Selective loss of PMA-stimulated expression of matrix metalloproteinase 1 in HaCaT keratinocytes is correlated with the inability to induce mitogen-activated protein family kinases

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Many cell types, including fibroblasts and primary keratinocytes, increase matrix metalloproteinase 1 (MMP-1) production in response to agonists such as growth factors and phorbol esters. However, the spontaneously transformed human keratinocyte cell line HaCaT, although it increases MMP-1 production in response to epidermal growth factor (EGF), does not respond similarly to stimulation with PMA. This phenomenon occurs even though HaCaT cells remain proliferatively responsive to both agonists, suggesting a HaCaT-specific defect in a PMA-mediated signal transduction pathway. Using an inside-out approach to elucidate the source of this defect, we found that EGF, but not PMA, stimulated MMP-1 promoter activity in transiently transfected HaCaT keratinocytes. In addition, an assessment of fibroblast and HaCaT c-*fos* and c-*jun* gene expression after exposure to EGF and PMA showed that although both agonists increased the expression of c-*fos* and c-*jun* mRNA in fibroblasts, only EGF did so in HaCaT keratinocytes. Finally, we looked at the activation of mitogen-activated protein (MAP) family kinases after stimulation with EGF or PMA and found that both agonists increased the phosphorylation and activation of fibroblast extracellular signal-regulated protein kinase and c-Jun N-terminal kinase, but only EGF activated the same kinase activities in HaCaT cells. Further, the EGF-mediated increase in MMP-1 gene expression was inhibited by the MAP kinase/ERK kinase (MEK)-specific inhibitor PD98059 and the p38 kinase-specific inhibitor SB203580. Our evidence indicates that although HaCaT MAP kinases are functional, they are not properly regulated in response to the activation of protein kinase C, and that the defect that bars HaCaT MMP-1 expression in response to stimulation with PMA lies before MAP kinase activation.

Key words: ERK, c-Fos, JNK, c-Jun, signal transduction, transcription factors.

INTRODUCTION

Extracellular matrix remodelling is a hallmark feature of many biological processes, ranging from uterine involution and angiogenesis [1,2] to disease states such as osteoarthritis and tumour metastasis [3,4]. Much of this catabolic activity can be attributed to the expression and activation of matrix metalloproteinases (MMPs), a family of endopeptidases with the combined capacity to degrade nearly all components of the extracellular matrix [5]. In fact, the successful degradation of native collagen, the most abundant protein in the body, requires an initial cleavage within the molecule's triple helical domain by specific MMPs, including interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), collagenase-3 (MMP-13) or MT1-MMP (MMP-14) [5,6]. However, owing to its widespread tissue distribution, MMP-1 is believed to provide most of the biologically relevant collagenase activity in humans.

Under normal resting conditions, MMP-1 expression is low or absent and requires a stimulating agent to increase its production. This inducible expression of MMP-1 has been shown in multiple cell types after exposure to various growth factors, cytokines and tumour promoters [5], and is tightly regulated, primarily at the level of transcription [7]. The major governing elements within the MMP-1 promoter include AP-1 and ETS transcription factor-binding sites, but it is through the PMA-responsive element (TRE) of the AP-1 binding site that most of the transcriptional control of MMP-1 expression is believed to be exerted [8]. The AP-1 transcription factor itself can be formed by either the dimerization of Jun family members or by the formation of Jun–Fos heterodimers [9]. However, AP-1 components can also form cross-family dimers with other transcription factors such as those from the activating transcription factor/cAMP response element-binding protein family [10]. Differential occupation of the AP-1 binding site by various transcription factor combinations is therefore likely to have a major role in determining whether particular stimuli increase or decrease MMP-1 gene expression.

Like many transcription factors, AP-1 activity can be regulated directly or indirectly via phosphorylation by intracellular kinases such as the mitogen-activated protein (MAP) family of kinases [11]. One potential regulatory mechanism involves the direct phosphorylation of AP-1 subunits, thereby altering their

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; FCS, fetal calf serum; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; MMP, matrix metalloproteinase; PKC, protein kinase C; TRE, PMA-responsive element.

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DNA binding and/or transactivating capacity. For example, c-Fos can be phosphorylated and activated by the novel MAP kinase FEK [12], whereas c-Jun is efficiently phosphorylated by the MAP kinase c-Jun N-terminal kinase (JNK) at sites within its N-terminal transactivating domain [13] and by extracellular signal-regulated protein kinases (ERKs) at an inhibitory site located within the C-terminal DNA-binding domain [14]. A second potential AP-1 regulatory mechanism involves increased transcription of its constituent genes by activating *fos* and *jun* promoter binding factors such as Elk-1 [15], SAP-1a [16] and activating transcription factor 2 [17], all of which are potential substrates of one or more MAP kinase family member. Either of these mechanisms would increase the transcription of TRE-containing promoters such as those found within c-*jun*, c-*fos* and most MMP genes. Therefore MAP family kinases are likely to have an important role in signalling processes that regulate MMP-1 gene expression.

Although much work has been done in elucidating the biochemical and molecular regulation of MMP-1 production, comparatively little is known about the signal transduction pathways utilized by various agonists to confer this control. Therefore, to gain insights into the signalling mechanisms underlying the inducible expression of MMP-1, we investigated a defect in the human keratinocyte cell line HaCaT that leads to their inability to increase MMP-1 production in response to stimulation with PMA. Using an inside-out approach, we found that this deficiency is correlated with the cells' failure to increase MMP-1 promoter activity, as well as their inability to induce c-*fos* and c-*jun* mRNA expression and to activate the MAP family kinases ERK and JNK. Taken together, our results suggest that MAP kinases have an essential role in up-regulating MMP-1 gene expression.

MATERIALS AND METHODS

Materials

β-Glycerophosphate, PMSF, Na_3VO_4, PMA and epidermal growth factor (EGF) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Aprotinin and *p*-nitrophenyl phosphate were purchased from Roth (Karlsruhe, Germany), cycloheximide was obtained from Aldrich (Steinheim, Germany), myelin basic protein (MBP) from Gibco BRL (Life Technologies, Eggenstein, Germany), Pefabloc™ from Serva (Heidelberg, Germany), and PD98059 and SB203580 were purchased from Calbiochem (San Diego, CA, U.S.A.).

Cell culture

Primary human keratinocytes were isolated from healthy, full-thickness skin obtained by reduction mammoplasty or abdominoplasty as described previously [18] and grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) fetal calf serum (FCS), 2 mM glutamine, 0.28 mM ascorbate, 100 i.u./ml penicillin and 100 μg/ml streptomycin. The human non-tumorigenic keratinocyte cell line HaCaT [19] was kindly provided by Dr. Norbert Fusenig (German Cancer Research Center; Heidelberg, Germany) and human skin fibroblasts were obtained by explant culture from healthy adult skin. Both cell types were grown and subcultured in DMEM containing 10% (v/v) FCS, 2 mM glutamine, 0.28 mM ascorbate, 100 i.u./ml penicillin and 100 μg/ml streptomycin. Where indicated, cultures were treated for various periods with 30 ng/ml EGF/10 nM PMA/15 μM cycloheximide or, as a control, with an equal concentration of vehicle [0.04% (v/v) DMSO].

Transfection of HaCaT cells and assessment of promoter activity

The expression construct pCLβgal was created by inserting the -2240 to $+34$ fragment of the MMP-1 promoter (kindly provided by Dr. Steve Frisch, Washington University, St. Louis, MO, U.S.A.) into the *Hin*dIII restriction site of the β-galactosidase expression vector pCH110 (Pharmacia, Uppsala, Sweden). For transfections, HaCaT keratinocytes (2×10^4 cells/cm^2) were transfected with 0.6 μg of pCLβgal DNA by using the lipid-mediated transfection reagent DMRIE-C (Gibco BRL). After they had formed a confluent monolayer, cells were stimulated for 48 h with 30 ng/ml EGF or 10 nM PMA or vehicle, after which cell lysates were isolated and β-galactosidase activity was assessed by using the Galacto-Light™ system (Tropix, Bedford, MA, U.S.A.). Samples were read on a Lumat luminometer (Berthold, Wildbad, Germany). The values obtained for β-galactosidase activity were normalized to identical amounts of protein in the cell lysates, which were determined with a commercial assay (Bio-Rad) and are expressed as averages for quadruplicate samples.

Northern blot analysis

Total RNA was isolated and used for Northern hybridization as described previously [18]. cDNA probes were prepared by labelling 2 kb cDNA fragments of human MMP-1 [20], c-*fos* [21] or c-*jun* [22] with [α-^{32}P]dCTP by using a High Prime DNA Labeling Kit™ (Boehringer Mannheim, Mannheim, Germany). Unincorporated nucleotides were removed by precipitation with ethanol/ammonium acetate in the presence of excess carrier DNA.

Immunostaining

To assess differentiation, HaCaT keratinocytes (2×10^4 cells/cm^2) were seeded and grown in 8-well chamber slides (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Cells were treated with 10 nM PMA or 2 mM sodium butyrate or vehicle for 48 h, then fixed with formalin [4% (v/v) formaldehyde/PBS] and processed for immunostaining with a 1:20 dilution of the antikeratin 10 monoclonal antibody K8.60 (Sigma, Deisenhofen, Germany). Bound primary antibody was detected using the APAAP System™ from DAKO (Hamburg, Germany) following the manufacturers recommended conditions. Negative controls consisted of using pre-immune murine serum or the omission of the primary antibody.

Western blot analysis

To assess secreted MMP-1, fibroblast and HaCaT monolayers were cultured in serum-free DMEM for 24 h and then stimulated with 30 ng/ml EGF or 10 nM PMA or vehicle. After an additional 24 h, conditioned medium was harvested for Western blotting with an affinity-purified rabbit anti-(human MMP-1) polyclonal antibody (kindly provided by Dr. Peter Angel, German Cancer Research Center, Heidelberg, Germany). To assess ERK phosphorylation, subconfluent HaCaT or fibroblast monolayers were serum-starved for 48 h, then stimulated for 10 min with 30 ng/ml EGF or 10 nM PMA or vehicle. Monolayers were washed with ice-cold PBS containing 1 mM Na_3VO_4, lysed at 88 °C in 500 μl of Laemmli sample buffer containing 5% (v/v) 2-mercaptoethanol and boiled for 10 min. Equal volumes of cell lysate were resolved on SDS/8% (w/v) polyacrylamide gels, transferred to Hybond-C Super™ (Amersham, Little Chalfont, Bucks., U.K.) and probed with a rabbit polyclonal ERK antibody (1:200 dilution) recognizing both the phosphorylated and non-phosphorylated forms of ERK1 and

ERK2 (Santa Cruz BioTechnology, Santa Cruz, CA, U.S.A.). Bound primary antibody was detected by using a horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) and detected by enhanced chemiluminescence (ECL™; Amersham).

MBP kinase assay

MBP kinase assays were performed as described [23], with modifications. In brief, fibroblasts and HaCaT cells (5×10^4 cells/cm^2) were seeded and cultured for 24 h in DMEM containing 1 % (v/v) FCS. Cells were then treated for 10 min with 30 ng/ml EGF or 10 nM PMA or vehicle, then washed with ice-cold PBS containing 1 mM Na_3VO_4, scraped into 1 ml of homogenization buffer [20 mM Hepes (pH 7.0)/1 mM dithiothreitol (DTT)/20 mM EGTA/15 mM magnesium acetate/40 mM *p*-nitrophenyl phosphate/0.1 mM PMSF] and disrupted with 25 strokes of a Dounce homogenizer. Cellular debris was removed by centrifugation in a tabletop ultracentrifuge (10 min, 30000 ***g***). For each 30 μl kinase assay, 5 μl of 'cleared' supernatant was added to 20 μl of reaction buffer [2 mM DTT/0.3 mM EDTA/50 mM β-glycerophosphate (pH 7.3)/0.33 mg/ml MBP/15 mM $MgCl_2$/7 mM NaF]. The phosphorylation reaction was initiated by the addition of 5 μl of ATP {0.33 mM ATP (pH 7.0)/33 μM $MgCl_2$/0.33 μl of 3000 Ci/mM [γ-^{32}P]ATP} and incubated at 30 °C for 10 min. Reactions were terminated by spotting 25 μl on 1 cm^2 phosphocellulose paper (Whatman, Maidstone, Kent, U.K.). Filters were washed five times in 0.85 % (v/v) phosphoric acid and once with 95 % (v/v) ethanol; the incorporated radioactivity was quantified by Čerenkov counting.

ERK and JNK immunoactivity assays

ERK and JNK activity assays were performed *in vitro* by using the immunoprecipitated kinase as described [23], with modifications. In brief, fibroblasts and HaCaT cells (5×10^4 cells/cm^2) were seeded and cultured for 48 h in serum-free DMEM. Where indicated, cells were pretreated for 30 min with the indicated concentration of either PD98059 or SB203580 before the addition of agonist. Cells were then stimulated either for 60 min with 13.5 μM cycloheximide or for 10 min with 30 ng/ml EGF or 10 nM PMA or vehicle, then washed with ice-cold PBS containing 1 mM Na_3VO_4, scraped into 1 ml of lysis buffer [20 mM Tris/HCl (pH 7.5)/2 mM EDTA/150 mM NaCl/1 % (v/v) Triton X-100/10 % (v/v) glycerol/25 mM β-glycerophosphate/5 μg/ml Aprotinin/0.25 mg/ml Pefabloc/1 mM Na_3VO_4/1 mM PMSF], transferred to an Eppendorf tube and incubated on ice for 30 min. Cellular debris was removed by centrifugation and equal volumes of 'cleared' lysate (500 μl for ERK assay and 750 μl for JNK assay) were brought to 1 ml with lysis buffer and incubated at 4 °C for 2 h with either 4 μl of anti-ERK antibody (UBI, Lake Placid, NY, U.S.A.) or 3 μl of anti-JNK antibody (Santa Cruz BioTech), both of which were precoupled to 20 μl of Protein A–agarose (Boehringer Mannheim). Immunocomplexes were collected by centrifugation and washed twice with lysis buffer and once with either ERK wash buffer (0.3 mM EDTA/50 mM β-glycerophosphate/15 mM $MgCl_2$/1 mM Na_3VO_4/7 mM NaF) or JNK wash buffer [25 mM Hepes (pH 7.4)/25 mM β-glycerophosphate/25 mM $MgCl_2$/0.1 mM Na_3VO_4]. After the final wash, kinase–agarose pellets used to determine ERK activity were resuspended in 20 μl of ERK reaction buffer (ERK wash buffer containing 0.33 mg/ml MBP and 2 mM DTT) and those used for JNK activity were resuspended in 25 μl of JNK reaction buffer [JNK wash buffer containing 2 μg of glutathione S-transferase (GST)–Jun (residues 1–165) and 1 mM DTT]. ERK reactions were initiated by the addition of 10 μl ERK/ATP mix {0.33 mM ATP/6.7 mM $MgCl_2$/0.33 μl of 3000 Ci/mM [γ-^{32}P]ATP} and JNK reactions were initiated by the addition of 5 μl of JNK/ATP mix {0.33 mM ATP/0.5 μl of 3000 Ci/mM [γ-^{32}P]ATP}. Both kinase reactions were incubated for 20 min at 30 °C and stopped by the addition of 6× Laemmli sample buffer. Reactions were then boiled for 3 min and 20 μl of each was resolved by SDS/PAGE [15 % (w/v) gel for ERK assays or 12 % (w/v) gel for JNK assays]. Gels were dried between cellophane sheets (Novex, San Diego, CA, U.S.A.) and exposed to X-ray film (Hyperfilm; Amersham). Radiographic bands were scanned and quantified with NIH IMAGE 1.61 software.

RESULTS

Resistance to PMA in HaCaT keratinocytes

Under non-stimulated conditions, MMP-1 expression is typically low or absent and increases only after challenge with agonist. In culture, both fibroblasts and primary keratinocytes increase MMP-1 mRNA and protein production in response to stimulation with EGF or PMA (Figure 1). However, comparable studies with HaCaT cultures indicate that although EGF increases the accumulation of HaCaT MMP-1 mRNA and protein, these cells were unique in being unable to augment either of these parameters in response to stimulation with phorbol ester (Figure 1). These results indicate that although the HaCaT MMP-1 gene is transcriptionally active in response to stimulation with EGF, it lacks the ability to respond similarly to stimulation by phorbol ester, suggesting that signalling pathways used to regulate certain PMA responses have become either blocked or disabled. Also, because of the apparent similarities in the regulated expression of MMP-1 in multiple cell types, fibroblasts were used as a comparison in subsequent experiments.

To test whether this loss of responsiveness to PMA was specific for MMP-1 regulation or was generalized, we investigated other phorbol ester-mediated cellular responses. Application of phorbol esters to mouse skin promotes murine keratinocyte hyperproliferation and tumour formation [24]. In culture, PMA can increase the proliferation of fibroblasts [25] and of susceptible keratinocytes, whereas it induces differentiation in others [26]. To test whether HaCaT keratinocytes remain proliferatively responsive to phorbol ester treatment, subconfluent cultures were stimulated with either PMA or, as a positive control, EGF [27], then harvested at various times for determination of cell numbers. Results clearly show that both agonists retained their ability to increase HaCaT proliferation over control conditions (Figure 2A). In addition, cultures treated with phorbol ester or sodium butyrate, a known keratinocyte-differentiating agent, induced the expression of the early keratinocyte differentiation marker keratin 10. However, in contrast with sodium butyrate, PMA induced keratin 10 expression only in a subpopulation of HaCaT cells (Figure 2B). Taken together, these results indicate that, whereas certain PMA-mediated signalling pathways are defective in HaCaT keratinocytes, others remain functional. Further, because PMA mediates many of its effects through the activation of protein kinase C (PKC), our results, along with those of others showing the presence of PMA-activatible PKC isoforms in HaCaT cells [28], indicate that PKC is functionally active in these cells and is therefore unlikely to mediate the observed non-responsiveness to PMA.

MMP-1 promoter activity inducible by EGF and by PMA

To elucidate the defect that leads to insensitivity of the HaCaT MMP-1 gene to PMA, we used an inside-out approach and assessed the effect of EGF and PMA on the expression or activity of likely MMP-1 gene regulators. MMP-1 promoter inducibility

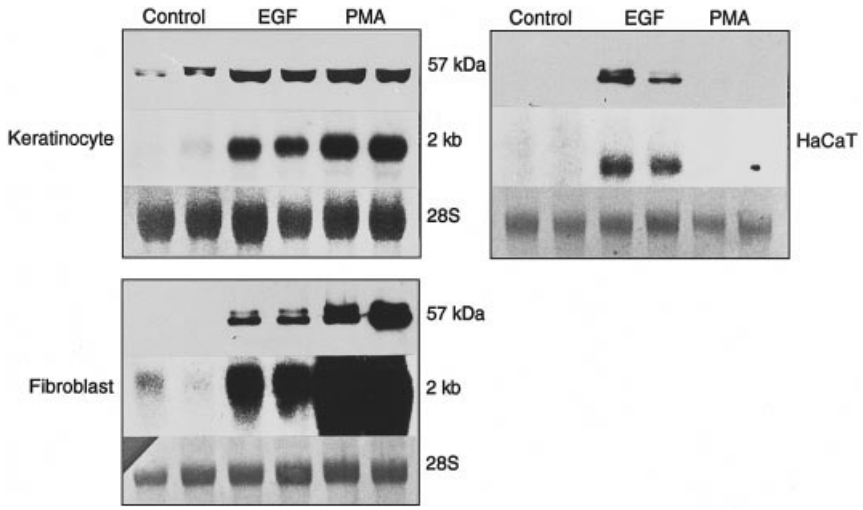

Figure 1 Effect of stimulation with EGF and with PMA on MMP-1 production in skin fibroblasts, primary keratinocytes and HaCaT keratinocytes

Confluent monolayers of each cell type were serum-starved for 24 h, then stimulated for an additional 24 h with either 30 ng/ml EGF, 10 nM PMA or vehicle (control). Conditioned media were harvested for the detection of MMP-1 by Western blot analysis by using an affinity purified rabbit polyclonal anti-(human MMP-1) antibody. Bound primary antibody was detected by using a horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence. Total mRNA was isolated from monolayers by using guanidine thiocyanate, then resolved through a formaldehyde-containing agarose gel and transferred to Hybond N^+. MMP-1 mRNA was detected by using a ^{32}P-labelled cDNA probe. Autoradiographic exposure was for 16 h. RNA loading was assessed visually by ethidium bromide staining of 28 S ribosomal RNA (28S). The RNA and protein data are both shown; 2 kb, indicates the mRNA size of MMP-1; 57 kDa, indicates the protein size.

in HaCaT keratinocytes was first tested by transient transfection with an MMP-1 promoter–β-galactosidase reporter gene construct, followed by stimulation with EGF or PMA [8,29]. The promoter–reporter gene construct spanning nucleotide positions -2240 to $+34$ contained the proximal TRE at position -73, which contributes to induction by PMA [8,29], and the adjacent PEA3 site, which has been shown to act co-operatively in mediating phorbol inducibility [8,30]. Quantification of β-galactosidase activity in stimulated HaCaT cell lysates showed that EGF caused a 1.6-fold increase in reporter gene activity, an increase comparable to that reported in transiently transfected fibroblasts [31]. PMA treatment, in contrast, had no effect on reporter gene activity in HaCaT cells, although this construct was strongly inducible by PMA in fibroblasts (results not shown). These results are not due simply to an increase in cell number (see Figure 2A), in which case a similar increase in reporter gene activity should have been detected in both EGF-stimulated and PMA-stimulated cell lysates. Taken together, our transfection and MMP-1 mRNA results indicate that the observed non-responsiveness of the HaCaT MMP-1 gene to PMA is not due to a transcriptional defect but precedes MMP-1 gene activation.

Inducibility of c-*fos* and c-*jun* mRNA expression by EGF and PMA

In many instances the transcriptional regulation of MMP genes involves the activation and/or expression of Fos and Jun family members [9,32], proteins that are capable of associating into a functional AP-1 transcription factor [33]. To determine the effect of treatment with EGF and with PMA on the expression of c-*fos* and c-*jun*, Northern blot analysis was performed with total RNA isolated from fibroblast and HaCaT cultures after stimulation with EGF or PMA. Results clearly show that, whereas both EGF and PMA induced c-*fos* expression in fibroblasts, EGF, but not PMA, caused a similar induction in HaCaT keratinocytes (Figure 3). Also, although c-*jun* mRNA was seen in fibroblast cultures under basal conditions, both EGF and PMA were able to increase this expression. Interestingly, c-*jun* mRNA was not detectable in unstimulated HaCaT cultures but was clearly induced after treatment with EGF. PMA, in contrast, had no comparable effect on the expression of HaCaT c-*jun* mRNA (Figure 3). These results indicate that although the c-*fos* and c-*jun* genes are transcriptionally regulated in HaCaT keratinocytes by agonists such as EGF, they are non-responsive to treatment with phorbol ester. Furthermore, these results suggest that the signalling defect that leads to the insensitivity of the HaCaT MMP-1 gene to PMA also precedes the induction of c-*fos* and c-*jun*.

Determination of ERK activation by EGF and PMA

The activity and expression of the transcription factor AP-1 can in turn be regulated by the phosphorylation of its constituent parts by MAP kinases [11], a family of enzymes including ERK, JNK and p38. Although various signal transduction pathways are activated in response to stimulation with EGF and with PMA, one major response shared by both agonists is the activation of MAP family kinases. To determine whether these agonists retained their ability to activate MAP kinases in HaCaT keratinocytes, we first investigated the effects of stimulation with EGF and with PMA on ERK activation. With the use of Western blot analysis, ERK phosphorylation and activation have been shown to be correlated with a decrease in the electrophoretic mobility of both the p42 and p44 ERK isoforms, causing an upward shift in the apparent molecular mass of both proteins. As shown in Figure 4, both EGF and PMA stimulated ERK phosphorylation in fibroblast cultures, with EGF causing a complete activation of both ERK isoforms. Interestingly, in HaCaT keratinocytes the p42 ERK seems to be expressed preferentially; very little p44 ERK was detected. However, EGF stimulation did increase the phosphorylation/activation of this enzyme, whereas PMA had no significant effect (Figure 4). These findings indicate that, although HaCaT ERK kinase is apparently

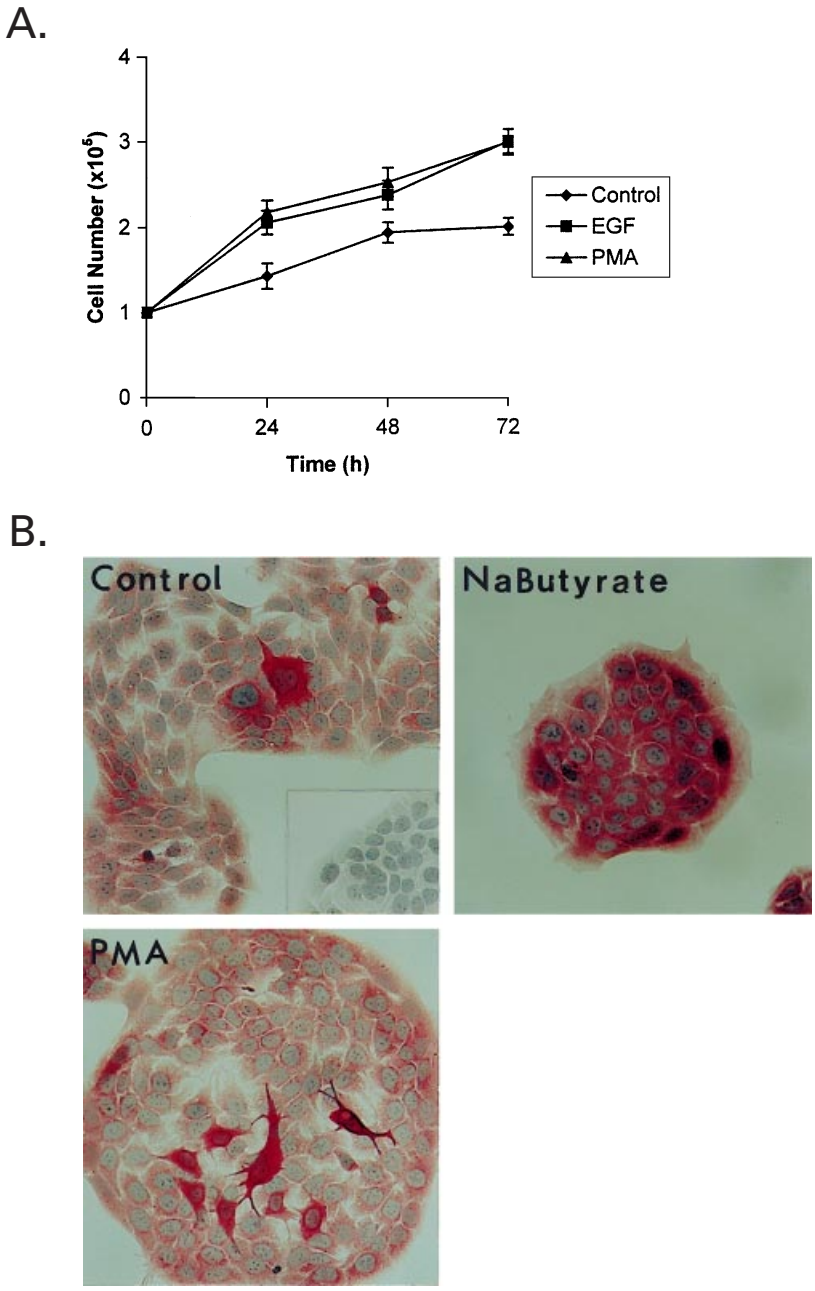

Figure 2 Effect of stimulation with EGF and with PMA on the proliferation of HaCaT keratinocytes

(**A**) HaCaT keratinocytes were seeded and incubated under serum-free conditions for 24 h, after which they were stimulated with 30 ng/ml EGF, 10 nM PMA or vehicle (control). At the indicated times, cells were harvested by treatment with trypsin and then counted. Results are means ± S.E.M. for three independent experiments performed in duplicate. (**B**) To assess HaCaT keratinocyte differentiation, the cells were treated for 48 h with 10 nM PMA or 2 mM sodium butyrate or vehicle (Control), then fixed with formalin and processed for immunostaining with a 1:20 dilution of the antikeratin 10 monoclonal antibody K8.60 or pre-immune murine serum (insert). Bound primary antibody was detected using the APAAP System.

activatable by EGF stimulation, the kinase lacks the ability to respond similarly to stimulation by phorbol ester.

To verify that the phosphorylation-induced shift in the molecular mass of ERK is indeed correlated with kinase activation, we also determined the ability of EGF-stimulated or PMA-stimulated whole-cell lysates to phosphorylate the exogenous ERK substrate MBP in an activity assay *in vitro*. Results with fibroblast cell lysates showed an 11.5-fold increase in MBP phosphorylation in response to EGF and an 8.5-fold increase in response to stimulation with PMA (Figure 5A). In HaCaT cell lysates, the basal level of MBP phosphorylation was approx. 2.7-fold that in fibroblast cell lysates. However, stimulation with EGF resulted in a 3.2-fold increase in MBP phosphorylation. Stimulation with PMA resulted in only a 1.6-fold increase (Figure 5A). Because multiple cytoplasmic kinases can use MBP as a potential substrate, we also immunoprecipitated ERK before assessing its kinase activity *in vitro*. By using this method to determine specifically the effect of EGF and stimulation with

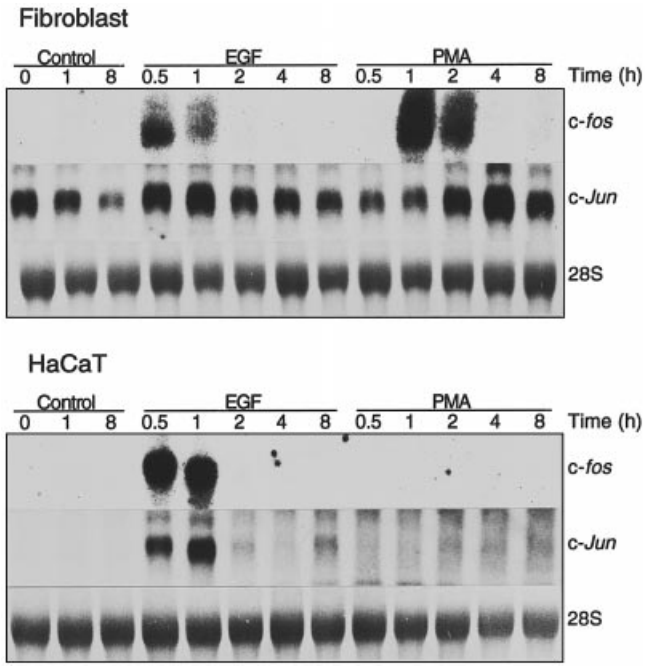

Figure 3 Time course of EGF- and PMA-stimulated c-*fos* and c-*jun* mRNA expression in fibroblast and HaCaT cultures

Confluent fibroblast and HaCaT cultures were serum-starved for 24 h, then stimulated with 30 ng/ml EGF or 10 nM PMA. Total RNA was isolated at the indicated times and processed for Northern blot hybridization. c-*fos* or c-*jun* mRNA were detected by using ^{32}P-labelled cDNA probes. Autoradiographic exposure was for 7 days. RNA loading was assessed visually by ethidium bromide staining of 28 S ribosomal RNA.

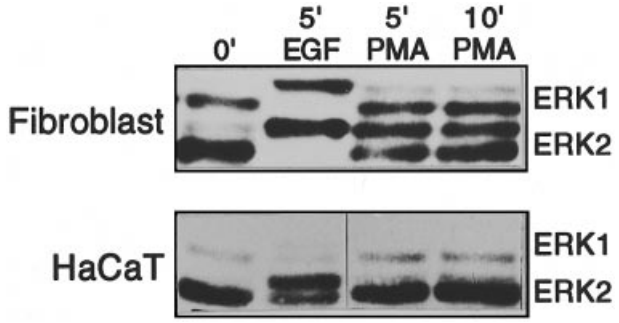

Figure 4 Western blot detection of ERK1 and ERK2 in fibroblast and HaCaT cell lysates after stimulation with EGF and with PMA

To assess ERK activation, subconfluent monolayers were serum-starved for 48 h, then stimulated for 5 or 10 min with 30 ng/ml EGF, 10 nM PMA or vehicle. Cultures were then washed, lysed in Laemmli sample buffer and processed for Western blot detection by using a rabbit polyclonal antibody (1:200 dilution) that recognized both the phosphorylated and non-phosphorylated forms of ERK1 and ERK2. Bound primary antibody was detected by using a horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence. The autoradiograph is representative of three experiments.

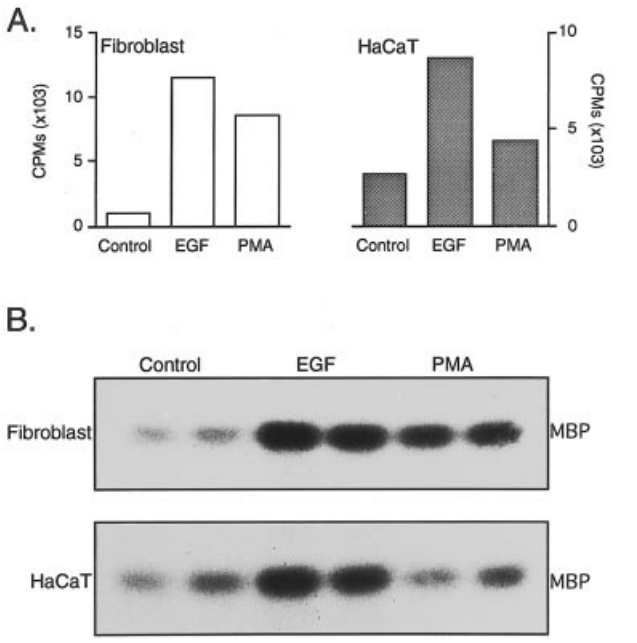

Figure 5 Detection of ERK activity in EGF- and PMA-stimulated fibroblast and HaCaT cell lysates

Subconfluent monolayers were serum-starved and then stimulated for 10 min with 30 ng/ml EGF, 10 nM PMA or vehicle (control). (**A**) Cell lysates were isolated and used to determine MBP phosphorylation as described in the Materials and methods section. Reaction mixtures were spotted on phosphocellulose paper and washed, then incorporation of radioactivity was quantified by Čerenkov counting. Values are averages from duplicate samples of two independent experiments. (**B**) ERK activity assays were performed *in vitro* by using immunoprecipitated kinase as described in the Materials and methods section. Reactions were resolved by SDS/15% (w/v) polyacrylamide gels, which were then dried and exposed to X-ray film for 3 h. The autoradiograph is representative of five experiments.

PMA on ERK activity, similar results to those from our MBP phosphorylation assay were obtained. EGF and PMA increased fibroblast ERK activity 3.6-fold and 2.2-fold respectively, but only EGF increased HaCaT ERK activity, by 2.1-fold (Figure 5B). In addition, we found that both EGF and serum stimulated the nuclear translocation of ERK [34] in both fibroblast and HaCaT cells (results not shown), indicating that HaCaT ERKs are functional not only *in vitro* but also *in vivo*. These results show convincingly that although HaCaT ERK is phosphorylated and activated in response to EGF stimulation, it lacks the ability to respond similarly to stimulation with PMA.

Effect of ERK and p38 inhibitors on MMP-1 mRNA expression

Pharmacological inhibitors of MAP family kinases are now available to test the involvement of these enzymes during various signalling processes. Using this approach, we examined whether the loss of ERK or p38 activity could affect the induction of MMP-1 mRNA. PD98059, an inhibitor of the upstream ERK activator MAP kinase/ERK kinase (MEK) [35], completely blocked the EGF-mediated activation of ERK in both HaCaT cells and fibroblasts, as well as the expression of MMP-1 mRNA (Figure 6). Another inhibitor, wortmannin, which irreversibly blocks phosphoinositide 3-kinase activity, has also been shown to block the activation of ERK after stimulation by certain agonists [36]. In the present study, however, this inhibitor had no effect on the EGF-mediated induction of MMP-1 mRNA in HaCaT keratinocytes and fibroblasts (results not shown). Finally, the addition of SB203580, which specifically inhibits p38 activity [37], was also found to block the EGF-mediated accumulation of MMP-1 mRNA but did so without affecting the EGF-mediated activation of ERK (Figure 6). Because neither inhibitor affected the PMA-mediated induction of MMP-1 in fibroblasts (results not shown), a different signalling pathway such as JNK seems to

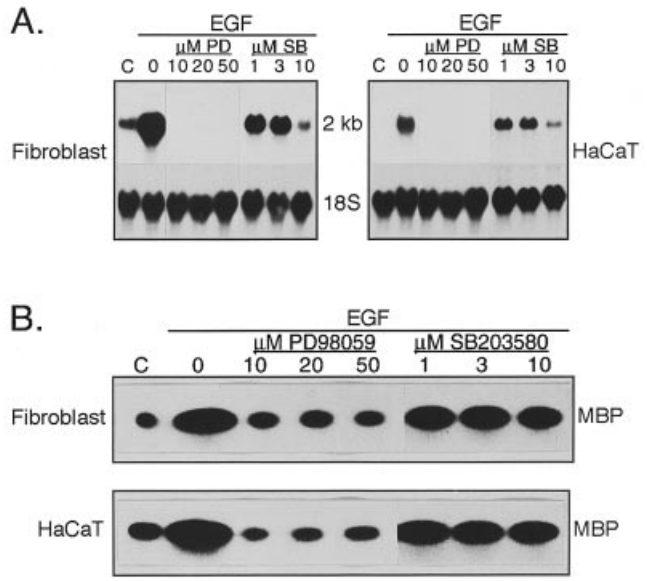

Figure 6 Effect of ERK and p38 inhibitors on kinase activity and expression of MMP-1

Subconfluent monolayers of dermal fibroblasts and HaCaT cells were serum-starved for 48 h before being pretreated with the indicated concentration of PD98059 or SB203580. Cells were then stimulated with either 30 ng/ml EGF or vehicle (C). (**A**) MMP-1 mRNA was detected in total RNA isolated from cells stimulated for 24 h by using a ^{32}P-labelled cDNA probe. RNA loading was assessed by hybridization with an 18 S ribosomal RNA-specific oligonucleotide. (**B**) ERK activity assays were performed *in vitro* by using immunoprecipitated kinase as described in the Materials and methods section. Reactions were resolved through SDS/15% (w/v) polyacrylamide gels, which were then dried and exposed to X-ray film for 3 h. The results are representative of three experiments.

be responsible for mediating the inductive response of PMA. Taken together, our results indicate that ERK and p38 activities are required in the EGF-initiated signalling pathway that leads to the elevated gene expression of MMP-1.

Determination of JNK activation by EGF and PMA

Growth and differentiation-inducing agonists seem to use the ERK pathway preferentially as a major effector, whereas stresses such as UV irradiation and cycloheximide activate the JNK and p38 pathways [38]. However, EGF and PMA have been shown to increase both ERK and JNK activities [39], indicating the presence of considerable cross-talk between these signalling cascades. To assess whether other MAP family kinases are activated in HaCaT keratinocytes by stimulation with phorbol ester, we determined JNK activity after agonist challenge by using GST–Jun as a substrate. As previously reported, EGF, PMA and cycloheximide stimulation each increased JNK activity in fibroblast cell lysates, by 2.5-fold, 3.7-fold and 5.4-fold respectively, but only EGF stimulated a similar 3-fold induction of HaCaT JNK activity (Figure 7). These results show that another MAP family kinase is properly regulated by mitogen stimulation in HaCaT cells but that other agonists, including PMA and cycloheximide, lack the ability to increase JNK activity of HaCaTs. Our evidence indicates that although multiple members of the HaCaT MAP family kinases are functional, they are not properly regulated in response to the activation of PKC, and that the defect that bars HaCaT MMP-1 expression in response to stimulation with PMA lies before the activation of MAP kinase.

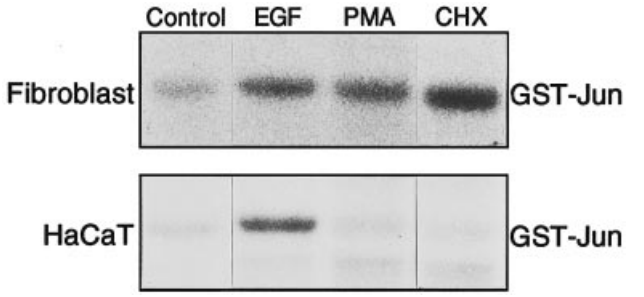

Figure 7 Detection of JNK activity in EGF- and PMA-stimulated fibroblast and HaCaT cell lysates

Subconfluent monolayers were serum-starved, then stimulated either for 60 min with 13.5 μM cycloheximide (CHX) or for 10 min with 30 ng/ml EGF, 10 nM PMA or vehicle (control). Subsequently, JNK was immunoprecipitated and used in a JNK activity assay *in vitro* by using GST–Jun as a substrate as described in the Materials and methods section. Reactions were resolved through SDS/12% (w/v) polyacrylamide gels, which were then dried and exposed to X-ray film for 16 h. The results are representative of three independent experiments.

DISCUSSION

MMP expression and activation are critical determinants during physiological and pathological events characterized by matrix degradation [5,6,9]. Although many studies have investigated the biochemical and molecular regulation of MMPs, comparatively little is known about the signal transduction pathways used to control their expression. In the present study, we used the human keratinocyte cell line HaCaT, which unlike primary keratinocytes and fibroblasts does not increase MMP-1 mRNA or protein synthesis in reaction to stimulation with phorbol ester. This observation is not, however, due to a general loss of PMA-mediated responses, because distinct HaCaT subpopulations either proliferate or differentiate in response to treatment with phorbol ester. Indeed, these cells have been shown to express and correctly regulate the activity of multiple PKC isoforms after stimulation with PMA [28]. Instead, our results suggest that certain signal transduction pathways used to increase MMP-1 gene expression are functional in HaCaT cells, whereas those used to mediate select phorbol ester responses are either blocked or disabled. Therefore further investigations were performed to gain insight into the potential role of MAP family kinases during signalling events that regulate MMP-1 gene expression. Results from these studies show clearly that although the MAP kinases ERK and JNK are activated in HaCaT cells after stimulation with EGF, they are not similarly regulated in response to treatment with PMA. Taken together, our results indicate that the defect that causes the HaCaT MMP-1 gene to be PMA-resistant lies before MAP kinase activation; further, they suggest that MAP kinase pathways have an important role in regulating MMP-1 gene expression in response to diverse stimuli.

Because of its potentially deleterious effects on tissue integrity, the expression and activity of MMP-1 is tightly regulated by both transcriptional and post-transcriptional mechanisms. Although PMA and EGF have been shown to increase MMP-1 promoter activity in a variety of cell types [8,9,30], we found that EGF, but not PMA, functioned similarly in transiently transfected HaCaT keratinocytes. Other workers have described a similar moderate increase in MMP-1 gene activity in both fibroblasts and primary keratinocytes [31]. In that study, the EGF-mediated induction of MMP-1 could be attributable to an increase in MMP-1 gene activity coupled with a marked increase in the half-life of MMP-1 mRNA. Our findings indicate that although the HaCaT MMP-1 gene is transcriptionally responsive to growth factor

stimulation, it lacks the ability to be up-regulated in response to stimulation with phorbol ester.

The basic controlling promoter elements within the MMP-1 promoter include the TRE- and PEA3-binding sites, sequences utilized by AP-1 and ETS family transcription factors [9,30,40]. Although the AP-1 site of the MMP-1 promoter was shown to be required for mediation of the stimulatory effects of both EGF and PMA [8], the signalling pathways initiated by PMA and EGF are distinct, at least initially. EGF binds its cell-surface receptor, whereas PMA directly activates PKC. These pathways probably converge, or at least cross-talk at the level of c-Raf. EGF and PMA are reported to increase c-*fos* and c-*jun* gene expression in both fibroblasts and keratinocytes [40,41]. However, we found that EGF, but not PMA, increased the expression of c-*fos* and c-*jun* mRNA in HaCaT cells. These findings again indicate that although the corresponding HaCaT genes are functionally responsive to EGF, they lack the ability to respond to stimulation with PMA. In addition, the inability of PMA to increase the expression of AP-1 component mRNA correlates with the agonist's failure to increase MMP-1 promoter activity and MMP-1 mRNA expression, suggesting that AP-1 is essential for the production of MMP-1 in HaCaT cells.

Like many transcription factors, AP-1 activity can be regulated directly or indirectly via phosphorylation by intracellular kinases such as the MAP kinases [11]. Although PMA and EGF have been shown to induce both ERK and JNK activities [33,39,42] and cycloheximide has been shown to increase JNK activity [42], we found that, of these three agonists, only EGF stimulated ERK and JNK activity in treated HaCaT cells. In addition, using the pharmacological inhibitors of MAP kinase/ERK kinase and p38, PD98059 and SB203580 respectively, we found that the EGF-mediated induction of MMP-1 in both fibroblast and HaCaT cells required ERK and p38 activities. However, the PMA-mediated induction of MMP-1 in fibroblasts was not altered in the presence of these inhibitors, suggesting that the JNK pathway is responsible for mediating the inductive response of PMA. Taken together, these results indicate a direct correlation between the agonist's ability to increase MAP family kinase activity and its ability to increase MMP-1 gene expression, suggesting a reliance on the activation of MAP kinase for increased MMP-1 gene expression.

On the basis of overall similarities in MMP regulatory mechanisms, one can speculate that the signalling pathways used by diverse agonists to regulate MMP expression might also share overlapping requirements. In fact, MAP kinases have been shown to be important in regulating the expression of MMP genes in several systems. For instance, the induction of MMP-1 by the multifunctional cytokine oncostatin M has been shown to depend on ERK activity [43], and ERK phosphorylation was found to increase during the collagen-mediated induction of fibroblast MMP-1 [44]. In addition, ERK can be activated in response to Ras-initiated signalling events such as growth factor binding. These results, together with our own, strongly suggest that MAP family kinases have a key role in the signalling cascades that regulate MMP-1 expression.

So far, our results suggest that the signalling defect within HaCaT keratinocytes that leads to their inability to regulate MMP-1 gene expression in response to stimulation with phorbol ester lies upstream of MAP kinase activation. Therefore further work will focus on signalling intermediates likely to be regulated by both agonists. EGF-mediated signalling is initiated at the plasma membrane after ligand–receptor binding [45], whereas PMA by-passes the cell membrane to activate cytoplasmic receptors such as PKC directly [46]. Both agonists, however, can lead to the activation of Raf kinase, a major co-ordinator of signals, leading to proliferation and differentiation [47,48]. Raf kinase activity has been shown to be required during mitogen-, phorbol ester- and oncogene-mediated activation of ERK in COSM6 and 293 cells [49,50] but additional Raf-independent pathways for the mitogen and phorbol ester-mediated activation of ERK have been reported in other cell types [51]. These results again indicate the importance of the cellular environment on whether or not a given agonist affects MAP kinase activity.

Before the activation of Raf kinase, EGF and PMA signalling processes are probably independent. In fact, using a dominant-negative Ras mutant, Ueda et al. [52] were able to block the EGF-mediated activation of ERK while maintaining activation by PMA. Conversely, down-regulation of PKC by pretreatment with phorbol esters does not block the EGF-mediated induction of c-*jun* and c-*fos* [53] nor the insulin-induced activation of ERK kinase in rat adipocytes [54]. In terms of MMP regulation, the activity of specific PKC isoforms has been linked to increased expression of MMP-3 [55]; the induction of keratinocyte and fibroblast MMP-1 by fibrillar collagen has been shown to require both tyrosine kinase and PKC activities [18,56,57]. One possible explanation for the insensitivity of the HaCaT MMP-1 gene to PMA would be a defect in PKC-mediated signalling events upstream of Raf activation. Although Geiges et al. [28] have shown that the expression of different PKC isoforms and their regulation by treatment with PMA are normal in HaCaT keratinocytes, the possibility that a single PKC isoform might be required for MMP-1 induction by PMA cannot be fully excluded. A defect in the expression or activity of this isoform would thereby lead to the observed effect on HaCaT MMP-1 gene expression.

Overall, the regulated expression of MMP family members will probably depend on a complex balance between the signalling cascades activated by individual agonists and their effect on the activity or expression of downstream transcription factors. The outcome of these competing events might therefore determine which MMP genes are expressed and for what length of time.

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