suspension was made, without the 3T3 feeder cells, and frozen in liquid nitrogen in small vials. For the experiments, the keratinocytes were thawed and grown in a serum-free defined keratinocyte growth medium supplemented with recombinant EGF (5 ng/ml) and 5 μg/ml of bovine pituitary extract in the absence of feeder cells. After two 1:4 passages, cells were starved for at least 16 hours by changing the medium to keratinocyte basal medium (KBM), which is a serum-free keratinocyte growth medium without EGF and other growth factors. The keratinocytes were then stimulated with either 10 % fetal calf serum, EGF (100 ng/ml), TNFα (50 ng/ml), or OsM (25 ng/ml). At time periods ranging between 5 min and 4 hrs after addition of the stimulants, the keratinocytes were washed once with PBS, harvested by scraping into 1.5 ml of PBS, pelleted by centrifugation (800 x g, 5 min) and the pellets washed twice with PBS. Cells were disrupted in 50 μl of extraction buffer (20 mM HEPES, pH 7.8, 450 mM NaCl, 0.4 mM EDTA, 0.5 mM DTT, 25 % Glycerol, 0.5 mM PMSF) by four cycles of freezing in liquid nitrogen, followed by thawing in a 37°C water bath. The clear supernatant obtained after centrifugation (2000 x g, 10 min) was aliquoted and stored at -70°C.

Ham's F-12 medium, DMEM, EGF, bovine pituitary extract, adenine, penicillin, streptomycin, hydrocortisone, insulin, cholera toxin, keratinocyte serum-free growth medium, keratinocyte basal medium and okadaic acid were purchased from Gibco BRL. The fetal bovine serum was from HyClone, while TNFα and OsM were obtained from Intergen.

Electrophoretic Mobility Shift Assays (EMSAs)

Whole cell extracts were prepared from keratinocytes at specified time points after treatment with growth factors and cytokines, as described previously (51-52). About 10 μg protein of the extracts was incubated for

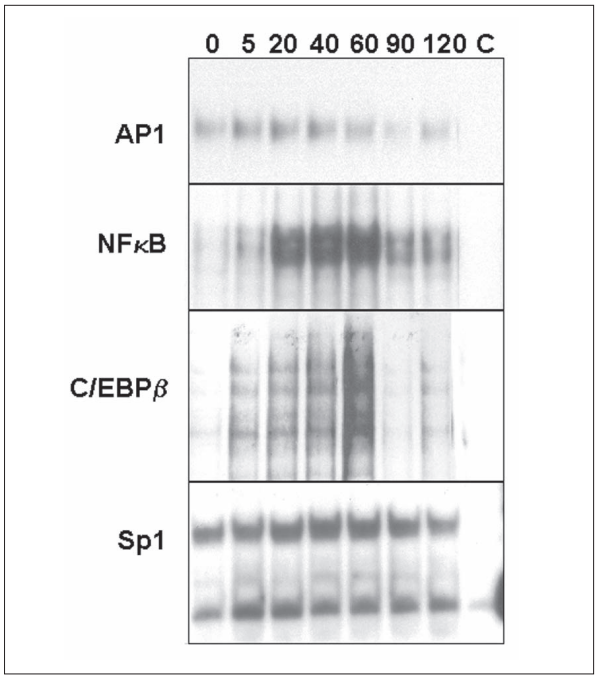

Figure 3. The effect of TNFα addition on the four transcription factors.
The conditions were equivalent to those in Fig. 1. Note the relatively sharp peak of NFκB activation from 20 to 60 min. The C/EBP binding probe in this particular gel derives from the K6 keratin gene promoter and contains four tandem C/EBPβ binding sites. The smearing seen at the 60 min time point is due, in part, to overloading. Multiple C/EBP proteins are evident, probably due to several proteins binding to the clustered C/EBP sites.

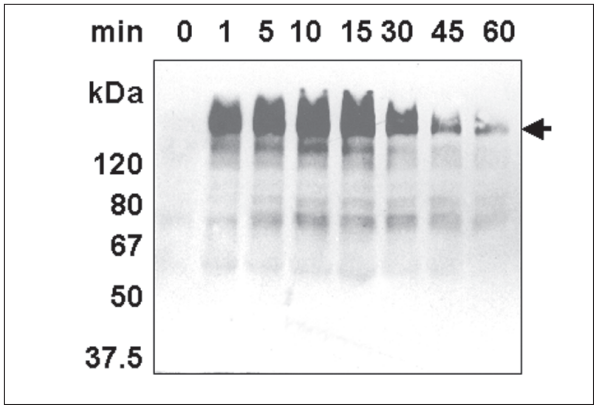

Figure 4. The time course of activation of the EGF receptor.
Keratinocytes were starved for 16 hours, then treated with EGF and harvested at indicated time points. Proteins were separated on acrylamide gels, transferred to nitrocellulose sheets and reacted with a phosphotyrosine-specific antibody. Note that the EGF receptor-sized band, arrow, is not visible at all in extracts harvested before the addition of EGF. The phosphorylation is prominent after 1 minute, peaks after 10 to 15, and recedes after an hour.

15 minutes on ice in the presence or absence of a 100-fold molar excess of an unlabeled competitor (double stranded synthetic oligomers) with 1.6 μg poly dI.dC (a nonspecific competitor), 2 % polyvinyl alcohol and binding buffer (20 mM Tris HCl, pH 7.6, 5 mM $MgCl_2$, 100 mM NaCl, 10 % Glycerol, 1 mM DTT and 0.1 mM EDTA). Double stranded oligomers were labeled using T4 polynucleotide kinase and (γP^{32})-dATP, and used as probes after purification through spin-columns. The sequences of the oligomers used are listed in Table 1. The labeled probes (50,000 cpm per lane) were added to the cell extracts and the incubation continued for an additional 30 minutes on ice. To separate the bound complexes from the free probe, we used a 5 % native polyacrylamide gel (29:1 = acrylamide:bis-acrylamide) run in TBE buffer at 200 V for two hours. The gels were dried and exposed overnight to XAR-5 film at -70°C with intensifying screens. All EMSAs were done at least twice with two different batches of cell extracts and separately labeled probes.

The poly dI.dC and polyvinyl alcohol were purchased from Pharmacia Biotech, while the T4 polynucleotide kinase and the consensus double stranded oligomers were from Promega. The C/EBPβ sequence from keratin K6 promoter was synthesized in the Kaplan Comprehensive Cancer Center Core Facility. Spin columns were from Clontech Lab. Inc., x-ray film and intensifying screens from Kodak and the radioactive label from Amersham.

Western Blotting

Keratinocytes were grown as described above. At their third passage, at 80% confluence, keratinocytes were incubated with KBM for 16 hours to starve the cells of growth factors, and then treated with EGF (100 ng/ml). At various time intervals, cells were harvested by scraping, washed twice with PBS and the cell pellet was resuspended in 100 μl of the lysis buffer (2 % SDS, 10 % Glycerol, 10 mM Tris HCl, pH 6.8). The cells were lysed by ultrasonication for 30 seconds using an ultrasonic homogenizer sonicator (Cole-Parmer Instrument Co.) and boiling for 10 minutes. Aliquots of the supernatant obtained after centrifugation (2000 x g, 10 min) were stored at -70°C. Protein content was determined using the Bradford reagent (Perkin-Elmer). Equal amounts of total protein were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (10 % gel) and electrophoretically transferred to nitrocellulose at 8 V for one hour. After blocking the membranes for one hour at room temperature on an orbital shaker with 5 % nonfat dry milk in Tris-buffered saline with 0.1% Tween (TBS-T), the blots were washed thrice (10 min each time) with TBS-T and incubated at 4°C with 1:1000 dilution of the anti-phosphotyrosine antibody. Blots were washed 16 hours later with TBS-T (3 x 10 min) and incubated at room temperature with 1:1000 dilution of anti-rabbit immunoglobin-horseradish peroxidase linked antibody for two hours. The chemiluminescence ECL reagent was used to develop the blots after washing the membranes (3 x 10 min) with TBS-T. The phosphotyrosine antibody was purchased from Transduction Lab, while the anti-rabbit immunoglobulin-horseradish peroxidase-linked antibodies and the ECL reagent were from Amersham.

Table 1. Oligonucleotides used as probes in EMSA.

Oligomer	Sequences (both strands).
AP1	CGCTTGATGAGTCAGCCGGAA GCGAACTACTCAGTCGGCCTT
NFkB	AGTTGAGGGGACTTTCCCAGGC TCAACTCCCCTGAAAGGGTCCG
Sp1	ATTCGATCGGGGCGGGGCGAGC TAAGCTAGCCCCGCCCCGCTAG
C/EBP Cons.	TGCAGATTGCGCAATCTGCA ACGTCTAACGCGTTAGACGT
K6	GACTAAAGGAAGCGAAAAATGCAATCT GGTATTTCATAACTTTTGTAA CTGATTTCCTTCGCTTTTTACGTTAGAGCC ATAAAGTATTGAAAACATT

Immunofluorescence Staining.

Human keratinocytes were grown on coverslips. Prior to the addition of TNFα (50 ng/ml), keratinocytes were starved for 16 hours in KBM. At different time points, cells were fixed with 4 % paraformaldehyde in PBS for 20 minutes at room temperature. This was followed by three washes with PBS and then permeabilization of the cells using methanol for 6 minutes at -20°C. After one hour incubation at 37°C with 1:500 diluted p65 polyclonal antibody, cells were washed thrice with PBS and treated with 1:200 diluted anti-rabbit immunoglobulin-fluorescein isothiocyanate conjugate for one hour at 37°C. After three washes with PBS, the coverslips were mounted, observed under the microscope and photographed. The p65 antibody was purchased from SantaCruz Biotechnology, Inc., and the anti-rabbit immunoglobulin-fluorescein isothiocyanate conjugate was from Sigma.

Results

Serum activates AP1, NFκB, C/EBPβ and Sp1 transcription factors.

Serum contains a complex mixture of various signaling molecules, growth factors, cytokines, hormones and vitamins, and is expected, therefore, to activate a large number of transcription factors. Indeed, we find that the addition of serum to keratinocytes activated AP1, NFκB, C/EBPβ and Sp1 proteins (Fig. 1). The factors were activated to various extents and with different kinetics. The fastest and most pronounced was the activation of AP1, which could be observed after 5 min, reached the peak at one hour and persisted for at least another hour. In EMSA using the AP1 consensus sequence as a probe and whole cell extracts, two bands are visible. The slower moving one is the true AP1 band because it was completely competed by the addition of the specific probe, lane C, but not by a nonspecific competitor (not shown). This band can also be reduced in intensity using a c-Jun specific antibody (e.g., in Fig. 5), although we find the effects of the antibody variable. The faster moving band is not specific and present in whole cell extracts, but not nuclear extracts. It can be reduced using any nonspecific competitor, including salmon sperm DNA, but not with poly dI.dC (M.B., not shown). Note that this band is observed with all labeled probes (Fig. 1). The intensity of the nonspecific band varies from preparation to preparation and therefore cannot be used as a measure of uniformity of loading the gels.

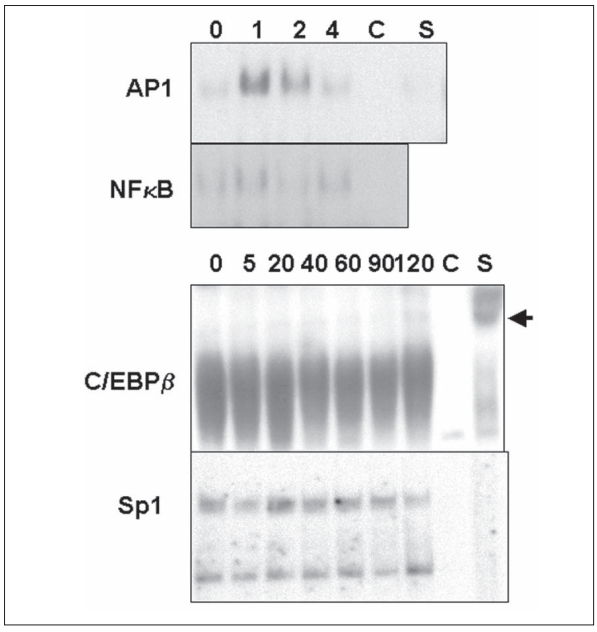

Figure 5. The effect of EGF addition on the four transcription factors.
The conditions were equivalent to those in Fig. 1. Note that AP1 is activated very strongly, while the activation of NFκB is relatively weak and delayed.

The NFκB activation was rather weak and delayed, seen after one to two hours. The activation of NFκB is more difficult to see because of the relatively strong nonspecific band. The activation of C/EBPβ proteins was relatively delayed, observable after 20 min, and reached a plateau after 90 min. Again, multiple C/EBP binding bands are visible, the slowest one being C/EBPβ supershifted by the antibody in lane S (arrow in Fig. 1).

There are three proteins that bind the Sp1 consensus sequence. All three were activated in parallel (Fig. 1). Only the top one is supershifted using an Sp1 specific antibody, note the disappearance of the top band and the appearance of a new, even slower moving band in lane S. The two lower bands may be due to Sp2 and Sp3, members of the Sp1 family. The earliest increase in the Sp1 binding activity was observed after 5 min and the bands grew steadily stronger throughout the two-hour progression of the experiment (Fig. 1).

TNFa activates AP1, NFκB and C/EBPβ, but not Sp1.

The signal transduction initiated by TNFα is very complex and after binding of the ligand to the receptor, it branches into a delta of cascades including ceramide production, PKCζ activation, IκB degradation and JNK phosphorylation. Transcription factors AP1, NFκB, C/EBPβ and others have been seen activated by TNFα. Focusing on NFκB, we first examined the time course of its nuclear translocation in keratinocytes. To this end, keratinocytes were grown on glass cover slips and, after addition of TNFα, fixed and stained with an antibody specific for the p65 component of NFκB. We found that the addition of TNFα causes nuclear translocation of NFκB visible at 20 min, the protein is completely nuclear at 40 min, but by 60 min it begins to leave the

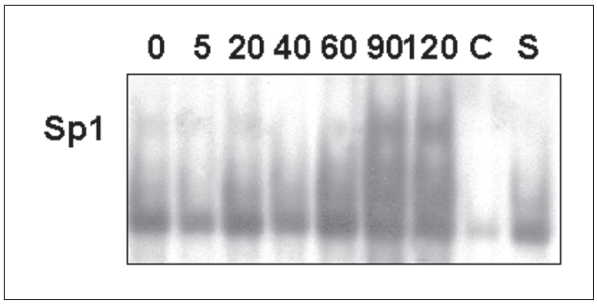

Figure 6. Oncostatin M activates the Sp1 transcription factor.
The time course of Sp1 activation shows the strongest activity after 90 to 120 min.

nucleus and reenter the cytoplasm, which continues at the 90 min time point (Fig. 2).

Gel shift experiments using NFκB consensus sequence concur with these results: specific bands are visible after 5 min, are very strong at 20 and 40 min, the DNA binding activity peaks at 60 min and then wanes, being much weaker at 90 min after the addition of TNFα (Fig. 3). The discrepancy between the two approaches, at 5 min, could be because we used whole cell extracts for the gel shift experiments, rather than nuclear extracts. The NFκB proteins can bind DNA as soon as they are released from the IκB inhibitor, in transit from the cytoplasm into the nucleus. At present, we do not know why the NFκB band appears as a doublet, possibly because multiple members of the family are activated by TNFα. We note that the bottom member of the doublet is supershifted by the p65 specific antibody.

When the cells are grown in the presence of EGF, normal component of the keratinocyte culture medium, the AP1 is already activated and there was no further activation by TNFα (see below and data not shown). The cells had to be pre-incubated without EGF for 16 hours in order to observe the effect of TNFα. Under these conditions, we saw a sustained activation of AP1 by TNFα (Fig. 3).

The C/EBP binding activity gets induced with a similar time course to that of NFkB (Fig. 3). In the experiment shown in Fig. 3, we used, instead of the consensus C/EBP DNA sequence, a naturally occurring C/EBP binding sequence found in the human K6 keratin gene promoter. This sequence contains a set of three C/EBPβ binding sites and confers regulation by co-transfected C/EBPβ to the K6 keratin gene (14,15). The increase in C/EBP binding activity is observed after 5 min grows for 60 min and after that wanes.

In contrast to the three transcription factors described above, the Sp1 activity remains only weakly affected by TNFα between 40 and 60 min and then slowly returning to the basal level. This results shows that not all transcription factors are activated by TNFα with same kinetics or intensity, i.e., its effects are specific.

The cytokine IL-1 is also a proinflammatory cytokine and the effects of IL-1 are to a large extent similar to those of TNFα, since the two cytokines activate some common branches of the signaling pathways (28). We tested the effects of IL-1 on activation of NFκB and C/EBPβ transcription factors in keratinocytes, at a single time point, 40 min, when the effects of TNFα are quite prominent. We found that the effects of IL-1 parallel the effects of TNFα, in that IL-1activates both NFκB and C/EBPβ transcription factors (data not shown).

EGF activates AP1 and NFκB, but not C/EBPβ and Sp1.

The initial step of EGF signaling, binding to the receptor, causes the receptor to dimerize, activate its cytoplasmic tyrosine kinase domain and phosphorylate a set of tyrosines in the cytoplasmic domain of the receptor itself. This self-phosphorylation reaction occurs very rapidly and its function is to assemble a set of phosphotyrosine binding proteins at the plasma membrane, which initiates the cytoplasmic signal transduction pathway (16). To examine the time course of the initial step in EGF-mediated signal transduction, we used an antibody specific for the phosphorylated form of the EGFR in Western blotting (Fig. 4). We found that cells starved for EGF have virtually undetectable amounts of phosphorylated EGFR. However, we observed the phosphorylation of EGFR within one minute after EGF addition. The phosphorylation peaks at 10-15 min and then decays over the one hour period that we followed it.

The signal transduction cascade proceeds through a series of protein phosphorylation steps that activate the AP1 transcription factors, *inter alia* (19). Indeed, we found strong and specific activation of AP1 binding activity culminating at one hour and getting weaker subsequently (Fig. 5). EGF also activates the NFκB transcription factors, but this occurs more slowly. Interestingly, the level of C/EBP binding activity remains unaffected by EGF, demonstrating that EGF effects on AP1 and NFκB are specific. The Sp1 activity is also not significantly affected by the addition of EGF (Fig. 5).

Activation of Sp1

Transcription factor Sp1 was activated weaker and later than the other transcription factors by serum, TNFα, IL-1 (not shown) and EGF, as presented in the above experiments. However, although Sp1 seems ubiquitously expressed, the modification of Sp1 activity has been implicated in regulation of gene expression in keratinocytes (50). Therefore, we decided to seek for an extracellular signal that can activate Sp1 preferentially or exclusively from the other three transcription factors. Surprisingly, we found that the Sp1 activity can

be increased by the treatment of keratinocytes with OsM (Fig. 6). This result is surprising because OsM is known to activate strongly the STAT family members (53). The increase in Sp1 binding activity due to OsM is slow but sustained, persisting for two hours. Importantly, OsM does not seem to affect NFκB, AP1 and C/EBP binding activities (not shown). The activation of Sp1 by OsM shows, on one hand, that Sp1 activity is not a constant, but can respond to extracellular signals, which implies that regulation of gene expression can proceed through Sp1 sites, and on the other, that only specific extracellular signals are capable of activating Sp1. Furthermore, these results demonstrate that different extracellular signals activate different subsets of transcription factors and thereby fine-tune changes in transcription of regulated genes.

Discussion

Using extracellular signals, we show here that different transcription factors are activated by distinct subsets of signals, with specific kinetics. We used serum as a source of many signaling molecules, TNFα and IL-1 as proinflammatory cytokines, EGF as a mitogen and OsM as an immunomodulator. Specifically, we find that AP1 is activated by serum, as well as by EGF and TNFα. NFκB can be also activated by the same signals, although TNFα and IL-1 activate NFκB strongly, but serum and EGF only weakly. On the other hand, C/EBPβ can be activated by serum, TNFα, and IL-1, but not by EGF. These agents activateSp1 transcription factor weakly and slowly, whereas OsM specifically activates Sp1, not the other three transcription factors.

Usually, even when two agents activate the same transcription factor, the timing of activation varies. For example, AP1 is quickly activated by serum and TNFa, slowly by EGF. On the other hand, NFkB is quickly activated by TNFα, and more slowly by serum and EGF. Activation of C/EBPβ by serum is sustained, but by TNFα it is transient. Therefore, it appears that individual signaling molecules activate specific subsets of transcription factors, each with a characteristic time course, to affect a specific, fine-tuned modulation of gene expression. Certain transcription factors can be activated very quickly, perhaps reflecting the need for quick responses to epidermal injury. Importantly, the activation of transcription factors is temporary and 2 to 4 hours after the addition of the extracellular stimulus, their activity returns to the normal, uninduced levels. It is not clear how deactivation of the transcription factor relates to the long-term levels of expression of the regulated genes.

Although some of the transcription factors studied in this work have been implicated in the process of epidermal differentiation, e.g., Sp1 and the AP1 family, our result concern the alternative pathway available to keratinocytes, their activation. *In vitro* differentiation is difficult to achieve, and certainly in our culture conditions, growth on plastic, low calcium concentration, absence of feeder calls and presence of growth factors, no phenotypic marker of differentiation is apparent. On the other hand, these conditions are in many ways similar to the wound healing response when keratinocytes are required to divide, migrate and respond to extracellular signals.

Multiple transcription factors activated by the extracellular reflect the specificities of the signals transduction mechanisms that respond to the corresponding growth factors and cytokines. For example, IL-1, which is present in healthy epidermis in inactive form (4), when released autocrinely, activates NFκB and C/EBPβ, thus initiating keratinocyte activation. Among the characteristics of activated keratinocytes is the production of TNFα (11), which maintains the two transcription factors activated. Activated keratinocytes produce ligands of the EGF receptor that cause activation of AP1, such as TGFα, amphiregulin and HB-EGF. Interestingly, all three, IL-1, TNFα and EGF, induce expression of the same set of keratin genes, albeit through separate pathways (14,15,54,55).

In summary, we found that environmental signals, such as growth factors and cytokines, modulate the activity of nuclear transcription factors in keratinocytes, thus regulating gene expression. This regulation is particularly important in skin where a large variety of signals appear, especially in disease states or in wound healing, to stimulate new gene expression. Different signaling pathways are activated in response to diverse extracellular signals, which orchestrates the activity of transcription factors at the promoters of regulated genes in epidermis and thus determines the level of gene expression.

Acknowledgments

Our research is supported by National Institutes of Health grants AR30682, AR39176, AR40522, AR41850 and DK16636. We thank M. Simon for the keratinocytes and members of our laboratory for help and reagents. Special thanks go to V. Milisavljevic for the early gel shift assays. We also thank members of our laboratory for advice, reagents and encouragement.

REFERENCES

1. Blumenberg M, Tomic-Canic M. Human epidermal keratinocyte: keratinization processes. In: P. Jolles HZaHH, editor. Formation and Structure of Human Hair. Volume 78. Basel: Birkhauser Verlag; 1997. p 1-29.

2. Fuchs E. Epidermal differentiation: The bare essentials. J Cell Biol 1990; 111: 2807-14.

3. Eckert RL. Structure, function, and differentiation of the keratinocyte. Physiol Rev 1989; 69: 1316-46.

4. Kupper TS. The activated keratinocyte: a model for inducible cytokine production by non-bone marrow-derived cells in cutaneous inflammatory and immune responses. J Invest Dermatol 1990; 94: 146S-150S.

5. Barker JN, Mitra RS, Griffiths CE, Dixit VM, Nickoloff BJ. Keratinocytes as initiators of inflammation. Lancet 1991; 337: 211-4.

6. Pittelkow MR, Coffey R, Jr., Moses HJ. Keratinocytes produce and are regulated by transforming growth factors. Ann New York Acad Sci 1988; 548: 211-24.

7. Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M. Keratins and the Keratinocyte Activation Cycle. J Invest Dermatol 2001; 116: 633-40.

8. Blumenberg M. Keratinocytes: Biology and Differentiation. In: al. KAe, editor. Cutaneous Medicine and Surgery. Volume 1. Philadelphia: W.B. Saunders Co.; 1994. p 58-74.

9. Grinnell F. Wound repair, keratinocyte activation and integrin modulation. J Cell Sci 1992; 101: 1-5.

10. Schafer IA, Kovach M, Price RL, Fratianne RB. Human keratinocytes cultured on collagen gels form an epidermis which synthesizes bullous pemphigoid antigens and alpha 2 beta 1 integrins and secretes laminin, type IV collagen, and heparan sulfate proteoglycan at the basal cell surface. Exp Cell Res 1991; 195: 443-57.

11. Barker JN, MacDonald DM. Cutaneous lymphocyte trafficking in the inflammatory dermatoses.. Brit J Dermatol 1992; 126: 211-5.

12. Cornelius LA, Taylor JT, Degitz K, Li LJ, Lawley TJ, Caughman SW. A 5' portion of the ICAM-1 gene confers tissue-specific differential expression levels and cytokine responsiveness. J Invest Dermatol 1993; 100: 753-8.

13. O'Keefe EJ, Woodley DT, Falk RJ, Gammon WR, Briggaman RA. Production of fibronectin by epithelium in a skin equivalent. J Invest Dermatol 1987; 88: 634-9.

14. Komine M, Rao LS, Kaneko T, Tomic-Canic M, Tamaki K, Freedberg IM, Blumenberg M. Inflammatory versus proliferative processes in epidermis. Tumor necrosis factor alpha induces K6b keratin synthesis through a transcriptional complex containing NF kappa B and C/EBP beta. J Biol Chem 2000; 275: 32077-88.

15. Komine M, Rao LS, Freedberg IM, Simon M, Milisavljevic V, Blumenberg M. Interleukin-1 induces transcription of keratin K6 in human epidermal keratinocytes. J Invest Dermatol 2001; 116: 330-8.

16. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell 1990; 61: 203-12.

17. Hill CS, Treisman R. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 1995; 80: 199-211.

18. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation.. Biochim Biophys Acta 1991; 1072: 129-57.

19. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. Philosl Trans R Soc of London: Biological Sciences 1996; 351: 127-34.

20. Klampfer L, Lee TH, Hsu W, Vilcek J, Chen-Kiang S. NF-IL6 and AP-1 cooperatively modulate the activation of the TSG-6 gene by tumor necrosis factor alpha and interleukin-1. Mol Cell Biol 1994; 14: 6561-9.

21. Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 1993; 7: 2135-48.

22. Dérijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ. JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 1994; 76: 1025-37.

23. Bernerd F, Magnaldo T, Freedberg IM, Blumenberg M. Expression of the carcinoma-associated keratin K6 and the role of AP-1 proto-oncoproteins. Gene Expression 1993; 3: 187-99.

24. Basset-Seguin N, Demoly P, Moles JP, Tesnieres A, Gauthier-Rouviere C, Richard S, Blanchard JM, Guilhou JJ. Comparative analysis of cellular and tissular expression of c-fos in human keratinocytes: evidence of its role in cell differentiation. Oncogene 1994; 9: 765-71.

25. Rutberg SE, Saez E, Glick A, Dlugosz AA, Spiegelman BM, Yuspa SH. Differentiation of mouse keratinocytes is accompanied by PKC-dependent changes in AP-1 proteins. Oncogene 1996; 13: 167-76.

26. Saez E, Rutberg SE, Mueller E, Oppenheim H, Smoluk J, Yuspa SH, Spiegelman BM. c-fos is required for malignant progression of skin tumors. Cell 1995; 82: 721-32.

27. Miyamoto S, Verma IM. Rel/NF-kappa B/I kappa B story. Advances in Cancer Res 1995; 66: 255-92.

28. Beg AA, Finco TS, Nantermet PV, Baldwin A, Jr. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. Mol Cell Biol 1993; 13: 3301-10.

29. Barnes PJ, Karin M. Nuclear factor-kappa B: a pivotal transcription factor in chronic inflammatory diseases. New England J Med 1997; 336: 1066-71.

30. Klement JF, Rice NR, Car BD, Abbondanzo SJ, Powers GD, Bhatt Ph, Chen Ch, Rosen CA, Stewart CL. I kappa B alpha deficiency results in a sustained NF-kappa B response and severe widespread dermatitis in mice. Mol Cell Biol 1996; 16: 2341-9.

31. Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S. Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc Natl Acad Sci U S A 1993; 90: 10193-7.

32. Stein B, Baldwin A, Jr., Ballard DW, Greene WC, Angel P, Herrlich P. Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J 1993; 12: 3879-91.

33. Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, Nakajima T, Hirano T, Kishimoto T. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J 1997; 9: 1897-906.

34. Trautwein C, Caelles C, van der Geer P, Hunter T, Karin M, Chojkier M. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. Nature 1993; 364: 544-7.

35. Nakajima T, Kinoshita S, Sasagawa T, Sasaki K, Naruto M, Kishimoto T, Akira S. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc Natl Acad Sci U S A 1993; 90: 2207-11.

36. Nishio Y, Isshiki H, Kishimoto T, Akira S. A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat alpha 1-acid glycoprotein gene via direct protein-protein interaction. Mol Cell Biol 1993; 13: 1854-62.

37. Chen P, Riley DJ, Chen Y, Lee WH. Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev 1996;10: 2794-804.

38. Mink S, Mutschler B, Weiskirchen R, Bister K, Klempnauer Kh. A novel function for Myc: inhibition of C/EBP-dependent gene activation. Proc Natl Acad Sci U S A 1996; 93: 6635-40.

39. Maytin EV, Habener JF. Transcription factors C/EBP alpha, C/EBP beta, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo. J Invest Dermatol 1998; 110: 238-46.

40. Stein B, Cogswell PC, Baldwin A, Jr. Functional and physical associations between NF-kappa B and C/EBP family members: a Rel domain-b ZIP interaction. Mol Cell Biol 1993; 13: 3964-74.

41. Brasier A, Ron D, Tate J, Habener J. A family of constitutive C/EBP-like DNA binding proteins attenuate the IL-1 alpha induced, NF kappa B mediated trans-activation of the angiotensinogen gene acute-phase response element. EMBO J 1990; 9: 3933-44.

42. Baldwin A, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Ann Rev Immunol 1996; 14: 649-83.

43. Kadonaga JT, Tjian R. Affinity purification of sequence-specific DNA binding proteins. Proc Natl Acad Sci U S A 1986; 83: 5889-93.

44. Kingsley C, Winoto A. Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. Mol Cell Biol 1992; 12: 4251-61.

45. Nehls MC, Rippe RA, Veloz L, Brenner DA. Transcription factors nuclear factor I and Sp1 interact with the murine collagen alpha 1 (I) promoter. Mol Cell Biol 1991; 11: 4065-73.

46. Janson L, Pettersson U. Cooperative interactions between transcription factors Sp1 and OTF-1. Proc Natl Acad Sci U S A 1990; 87: 4732-6.

47. Gegonne A, Bosselut R, Bailly RA, Ghysdael J. Synergistic activation of the HTLV1 LTR Ets-responsive region by transcription factors Ets1 and Sp1. EMBO J 1993; 12: 1169-78.

48. Mastrangelo IA, Courey AJ, Wall JS, Jackson SP, Hough PVC. DNA looping and Sp1 multimer links: A mechanism for transcriptional synergism and enhancement. Proc Natl Acad Sci U S A 1991; 88: 5670-4.

49. Ohtsuki M, Flanagan S, Freedberg IM, Blumenberg M. A cluster of five nuclear proteins regulates keratin transcription. Gene Expression 1993; 3: 201-13.

50. Fischer DF, Gibbs S, van De Putte P, Backendorf C. Interdependent transcription control elements regulate the expression of the SPRR2A gene during keratinocyte terminal differentiation. Mol Cell Biol 1996; 16: 5365-74.

51. Milisavljevic V, Freedberg IM, Blumenberg M. Characterization of nuclear protein binding sites in the promoter of keratin K17 gene. DNA & Cell Biol 1996;15:65-74.

52. Magnaldo T, Bernerd F, Freedberg IM, Ohtsuki M, Blumenberg M. Transcriptional regulators of expression of K#16, the disease-associated keratin. DNA and Cell Biology 1993; 12: 911-23.

53. Schindler C, Darnell J, Jr. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Ann Rev Biochem 1995; 64: 621-51.

54. Pittelkow MR, Lindquist PB, Abraham RT, Graves-Deal R, Derynck R, Coffey R, Jr. Induction of transforming growth factor-alpha expression in human keratinocytes by phorbol esters. J Biol Chem 1989; 264: 5164-71.

55. Jiang CK, Magnaldo T, Ohtsuki M, Freedberg IM, Bernerd F, Blumenberg M. Epidermal growth factor and transforming growth factor alpha specifically induce the activation- and hyperproliferation-associated keratins 6 and 16. Proc Natl Acad Sci U S A 1993; 90: 6786-90.

A U T H O R S ' A D D R E S S E S

Laxmi S. Rao, PhD, Department of Dermatology, New York University Medical Center, 550 First Avenue, New York, 10016 USA
Wendy S. Long, MD, same address
Takehiko Kaneko, MD, New York University Medical Center, 550 First Avenue, New York, 10016 USA. Permanent address: The department of Dermatology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 Japan.
Miroslav Blumenberg , PhD, Professor of biochemistry and dermatology, Dept Dermatology and Biochemistry, and the NYU School of Medicine Cancer Institute, New York University Medical Center, 550 First Avenue, New York, 10016 USA.
Please address correspondence to M. Blumenberg
E-mail: blumem01@ med.nyu.edu