Journal of Cellular Biochemistry 104:606–619 (2008)

Two Modes of ERK Activation by TNF in Keratinocytes: Different Cellular Outcomes and Bi-Directional Modulation by Vitamin D

Ester Ziv,[1,2] Carmela Rotem,[1] Mor Miodovnik,[1,2] Amiram Ravid,[1,3] and Ruth Koren[1,2]*

[1]The Basil and Gerald Felsenstein Medical Research Center, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[2]Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[3]Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Abstract Inflammation, elicited in the skin following tissue damage or pathogen invasion, may become chronic with deleterious consequences. Tumor necrosis factor (TNF) is a key mediator of cutaneous inflammation and the keratinocyte an important protagonist of skin immunity. Calcitriol, the hormonally active vitamin D metabolite, and its analogs attenuate epidermal inflammation and inhibit the hyperproliferation of keratinocytes associated with the inflammatory disorder, psoriasis. Since activation of extracellular signal-regulated kinase (ERK) promotes keratinocyte proliferation and mediates epidermal inflammation, we studied the effect of calcitriol on ERK activation in HaCaT keratinocytes exposed to the ubiquitous inflammatory cytokine TNF. By using the EGF receptor (EGFR) tyrosine kinase inhibitor, AG1487 and the Src family inhibitor, PP-1, we established that TNF activated ERK in an EGFR and Src dependent and an EGFR and Src independent modes. EGFR dependent activation resulted in the upregulation of the transcription factor, c-Fos, while the EGFR independent activation mode was of a shorter duration, did not affect c-Fos expression but induced IL-8 mRNA expression. Pretreatment with calcitriol, enhanced TNF-induced EGFR-Src dependent ERK activation and tyrosine phosphorylation of the EGFR, but abolished the EGFR-Src independent ERK activation. These effects were mirrored by enhancement of c-Fos and inhibition of IL-8 induction by TNF. Treatment with calcitriol increased the rate of the de-phosphorylation of activated ERK, accounting for the inhibition of EGFR-Src independent ERK activation by TNF. It is possible that effects on the ERK cascade contribute to the effects of calcitriol and its synthetic analogs on cutaneous inflammation and keratinocyte proliferation. J. Cell. Biochem. 104: 606–619, 2008.

Key words: extracellular signal-regulated kinase; tumor necrosis factor; epidermal growth factor receptor; vitamin D; keratinocytes

The skin functions as a barrier between the body and the exterior and therefore encounters multiple exogenous threats that must be counteracted by effective inflammatory and immune responses. Inflammation is elicited in the skin in response to disruption of the integrity of the epidermal barrier, tissue damage or pathogen invasion. However in some disorders, the inflammatory process—which under normal conditions is self-limiting—becomes continuous and chronic with deleterious consequences. Evidence accumulated in recent years points to the keratinocyte, the most abundant epidermal cell, as a protagonist of the innate skin immunity, which may control even acquired skin immune responses [Schroder et al., 2006]. The ubiquitous inflammatory cytokine tumor necrosis factor α (TNF) is considered to be a key mediator of cutaneous inflammation [Piguet et al., 1987, 1991; LaDuca and Gaspari, 2001; Trent and Kerdel, 2005] and was suggested to

Amiram Ravid contributed equally as senior author.

Grant sponsor: The Israel Science Foundation; Grant number: 703/04; Grant sponsor: Tel Aviv University Faculty of Medicine; Grant sponsor: Katzenelbogen Grant.

*Correspondence to: Ruth Koren, Felsenstein Medical Research Center, Beilinson Campus, Rabin Medical Center, Petah-Tikva 49100, Israel.
E-mail: rkoren@post.tau.ac.il

Received 28 July 2007; Accepted 30 October 2007
DOI 10.1002/jcb.21650

contribute also to the hyperproliferation of keratinocytes in the inflammatory skin disease, psoriasis [Piguet et al., 1990]. TNF was also shown to negatively affect wound healing [Buck et al., 1996; Mori et al., 2002]. It was suggested that modulation of TNF activity may provide therapeutic benefits in a variety of inflammatory skin conditions such as allergic and irritant contact dermatitis, cutaneous lupus, chronic photosensitive disease, and psoriasis [LaDuca and Gaspari, 2001; Trent and Kerdel, 2005]. Indeed, the TNF inhibitors Infliximab and Etanercept are employed with success in moderate to severe psoriasis [Gisondi et al., 2004].

The hormonal form of vitamin D, calcitriol ($1,25(OH)_2D_3$), is derived from the parent compound by two consecutive hydroxylations [Wikvall, 2001]. Epidermal keratinocytes contain the full enzymatic complement required for the synthesis of calcitriol from vitamin D_3 [Lehmann et al., 2004; Bikle, 2005] that is produced in the epidermis by a photochemical reaction from 7-dehydrocholesterol. Keratinocytes are also able to degrade calcitriol and are target to its action via their nuclear vitamin D receptor [Bikle, 2005]. Therefore, the epidermis can be viewed as a self-contained vitamin D endocrine system, where the hormone acts in an autocrine manner. Hormonally active vitamin D derivatives promote keratinocyte differentiation and modulate their proliferation [Levy et al., 1994; Van de Kerkhof, 1995; Lutzow-Holm et al., 1996; Gniadecki, 1996a; Bikle, 2005]. They also attenuate epidermal inflammation and this effect may at least partially account for the known beneficial action of these compounds in the treatment of psoriasis [Van de Kerkhof, 1995; Reichrath et al., 1997; Reichrath, 2007]. The anti-inflammatory effect of hormonally active vitamin D derivatives has been attributed both to inhibition of infiltrating immune cells and to inhibition of the pro-inflammatory action of the keratinocytes themselves [Barna et al., 1997; Romer et al., 2003; Vissers et al., 2004; Nagpal et al., 2005].

The extracellular signal-regulated kinase (ERK1/2) plays a key role in the mediation of signaling pathways that respond to extracellular stimuli and determine keratinocyte fate: proliferation, differentiation, and survival [Eckert et al., 2002]. The key role of ERK in regulation of epidermal cell proliferation was elegantly demonstrated by the growth promoting effect of constitutively expressed active ERK in human keratinocytes and by the dramatic inhibition of keratinocyte proliferation induced by overexpression of dominant negative ERK [Haase et al., 2001].

The ERK signaling pathway also regulates the induction of chemokines, cytokines, and adhesion molecules that trigger and maintain the inflammatory response [Zhang and Dong, 2005]. Indeed, interference with TNF-induced ERK activation was suggested as a target for anti-inflammatory drug development [Reimold, 2002]. The potential mediatory role of ERK in epidermal inflammation in vivo was demonstrated by Hobbs et al. [2004] who showed that expression of activated MAPK-ERK kinase (MEK1) in the epidermis of transgenic mice was sufficient to generate hyperproliferative and inflammatory skin lesion.

The activation of ERK through signaling via receptor tyrosine kinases (RTKs), including the EGF receptor (EGFR), is well characterized and involves the consecutive engagement of adaptor molecules such as Grb2 and Shc, the guanine nucleotide exchange factor Sos, the G protein Ras, the MAPK kinase kinase Raf, and the MAPK kinase MEK1/2 that phosphorylates and activates ERK. However, recent evidence points to an alternative mode of ERK activation by RTKs in some cellular contexts. This pathway involves the non-RTK c-Src, phspholipase Cγ1, RasGRP1, Ras, Raf, and MEK [Bivona et al., 2003; Philips, 2004]. TNF is known to activate the ERK cascade in many cellular scenarios [MacEwan, 2002]. However, the mode of activation is much less characterized and varies greatly in a cell type specific manner. In some systems upon binding to the cytokine, TNF receptor (TNFR1) similarly to RTKs, directly interacts with Grb2 leading to the activation of the ERK cascade. However, multiple different adaptor proteins that interact with TNFR1 are implicated in the activation of Raf such as MADD, FAN, FADD, and RIP. Some of these interactions lead to ERK activation mediated by ceramide generated by neutral sphingomyelinase. Notable among the pathways that can lead to ERK activation by TNF in epidermal cells is the rapid transactivation of the EGFR probably due to increased availability of EGFR autocrine ligands [Mascia et al., 2003; Pastore et al., 2005].

In this study, we investigated the activation of the ERK cascade in keratinocytes by TNF. Our main findings are that in the same cell TNF

activates ERK via two pathways that differ in the involvement of the EGFR and c-Src. These two modes of activation have different cellular consequences and are oppositely regulated by the hormonal form of vitamin D.

MATERIALS AND METHODS

Materials

Minimal essential medium (MEM), fetal calf serum (FCS), L-glutamine, antibiotics mixture (penstrepnystatin) and trypsin-EDTA solution B were purchased from Biological Industries (Beit Haemek, Israel). Tissue culture dishes were purchased from Corning Glass Work (Corning, NY). $1,25(OH)_2D_3$ was obtained from Hoffman-LaRoche (Nutley, NJ, the generous gift of Dr. M. Uskokovic). BSA fraction V was purchased from MP Biomedicals, Inc. (Irvine, CA). BCA Protein Assay Kit was obtained from Pierce Biotechnology, Inc. (Rockford, IL). Human recombinant TNFα was obtained from Pepro-Tech (Rocky Hill, NJ). Tyrphostin AG1478 is a gift from Professor A. Levitzki, The Hebrew University, Jerusalem, Israel. U0126 and PP1 were purchased from Alexis Biochemicals (Lausen, Switzerland). Mouse monoclonal antibody to the dually phosphorylated ERK and rabbit anti ERK1/2 polyclonal antibody were purchased from Sigma Chemical Co. (St. Louis, MO); rabbit polyclonal anti-EGFR and anti-ErbB3 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phosphotyrosine mouse monoclonal antibody, clone 4G10, was from Upstate Biotechnology (Lake Placid, NY); rabbit monoclonal anti c-Fos antibody was obtained from Cell Signaling Technology (Beverly, MA). Peroxidase-conjugated goat anti mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and peroxidase-conjugated goat anti rabbit IgG was purchased from Sigma Chemical Co. All other reagents are of analytical grade.

Cell Culture

The human keratinocyte cell line HaCaT was kindly provided by Professor N. Fusenig, German Cancer Research Center, Heidelberg, Germany. Cells were grown in MEM containing 0.075 mM Ca^{+2} (MEM-75) supplemented with 10% FCS and antibiotics. Cells were cultured in 6 cm Petri dishes and subcultured every 4 days. For Western blot analysis cells were plated in 3.5 cm Petri dishes (250,000 cells/dish). For mRNA analysis and dephosphorylation assays cells were plated in 6 cm dishes (500,000 cells/dish).

Cell Treatments

Autonomously proliferating cultures were prepared as follows: cells were plated in MEM-75 containing 10% FCS. Twenty-four hours after seeding the medium was replaced with serum-free MEM-75 containing 0.5 mg/ml BSA. The cultures were exposed to TNF (10 ng/ml) 48 h later. Where indicated, cells were treated with the various inhibitors (or vehicles) 1 h before exposure to TNF and until harvesting. Cultures were treated with $1,25(OH)_2D_3$ or its vehicle, ethanol, 48 h before exposure to TNF (ethanol concentration in cultures never exceeded 0.06%).

Western Blot Analysis

Cell extracts were prepared by lysing the cells with SDS-sample buffer without mercaptoethanol and bromophenolblue and boiled for 15 min. Mercaptoethanol and bromophenolblue were added after determination of protein content of the cell extracts by the BCA Protein Assay Kit. Samples were centrifuged before electrophoresis and subjected to SDS–PAGE under reducing conditions using 10% polyacrylamide gels (10–20 μg protein/lane). Proteins were transferred to nitrocellulose membranes and probed with the appropriate antibodies. Detection was carried out by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence and quantification by densitometry employing the VersaDoc Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA).

Dephosphorylation Assay

Cell cultures in 6 cm Petri dishes were washed with cold PBS and scraped into 0.25 ml of phosphatase buffer (HEPES 20 mM, sucrose 350 mM, KCl 150 mM, Triton ×100 0.1%, leupeptin 10 μg/ml, Aprotinin 10 μg/ml, PMSF 1 mM, pH 7.0). Cell suspensions were then sonicated in ice and transferred to glass tubes in a water bath preheated to 37°C. Twenty microliters aliquots were transferred at different time intervals (0–60 min) to microfuges containing 5 μl complete sample buffer X5 and boiled for 3 min. An aliquot of each sonicate was incubated in parallel at 37°C for 60 min in the presence of the phosphatase inhibitors vanadate (1 mM)

and NaF (0.1 M) and processed as described above. pERK levels were determined by Western blotting.

Total RNA Isolation and mRNA Determination by Real-Time PCR

Total RNA was isolated using the EZ-RNA total RNA isolation kit (Biological Industries) according to the manufacturer's instructions. Total RNA (1 μg) was then reverse transcribed by EZ-First Strand cDNA Synthesis Kit for RT-PCR (Biological Industries) using random hexamer primers according to the manufacturer's instructions. Transcribed cDNA were then amplified using TaqMan gene expression assay (Hs00174103-m1 for IL-8 and Hs9999902-m1 for the endogenous control gene, ribosomal protein large p-Zero, RPLP0) supplied by Applied Biosystems (Forster City, CA) according to the manufacturer's instructions by means of the Applied Biosystems Prism 7000 Sequence Detector (Applied Biosystems).

RESULTS

The Experimental System: Autonomously Proliferating HaCaT Keratinocytes

The objective of this work was to examine the activation of the ERK cascade by the inflammatory cytokine TNF in keratinocytes and the modulation of this process by the hormonal metabolite of vitamin D, calcitriol. The human immortalized non-tumorigenic HaCaT keratinocyte cell line was found suitable for this purpose. We have shown previously [Garach-Jehoshua et al., 1999] that in contrast to primary keratinocytes, these cells are able to grow in the absence of serum or other exogenous growth factors and active mediators that can initiate or affect intracellular signaling pathways particularly the ERK cascade. Under such conditions, used throughout this study, the proliferation of HaCaT cells is driven by the autocrine proteoglycan dependent EGFR ligands, amphiregulin, and HB-EGF. Notably, the same growth factors also support the proliferation of epidermal keratinocytes in vivo [Piepkorn et al., 1998].

Activation of the ERK Pathway by TNF

Autonomously proliferating HaCaT cells were treated with TNF in the presence or absence of the EGFR tyrosine kinase (EGFR-TK) inhibitor AG1478. Figure 1A shows the effects of TNF and AG1478 on the level of dually phosphorylated, activated ERK (pERK). In accordance with our previous findings [Garach-Jehoshua et al., 1999; Gamady et al., 2003], detectable levels of pERK are present in control cultures in the absence of any exogenous growth factor or other stimulator (see lane 1). This steady state activity of the ERK pathway is abolished by treatment with AG1487 attesting to its dependence on EGFR-TK activity (compare lane 1 to lane 2). The levels of pERK markedly increased upon 15 min treatment with TNF (lane 3). Co-treatment with AG1487 brought about only partial inhibition of ERK activation by TNF (compare lane 3 to lane 4), indicating that TNF can activate the ERK pathway in an EGFR independent manner. This partial inhibition of

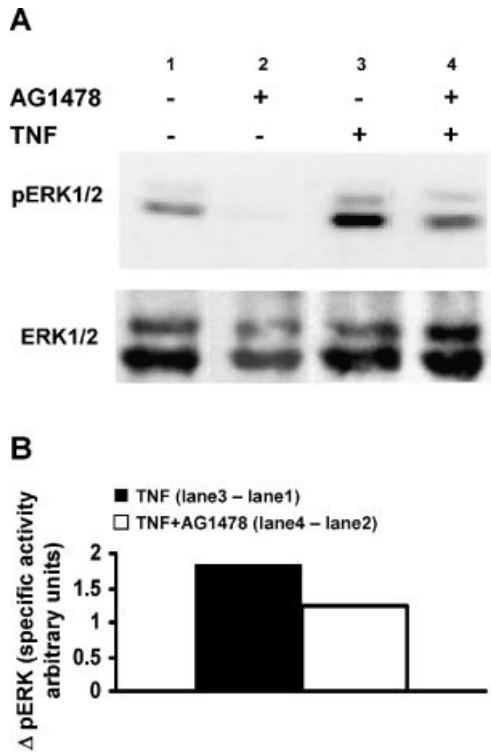

Fig. 1. Activation of ERK by TNF in the presence and absence of AG1478. HaCaT cells were treated with TNF (10 ng/ml) for 15 min after 48 h culturing in serum-free medium containing albumin. AG1478 (1 μM) was added to the cultures 1 h before treatment with TNF. Cell extracts were subjected to Western blot analysis using antibodies recognizing dually phosphorylated ERK (pERK) and total ERK (**A**). **B**: Densitometric quantification of the results in A; ΔpERK is the increase in pERK specific activity (pERK/total ERK) brought about by TNF treatment as compared with the relevant control. The results are of one representative experiment out of six.

ERK activation was greater than that expected assuming that AG1478 inhibited only the steady state activity of the ERK pathway (Fig. 1B), suggesting that TNF also stimulates the ERK pathway via an EGFR dependent mechanism. This finding was corroborated by five similar experiments showing that the EGFR independent activation ranged between 30 and 90% and was on the average $66 \pm 11\%$ (mean $\pm$ SEM) of the overall stimulation of ERK by TNF in HaCaT cells.

Figure 2 depicts the time courses of ERK stimulation by TNF: the EGFR independent mode (in the presence of AG1487) and the overall stimulation, which consists of both the EGFR dependent and independent modes. Comparison of the two time courses was facilitated by assigning an equal arbitrary value to the highest level of pERK (attained 15 min following exposure to TNF) in both experimental setups (Fig. 2B). It is evident that the duration of the stimulation is longer in the presence of an active EGFR-TK. The difference between the EGFR dependent and independent modes is probably even more pronounced than the difference between the two time courses in Figure 2B, since ERK activation in the absence of AG1487 (full triangles) is due to the cumulative effects of both modes of TNF action. An example of the possible functional consequences of the difference between the two modes of ERK activation is their effect on c-Fos protein level (Fig. 2C). Steady state levels of c-Fos are present in autonomously proliferating HaCaT cells. c-Fos protein expression is completely dependent upon the activity of both ERK and the EGFR-TK as the transcription factor is undetectable in cells treated with either U0126, the inhibitor of ERK activation, or AG1487. Whereas 1-h treatment of HaCaT cells with TNF was sufficient to markedly increase the level of c-Fos protein in an ERK dependent manner (Fig. 2C), this induction was abolished by co-treatment with AG1487. In other words, the EGFR independent activation of ERK by TNF does not support the upregulation of c-Fos protein.

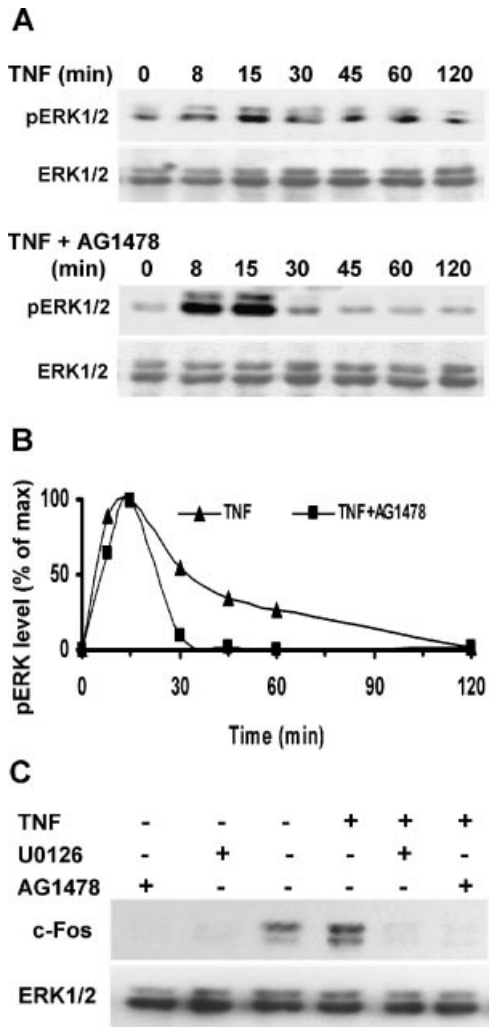

Fig. 2. Duration of ERK activation by TNF in the presence and absence of AG1478. HaCaT cells were treated with TNF (10 ng/ml) for 8–120 min following 48 h culture in serum-free MEM-75 containing BSA. AG1478 (1 μM) was added to the cultures 1 h before treatment with TNF. pERK levels were determined in cell extracts by Western blot analysis (**A**) and quantified by densitometry (**B**). The results in B are presented as percent of maximal ERK activation according to the equation: $100 \times (X_i - X_o)/(X_{max} - X_o)$, where, X_i is the specific activity (pERK/total ERK) of pERK at a specific time point; X_o is the specific activity at time 0; and X_{max} is the maximal specific activity (at 15 min). C, HaCaT cells were treated with TNF for 60 min following pretreatment with AG1478 (1 μM) or U0126 (5 μM) as described in A. c-Fos levels were determined in cell extracts by Western blot analysis. The results in A and C are of one representative experiment out of three.

The Effect of Calcitriol on the Activation of ERK by TNF

We have previously shown [Garach-Jehoshua et al., 1999] that the steady state level of ERK activity in autonomously proliferating HaCaT cells is dependent on a heterodimer of two EGFR family members (ErbB1 (EGFR) and ErbB3) that are activated by autocrine ligands. We also showed that treatment with calcitriol increases the steady state levels of pERK and that the enhancing effect of calcitriol is associated with increased signaling activity of the ErbB1-ErbB3 heterodimer. The results depicted in Figure 3A

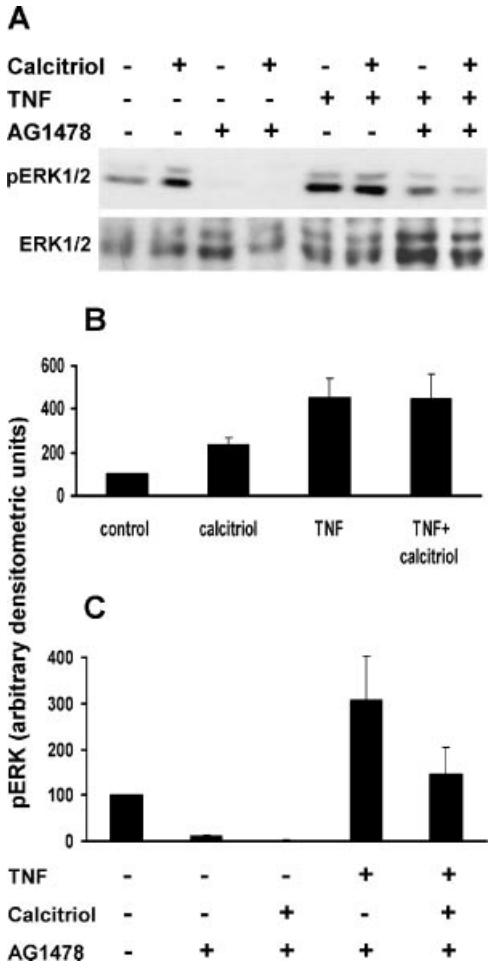

Fig. 3. Effect of calcitriol on ERK activation by TNF in the presence or absence of AG1478. **A**: HaCaT cells were cultured in serum-free MEM-75 containing BSA with or without calcitriol (100 nM) for 48 h. Cultures were then treated for 1 h with AG1478 (1 μM) or its vehicle, further treated with TNF (10 ng/ml) for 15 min and harvested. pERK and total ERK levels were determined in cell extracts by Western blot analysis. **B**: Densitometric quantification of pERK specific activity in cultures treated with TNF and calcitriol. The results represent the mean ± SEM of eight independent experiments. **C**: Densitometric quantification of pERK specific activity (pERK/total ERK) in cultures treated with calcitriol and with TNF in the presence of AG1478. The results represent the mean ± SEM of eight independent experiments. The results in B and C are presented as percent of pERK specific activity in untreated cultures.

illustrate the effect of 48-h pretreatment with calcitriol on the level of pERK in HaCaT cells treated for 15 min with TNF. In accordance with our previous findings, treatment with calcitriol as a single agent markedly increased the levels of pERK (compare lane 1 and lane 2). This increase was observed in eight independent experiments and found to be 137% ± 28% (mean ± SEM, $P < 0.002$ paired t-test) above control levels. The activation of ERK under these conditions was completely dependent on EGFR-TK activity as evidenced by the eradication of the pERK electrophoretic band following treatment with AG1478 (compare lanes 1–2 to lanes 3–4). As shown above (Figs. 1 and 2), pERK levels increased markedly following a 15-min treatment with TNF. However, it was similar in calcitriol-treated and untreated cells. This observation was confirmed in eight independent experiments (Fig. 3B). It should be recalled that in autonomously proliferating HaCaT cells treated with TNF three different signaling mechanisms contribute to the observed activity of the ERK cascade: the constitutive, steady state activity due to signaling via the ErbB1-ErbB3 heterodimer; and the two, EGFR dependent and independent, TNF-induced pathways. The response of the ERK cascade to the combined action of TNF and calcitriol is clearly sub-additive indicating that in contrast to its stimulatory action on the autonomous constitutive ERK activity, the hormone inhibits at least one of the TNF-mediated pathways. We can assess experimentally the effect of calcitriol on TNF-induced EGFR independent activation of ERK by inhibiting the two other signaling pathways with AG1478. Under these conditions calcitriol markedly inhibited ERK activation (Fig. 3A, lanes 7,8). In other words, calcitriol markedly inhibited the EGFR independent mode of ERK activation by TNF. As seen in Figure 3C this effect is reproducible and highly significant ($P = 0.001$, paired t-test, eight independent experiments).

The Involvement of Src Family Members in the Activation of ERK

The existence of two modes of ERK activation by TNF was inferred on the basis of the employment of the specific EGFR-TK inhibitor, AG1478. To further characterize the two activation processes, we examined also the involvement of Src family members in both, since protein kinases belonging to this family were shown to be involved in ERK activation by RTKs, including the EGFR, and in transactivation of the EGFR by various agents [Biscardi et al., 2000; Bivona et al., 2003; Philips, 2004]. To this end cells were treated with TNF in the presence or absence of the Src inhibitor PP-1. The effects of PP-1 (Fig. 4A) were very similar to those obtained with AG1478 (Fig. 3A). As with AG1478, treatment with PP-1 completely

abolished the constitutive steady state activity of ERK, but only partially inhibited ERK activation in TNF-treated cultures, indicating that TNF can activate the ERK cascade in a Src independent manner. However, as shown for AG1478 in Figure 1, the partial inhibition of ERK activation by PP-1 in the presence of TNF was greater than that expected assuming that Src mediates only the constitutive, steady state activity of the ERK pathway (Fig. 4B). This suggests that TNF also stimulates the ERK pathway via a Src dependent mechanism. The overlap between the EGFR independent and the Src independent modes of TNF-induced ERK activation is demonstrated by the inability of PP-1 to inhibit ERK activation in cultures treated simultaneously with TNF and the EGFR-TK inhibitor (Fig. 4C). In accordance with this notion, we find that treatment with calcitriol, while enhancing ERK activity in untreated cultures, completely abrogates the Src independent mode of TNF-induced ERK activation.

The Time and Dose Dependence of the Effect of Calcitriol on ERK Activation

Both the enhancing effect of the hormone on the steady state levels of pERK in autonomously proliferating cells and the inhibitory effect on TNF-induced EGFR independent ERK activation increase dose dependently and are already apparent at a concentration of 0.1 nM (Fig. 5). The time courses of these two opposing effects of calcitriol are presented in Figure 6. A slight increase in ERK activity can be observed as early as 30 min after treatment with calcitriol. The level of pERK increases further with time for at least 48 h. The inhibitory effect is around 60% following a 16-h treatment and attains its full extent following a 24-h treatment with the hormone. A non-genomic, effect of the hormone could be responsible for the rapid ERK stimulation observed following the 30-min treatment of autonomously proliferating cells [Gniadecki, 1996b]. However, the pronounced enhancing and inhibitory effects of calcitriol at later times are probably mediated by the well-known genomic mode of action of VDR.

Transactivation of EGFR by TNF and Its Modulation by Calcitriol

Direct examination of the EGFR dependent activation of ERK by TNF cannot be done because of lack of experimental tools that specifically and exclusively inhibit the two other signaling pathways. We therefore opted to assess directly the transactivation of EGFR by TNF. To this end, we examined the tyrosine phosphorylation of proteins in the molecular weight range (170–200 k) corresponding to the molecular mass of the ErbB family members. As seen in Figure 7, in autonomously proliferating HaCaT cells (lane 1) there is a ~180 k tyrosine-phosphorylated protein that co-migrates with

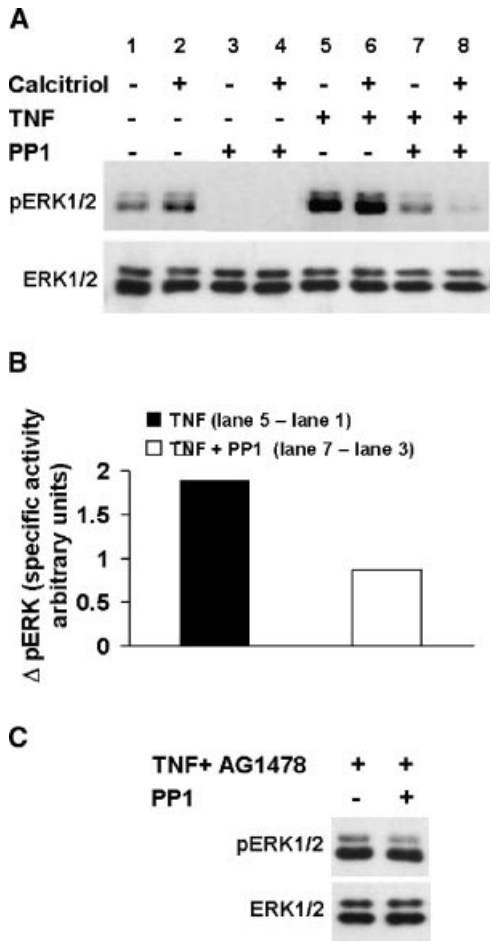

Fig. 4. The effect of PP-1 on ERK activation by TNF in the presence and absence of calcitriol. **A**: HaCaT cells were cultured in serum-free MEM-75 containing BSA with or without calcitriol (100 nM) for 48 h. Cultures were then treated for 1 h with PP-1 (10 μM) or its vehicle, further treated with TNF (10 ng/ml) for 15 min and harvested. pERK and total ERK levels were determined in cell extracts by Western blot analysis. **B**: Densitometric quantification averaging the results of two experiments like the one shown in A; ΔpERK is the increase in pERK specific activity (pERK/total ERK) brought about by TNF treatment as compared with the relevant control. **C**: Cultures were treated with AG1478 (1 μM) with or without PP-1 (10 μM) for 1 h and then treated with TNF (10 ng/ml) for 15 min. pERK and total ERK levels were determined in cell extracts by Western blot analysis.

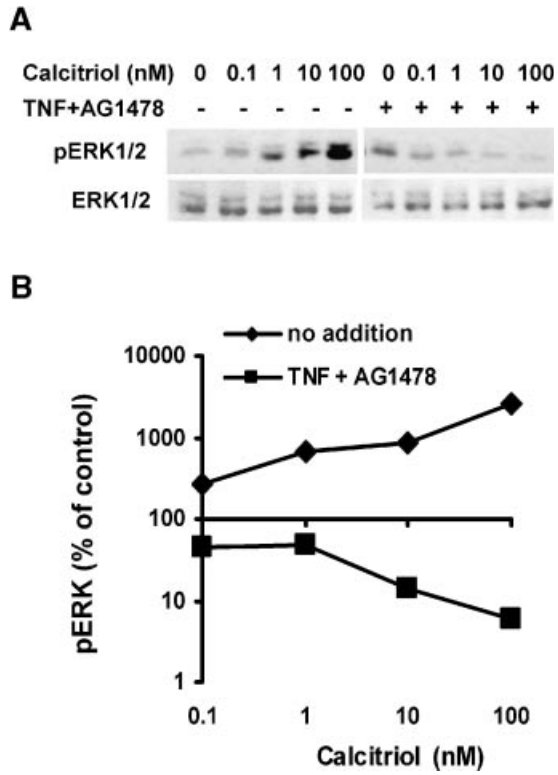

Fig. 5. The dose dependence of the effects of calcitriol on pERK level. HaCaT cells were cultured in serum-free MEM-75 containing BSA with or without calcitriol (0.1–100 nM) for 48 h and then treated for 1 h with AG1478 (1 μM) or its vehicle before treatment with TNF (10 ng/ml) for 15 min. pERK and total ERK levels were determined in cell extracts by Western blot analysis (**A**). **B**: Densitometric quantification of pERK specific activity (pERK/total ERK) in cultures treated with calcitriol in the absence or in the presence of TNF and AG1478. The results are presented as percent of pERK values in untreated cultures. The results are of one out of two independent experiments.

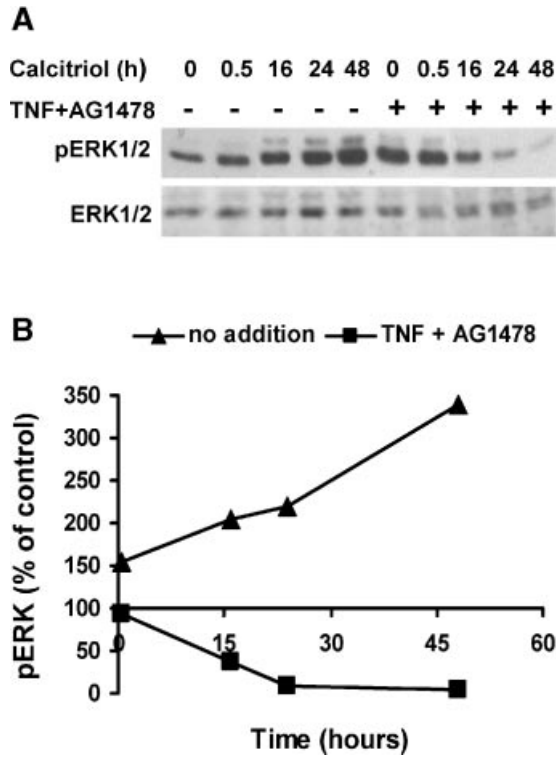

Fig. 6. The time course of the effects of calcitriol on pERK levels. HaCaT cells were cultured in serum-free MEM-75 containing BSA with or without calcitriol (100 nM) for different periods of time (0.5–48 h) and then treated for 1 h with AG1478 (1 μM) or its vehicle before treatment with TNF (10 ng/ml) for 15 min. pERK and total ERK levels were determined in cell extracts by Western blot analysis (**A**). **B**: Densitometric quantification of pERK specific activity (pERK/total ERK) in cultures treated with calcitriol in the absence or in the presence of TNF and AG1478. The results are presented as percent of pERK values in untreated cultures. The results are of one out of two independent experiments.

ErbB3 (lane 9). There is no detectable tyrosine phosphorylation in any protein that co-migrates with ErbB1. This finding is concordant with the known defective tyrosine kinase activity of ErbB3 [Alroy and Yarden, 1997]. Also in accordance with our previous report [Garach-Jehoshua et al., 1999], tyrosine phosphorylation of ErbB3 is completely dependent upon the activity of the EGFR tyrosine kinase (compare lane 1 to lane 3) and is enhanced following treatment with calcitriol (compare lane l to lane 5). Tyrosine phosphorylation of the presumed ErbB3 protein is not affected by treatment with TNF, however, a new tyrosine-phsophorylated protein appears, with a molecular weight of 170 k that corresponds to that of ErbB1 (see lanes 1,2,10). Tyrosine phosphorylation of this protein is also abolished by treatment with AG1478 (compare lane 2 to lane 4). Taken together we can conclude that treatment with TNF elicits a new and qualitatively different mode of EGFR activation that may be due to transactivation of the EGFR by TNF. Forty-eight hour pretreatment with calcitriol further enhanced tyrosine phosphorylation of the 170 k protein (compare lane 6 to lane 2), indicating that the hormone increases this transactivation process.

The Effect of Calcitriol on the EGFR Dependent Upregulation of c-Fos by TNF

Our evidence strongly suggests that calcitriol has opposing effects on ERK activation by TNF: inhibiting the EGFR independent activation while enhancing the EGFR dependent one. This interpretation is further supported by the finding that pretreatment with calcitriol enhanced the upregulation of c-Fos by TNF

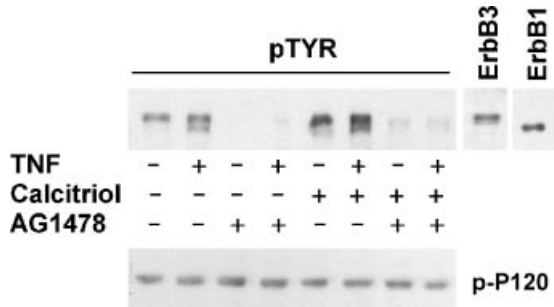

Fig. 7. Effect of TNF and calcitriol on EGFR dependent protein tyrosine phosphorylation. HaCaT cells were cultured in serum-free MEM-75 containing BSA with or without calcitriol (100 nM) for 48 h. Cultures were then treated for 1 h with AG1478 (1 μM) or its vehicle, further treated with TNF (10 ng/ml) for 15 min and harvested. Protein tyrosine phosphorylation was assessed by immunoblotting with antiphosphotyrosine antibodies and the position of ErbB1 and ErbB3 was determined with the appropriate antibodies on the same blot. A 120 k tyrosine phosphorylated protein band from the same blot is provided as a loading reference. The results are of one out of three similar experiments.

(Fig. 8) that was shown above to be completely due to the EGFR dependent activation of the ERK cascade.

EGFR Independent Upregulation of IL-8 by TNF and Its Inhibition by Calcitriol

The results depicted in Figure 9 exemplify the consequences of inhibition of the EGFR

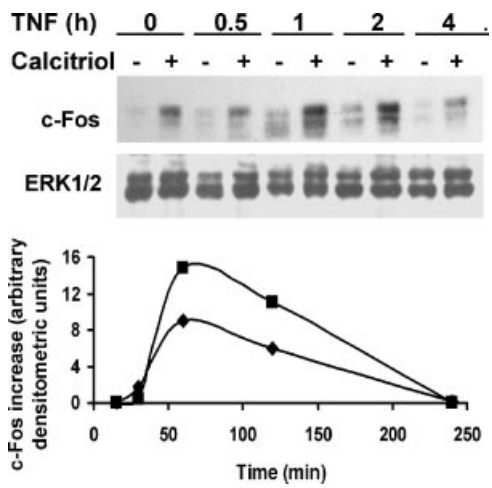

Fig. 8. The effect of calcitriol on the upregulation of c-Fos by TNF. HaCaT cells were treated with TNF (10 ng/ml) for 30–240 min following a 48 h culture with or without calcitriol (100 nM) in serum-free MEM-75 containing BSA. c-Fos levels were determined in cell extracts by Western blot analysis (**upper panel**) and quantified by densitometry (**lower panel**). c-Fos values are presented in the lower panel as the net difference between c-Fos levels in TNF-treated cultures and the appropriate control cultures treated identically except for exposure to TNF. The results are of one out of three similar experiments.

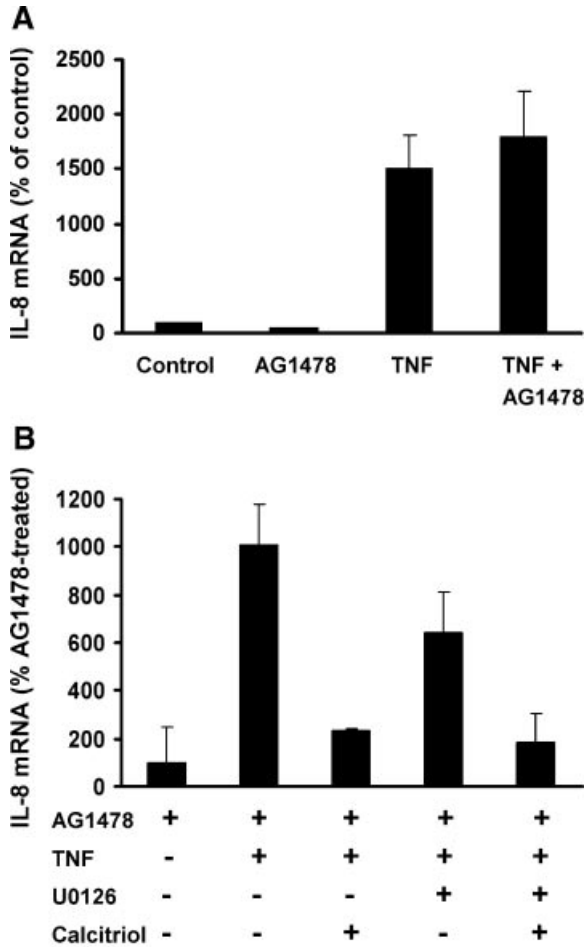

Fig. 9. Effect of calcitriol on induction of IL-8 mRNA by TNF in the presence or absence of AG1478 and U0126. HaCaT cells were treated with TNF (10 ng/ml) for 2 h following a 48 h culture with or without calcitriol (100 nM) in serum-free MEM-75 containing BSA and 1 h pretreatment with the inhibitors AG1478 (1 μM) or U0126 (5 μM). Cells were harvested and mRNA levels of IL-8 were measured by quantitative real-time PCR and normalized to the level of the endogenous control gene RPLP0. Normalized values for untreated cultures (**A**) and cultures treated with AG1478 (**B**) were assigned the arbitrary value of 100. Data are presented as A: mean ± SEM of three experiments each with three independent cultures. B: mean ± SD of three independent cultures.

independent activation of ERK by TNF. A 2 h treatment of HaCaT cells with TNF brings about a dramatic increase in mRNA of the chemokine IL-8. As seen in Figure 9A this increase is not affected by treatment with AG1478, indicating that the upregulation of IL-8 is EGFR independent. As seen in Figure 9B, co-treatment with the ERK inhibitor U0126 attenuated IL-8 induction in the presence of AG1478, demonstrating the role of EGFR

independent ERK activation in this process. The marked inhibitory effect of calcitriol on IL-8 induction (Fig. 9B) concords with the inhibition of EGFR independent ERK activation by the hormone. The residual inhibitory activity of calcitriol in the presence of U0126 may be attributed to its previously reported interference with NFκB signaling [Komine et al., 1999].

The Effect of Calcitriol on ERK Dephosphorylation

The interplay between kinases and phosphatases determines the strength and duration of ERK activation. MAPK phosphatases, members of the dual-specificity phosphatase family, contain a specific D docking domain that enables them to bind directly to pERK and dephosphorylate the threonine and tyrosine residues in its activation loop [Tonks and Neel, 2001]. We have assessed the effect of a 48-h treatment with calcitriol on the rate of ERK dephosphorylation mediated by these MAPK phosphatases. Our method relies on the stability of the complex between ERK and its associated phosphatase(s) [Zhou et al., 2001]. Following cell lysis and dilution of cytosol that stops further ERK activation, the level of pERK is determined solely by the rate of dephosphorylation. As can be seen from the results in Figure 10 pERK levels remained constant for the whole assay duration in the presence of phosphatase inhibitors (vanadate and NaF). This observation rules out any possible loss of pERK in the cell extracts due to protein degradation or non-specific dephosphorylation. On the other hand, in the absence of these phosphatase inhibitors the levels of pERK decayed in a time dependent manner. In preliminary experiments, we ascertained that the rate of dephosphorylation was independent upon the extent of cytosol dilution confirming the high affinity of the binding of pERK to the MAPK phosphatase(s). As can be seen from the logarithmic transformation of pERK levels at different time points, the decay followed first order kinetics as would be expected in the case of a preformed high affinity complex between pERK and its attached phosphatases. Comparison of the decay curves clearly demonstrated that treatment with calcitriol increased the rate constant of the dephosphorylation reaction. In control cultures this rate constant was: 0.019 ± 0.002 min^{-1} (mean $\pm$ SEM, 95% confidence

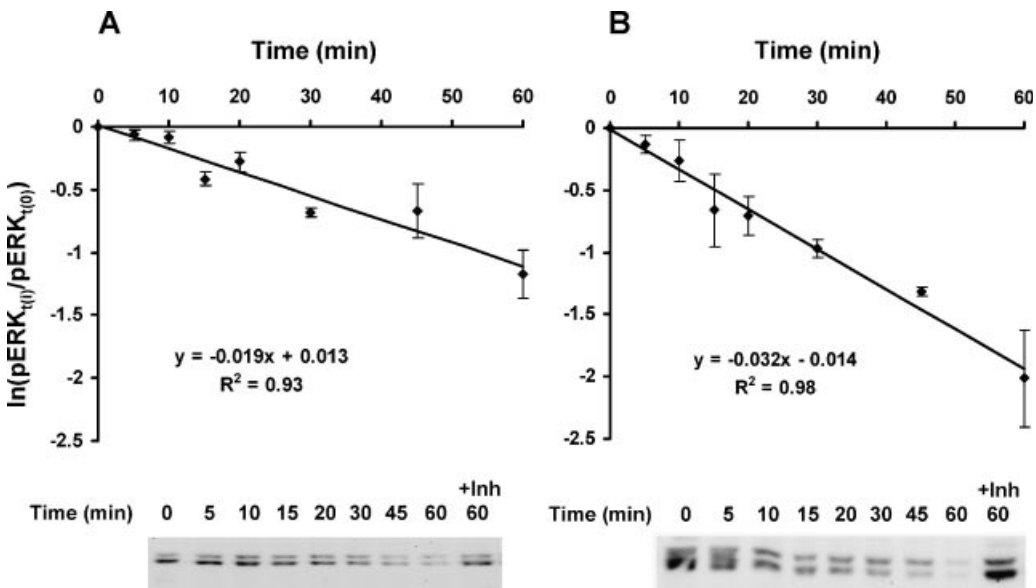

Fig. 10. The effect of calcitriol on the rate of pERK dephosphorylation. HaCaT cells were treated with calcitriol (100 nM) or its vehicle for 48 h in serum-free MEM-75 containing BSA. Cells were sonicated in lysis buffer and incubated at 37°C. Aliquots were taken at various time intervals and boiled immediately in sample buffer. Cell extracts containing the phosphatase inhibitors (Inh) vanadate and NaF were incubated in parallel. pERK levels in cell extracts were determined by Western blot analysis and quantified by densitometry. Each panel includes a Western blot illustrating the levels of pERK at all time points in the presence and absence of phosphatase inhibitors. Such experiments served as data source for the semilogarithmic presentation of the decay curves shown in the same panel in which each data point represents the mean $\pm$ SD of three experiments. **A**: Untreated cultures. **B**: Calcitriol-treated cultures. The details of the statistical analysis by linear regression of the semilogarithmic curves is given in the text.

intervals: 0.013 min^{-1}, lower; 0.024 min^{-1}, upper) and in calcitriol-treated cultures 0.032 ± 0.002 min^{-1} (mean $\pm$ SEM, 95% confidence intervals: 0.027 min^{-1}, lower; 0.037 min^{-1}, upper). This increased rate of phosphorylation could lead to decreased intensity and duration of ERK activation in calcitriol-treated cells.

DISCUSSION

The main findings of this study are that the activation of ERK by TNF in keratinocytes occurs by two distinct mechanisms, one that is mediated by EGFR and in addition requires the activity of a member of the tyrosine kinase Src family, and another mode, that is, EGFR and Src independent. Calcitriol, the hormonal form of vitamin D, exerts a dual effect on this activation: a stimulatory effect when ERK is activated in an EGFR-Src dependent manner but an inhibitory effect when ERK is activated by TNF via an EGFR-Src independent pathway.

The distinction between the pathways was based on a pharmacological approach applying the specific inhibitor of the EGFR tyrosine kinase (AG1487) and the Src family inhibitor PP-1. The activation of ERK by TNF in an EGFR and Src independent manner could be demonstrated by a straightforward experiment using these inhibitors. Existence of an additional mode of activation is inferred from the following three lines of evidence: (a) the observed levels of activated ERK in TNF-treated cultures are higher than the sum of the steady state levels due to activation by the autocrine EGFR ligands [Garach-Jehoshua et al., 1999] and the levels of pERK induced by TNF in the presence of either the EGFR-TK or the Src inhibitors. The implicit assumption behind this reasoning is that activation of ERK increases linearly with the intensity of the upstream signal; (b) the kinetics of ERK activation by TNF is different in the presence or absence of AG1478 indicating the involvement of qualitatively different activation pathways; (c) the obligatory requirement of both activated ERK and active EGFR-TK for the upregulation of the transcription factor c-Fos by TNF. Taken together the three lines of evidence support the notion of the presence of two distinct modes of ERK activation by TNF in keratinocytes: one in which the EGFR and Src tyrosine kinases are obligatory mediators, and another that is Src and EGFR independent. Src tyrosine kinase family members have been sown to partake in the transactivation of the EGFR by various agents [Biscardi et al., 2000] and also in signal transduction between RTKs and ERK [Bivona et al., 2003; Philips, 2004]. The same set of experiments also revealed that activity of a Src family member is obligatory for the steady state activation of ERK driven by endogenous EGFR ligands [Piepkorn et al., 1998; Garach-Jehoshua et al., 1999], indicating that in this experimental system a Src family tyrosine kinase is probably involved in EGFR signaling to ERK. The same can be true also following transactivaiton of the EGFR by TNF, although it should be noted that a different signaling ErbB dimer may be responsible for this mode of ERK activation.

Direct evidence for transactivation of the EGFR was obtained by looking at the TNF-induced tyrosine phosphorylation of proteins in the molecular weight range of the ErbB family members (Fig. 7). The appearance of an EGFR-TK dependent new tyrosine-phosphorylated protein that co-migrates with EGFR following treatment with TNF is consistent with the formation and activation of a new signaling dimer probably an EGFR homodimer. This new signaling mode could affect not only the intensity but also the quality of the signal emanating from the EGFR [Alroy and Yarden, 1997].

Here, we demonstrate that pretreatment with calcitriol dramatically inhibited the EGFR-Src independent activation of ERK. On the other hand, calcitriol increased the intensity of the TNF-induced EGFR transactivation that most probably leads to enhanced ERK activation. In agreement with this notion calcitriol also increased the TNF-induced expression of c-Fos that is entirely due to the EGFR dependent activation of ERK by TNF. This effect could result from the increase in the levels of the EGFR following treatment of autonomously proliferating cells with calcitriol [Garach-Jehoshua et al., 1999].

In addition to regulation by upstream signaling pathways, pERK levels may be modulated by dephosphorylation. A principal mechanism for ERK inactivation involves the action of dual-specificity MAP kinase phosphatases (MKPs) that dephosphorylate both the Thr and Tyr residues in the activation loop motif of ERK, TEY. There is abundant evidence to suggest that some MKPs contain specific kinase interaction motives that enable them to form stable, high affinity complexes with docking sites on

ERK and dephosphorylate it [Tonks and Neel, 2001; Zhou et al., 2001]. Taking advantage of this special feature of the dephosphorylation system, we have been able to examine the effect of calcitriol on the rate of ERK dephosphorylation in cell extracts. Using this methodology, we have been able to show that the first order rate constant of ERK dephosphorylation increases following treatment with calcitriol. Such an increase, reflecting a higher dephosphorylation capacity in calcitriol-treated cells, could lead to attenuation of ERK activation. Clearly, this effect of calcitriol affects both EGFR-dependent and independent manners of ERK activation by TNF. However, presumably, when the EGFR is mediating ERK activation (either by autocrine ligands in control cultures or following transactivation by TNF) the more intense upstream input to ERK overrides the increased rate of dephosphorylation.

The effects of calcitriol on ERK activation were observed at a concentration of 0.1 nM and above. This concentration is the circulating systemic concentration of calcitriol, but since the hormone is produced in vivo by the keratinocyte itself [Lehmann et al., 2004], its local concentration in the epidermis most probably exceeds that in the circulation. Therefore, it is plausible that the effects of the hormone described in this study do occur in vivo under physiological conditions.

It is becoming increasingly clear that the effects of ERK on cell fate and various cellular functions depend not only on the concentration of activated ERK molecules but also on the temporal and spatial characteristics of the activation of the ERK cascade [Murphy and Blenis, 2006; Kolch, 2005]. Differences in duration of activation and localization of the activated MAPK may lead to marked differences in the consequences of activation due to a different repertoire of substrates affected by the activated ERK molecules [Kolch, 2005]. These substrates include both cytosolic and nuclear proteins. In addition, prolonged but not transient activation of ERK leads to stabilization of some newly formed transcription factors including c-Fos [Murphy and Blenis, 2006]. In this study, we provided two examples that demonstrate the possible regulatory consequences of these differences. The sustained activation of ERK that requires transactivation of the EGFR is obligatory for upregulation of c-Fos. As c-Fos is a partner in the transcription factor complex AP-1 such upregulation may lead to modulation of transcription of numerous genes responsible for diverse biological activities. On the other hand, the induction of the chemokine IL-8 by TNF is partially ERK dependent but EGFR independent in our experimental model, and thus requires the activation of ERK by TNF in an EGFR independent manner. As predicted by our analysis, calcitriol increases the induction of-c-Fos by TNF while inhibiting the induction of IL-8 by the same cytokine, contradictory effects that presumably stem from the dual effect of the hormone on ERK activation.

The results of this study demonstrate the dual activity of calcitriol, both stimulatory and inhibitory, on one process in the multifaceted response of keratinocytes to inflammatory challenges, the activation of ERK by TNF. It is clear from our findings that the hormone may have both pro-inflammatory and anti-inflammatory effects in the epidermis and that its overall action may be strongly context specific. This study revealed the pivotal role of the EGFR in determining the extent and direction of the effect of calcitriol on the ERK cascade. Our results suggest that calcitriol may also enhance ERK activation mediated by transactivation of the EGFR in keratinocytes by other agents beside TNF. Some relevant examples are keratinocytes exposed in vitro to UV radiation [Peus et al., 1999; Xu et al., 2006], the antimicrobial peptide LL-37 [Tokumaru et al., 2005] or following sterile epidermal injury in vivo [Sorensen et al., 2006]. This insight may lead to judicious employment of vitamin D derivatives in hitherto unexplored pathological situations involving epidermal keratinocytes.

REFERENCES

Alroy I, Yarden Y. 1997. The ErbB signaling network in embryogenesis and oncogenesis: Signal diversification through combinatorial ligand-receptor interactions. FEBS Lett 410:83–86.

Barna M, Bos JD, Kapsenberg ML, Snijdewint FG. 1997. Effect of calcitriol on the production of T-cell-derived cytokines in psoriasis. Br J Dermatol 136:536–541.

Bikle DD. 2005. Vitamin D: Role in skin and hair. In: Feldman D, Pike JW, Glorieux FH, editors. Vitamin D. 2nd edition. Boston, San Diego, London: Elsevier Academic Press. pp. 609–630.

Biscardi JS, Ishizawar RC, Silva CM, Parsons SJ. 2000. Tyrosine kinase signalling in breast cancer: Epidermal growth factor receptor and c-Src interactions in breast cancer. Breast Cancer Res 2:203–210.

Bivona TG, Perez De Castro I, Ahearn IM, Grana TM, Chiu VK, Lockyer PJ, Cullen PJ, Pellicer A, Cox AD, Philips

MR. 2003. Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasG RP1. Nature 424 (6949):694–698.

Buck M, Houglum K, Chojkier M. 1996. Tumor necrosis factor-alpha inhibits collagen alpha1(I) gene expression and wound healing in a murine model of cachexia. Am J Pathol 149:195–204.

Eckert RL, Efimova T, Dashti SR, Balasubramanian S, Deucher A, Crish JF, Sturniolo M, Bone F. 2002. Keratinocyte survival, differentiation, and death: Many roads lead to mitogen-activated protein kinase. J Invest Dermatol Symp Proc 7:36–40.

Gamady A, Koren R, Ron D, Liberman UA, Ravid A. 2003. Vitamin D enhances mitogenesis mediated by keratinocyte growth factor receptor in keratinocytes. J Cell Biochem 89:440–449.

Garach-Jehoshua O, Ravid A, Liberman UA, Koren R. 1999. 1,25-Dihydroxyvitamin D3 increases the growth-promoting activity of autocrine epidermal growth factor receptor ligands in keratinocytes. Endocrinology 140: 713–721.

Gisondi P, Gubinelli E, Cocuroccia B, Girolomoni G. 2004. Targeting tumor necrosis factor-alpha in the therapy of psoriasis. Curr Drug Targets Inflamm Allergy 3:175–183.

Gniadecki R. 1996a. Stimulation versus inhibition of keratinocyte growth by 1,25-Dihydroxyvitamin D3: Dependence on cell culture conditions. J Invest Dermatol 106:510–516.

Gniadecki R. 1996b. Activation of Raf-mitogen-activated protein kinase signaling pathway by 1,25-dihydroxyvitamin D3 in normal human keratinocytes. J Invest Dermatol 106:1212–1217.

Haase I, Hobbs RM, Romero MR, Broad S, Watt FM. 2001. A role for mitogen-activated protein kinase activation by integrins in the pathogenesis of psoriasis. J Clin Invest 108:527–536.

Hobbs RM, Silva-Vargas V, Groves R, Watt FM. 2004. Expression of activated MEK1 in differentiating epidermal cells is sufficient to generate hyperproliferative and inflammatory skin lesions. J Invest Dermatol 123:503–515.

Kolch W. 2005. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat Rev Mol Cell Biol 6:827–837.

Komine M, Watabe Y, Shimaoka S, Sato F, Kake K, Nishina H, Ohtsuki M, Nakagawa H, Tamaki K. 1999. The action of a novel vitamin D3 analogue, OCT, on immunomodulatory function of keratinocytes and lymphocytes. Arch Dermatol Res 291:500–506.

LaDuca JR, Gaspari AA. 2001. Targeting tumor necrosis factor alpha. New drugs used to modulate inflammatory diseases. Dermatol Clin 19:617–635.

Lehmann B, Querings K, Reichrath J. 2004. Vitamin D and skin: New aspects for dermatology. Exp Dermatol 13 (Suppl 4):11–15.

Levy J, Gassmuller J, Schroder G, Audring H, Sonnichsen N. 1994. Comparison of the effects of calcipotriol, prednicarbate, and clobetasol 17-propionate on normal skin assessed by ultrasound measurement of skin thickness. Skin Pharmacol 7:231–236.

Lutzow-Holm C, De Angelis P, Clausen OP. 1996. Calcitriol and its analog KH 1060 induce similar changes in keratinocyte cell cycle progression after topical application to mouse skin. A bromodeoxyuridine pulse-chase flow cytometric study. J Invest Dermatol Symp Proc 1:54–59.

MacEwan DJ. 2002. TNF receptor subtype signalling: Differences and cellular consequences. Cell Signal 14: 477–492.

Mascia F, Mariani V, Girolomoni G, Pastore S. 2003. Blockade of the EGF receptor induces a deranged chemokine expression in keratinocytes leading to enhanced skin inflammation. Am J Pathol 163:303–312.

Mori R, Kondo T, Ohshima T, Ishida Y, Mukaida N. 2002. Accelerated wound healing in tumor necrosis factor receptor p55-deficient mice with reduced leukocyte infiltration. FASEB J 16:963–974.

Murphy LO, Blenis J. 2006. MAPK signal specificity: The right place at the right time. Trends Biochem Sci 31:268–275.

Nagpal S, Na S, Rathnachalam R. 2005. Noncalcemic actions of vitamin D receptor ligands. Endocr Rev 26: 662–687.

Pastore S, Mascia F, Mariotti F, Dattilo C, Mariani V, Girolomoni G. 2005. ERK1/2 regulates epidermal chemokine expression and skin inflammation. J Immunol 174: 5047–5056.

Peus D, Meves A, Vasa RA, Beyerle A, O'Brien T, Pittelkow MR. 1999. H_2O_2 is required for UVB-induced EGF receptor and downstream signaling pathway activation. Free Radic Biol Med 27:1197–1202.

Philips MR. 2004. Sef: A MEK/ERK catcher on the Golgi. Mol Cell 15:168–169.

Piepkorn M, Pittelkow MR, Cook PW. 1998. Autocrine regulation of keratinocytes: The emerging role of heparin-binding, epidermal growth factor-related growth factors. J Invest Dermatol 111:715–721.

Piguet PF, Grau GE, Allet B, Vassalli P. 1987. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. J Exp Med 166:1280–1289.

Piguet PF, Grau GE, Vassalli P. 1990. Subcutaneous perfusion of tumor necrosis factor induces local proliferation of fibroblasts, capillaries, and epidermal cells, or massive tissue necrosis. Am J Pathol 136:103–110.

Piguet PF, Grau GE, Hauser C, Vassalli P. 1991. Tumor necrosis factor is a critical mediator in hapten induced irritant and contact hypersensitivity reactions. J Exp Med 173:673–679.

Reichrath J. 2007. Vitamin D and the skin: An ancient friend, revisited. Exp Dermatol 16:618–625.

Reichrath J, Perez A, Muller SM, Chen TC, Kerber A, Bahmer FA, Holick MF. 1997. Topical calcitriol (1,25-dihydroxyvitamin D3) treatment of psoriasis: An immunohistological evaluation. Acta Derm Venereol 77:268–272.

Reimold AM. 2002. TNF alpha as therapeutic target: New drugs, more applications. Curr Drug Targets Inflamm Allergy 1:377–392.

Romer J, Hasselager E, Norby PL, Steiniche T, Thorn Clausen J, Kragballe K. 2003. Epidermal overexpression of interleukin-19 and -20 mRNA in psoriatic skin disappears after short-term treatment with cyclosporine A or calcipotriol. J Invest Dermatol 121:1306–1311.

Schroder JM, Reich K, Kabashima K, Liu FT, Romani N, Metz M, Kerstan A, Lee PH, Loser K, Schon MP, Maurer M, Stoitzner P, Beissert S, Tokura Y, Gallo RL. 2006. Who is really in control of skin immunity under physiological circumstances -lymphocytes, dendritic cells or keratinocytes? Exp Dermatol 15:913–929.

Sorensen OE, Thapa DR, Roupe KM, Valore EV, Sjobring U, Roberts AA, Schmidtchen A, Ganz T. 2006. Injury-

induced innate immune response in human skin mediated by transactivation of the epidermal growth factor receptor. J Clin Invest 116:1878–1885.

Tokumaru S, Sayama K, Shirakata Y, Komatsuzawa H, Ouhara K, Hanakawa Y, Yahata Y, Dai X, Tohyama M, Nagai H, Yang L, Higashiyama S, Yoshimura A, Sugai M, Hashimoto K. 2005. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. J Immunol 175:4662–4668.

Tonks NK, Neel BG. 2001. Combinatorial control of the specificity of protein tyrosine phosphatases. Curr Opin Cell Biol 13:182–195.

Trent JT, Kerdel FA. 2005. Tumor necrosis factor alpha inhibitors for the treatment of dermatologic diseases. Dermatol Nurs 17:97–107.

Van de Kerkhof PC. 1995. Biological activity of vitamin D analogues in the skin, with special reference to antipsoriatic mechanisms. Br J Dermatol 132:675–682.

Vissers WH, Berends M, Muys L, van Erp PE, de Jong EM, van de Kerkhof PC. 2004. The effect of the combination of calcipotriol and betamethasone dipropionate versus both monotherapies on epidermal proliferation, keratinization and T-cell subsets in chronic plaque psoriasis. Exp Dermatol 13:106–112.

Wikvall K. 2001. Cytochrome P450 enzymes in the bioactivation of vitamin D to its hormonal form. Int J Mol Med 7:201–209.

Xu Y, Voorhees JJ, Fisher GJ. 2006. Epidermal growth factor receptor is a critical mediator of ultraviolet B irradiation-induced signal transduction in immortalized human keratinocyte HaCaT cells. Am J Pathol 169:823–830.

Zhang YL, Dong C. 2005. MAP kinases in immune responses. Cell Mol Immunol 2:20–27.

Zhou B, Wu L, Shen K, Zhang J, Lawrence DS, Zhang ZY. 2001. Multiple regions of MAP kinase phosphatase 3 are involved in its recognition and activation by E RK2. J Biol Chem 276:6506–6515.