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EPIDERMAL GROWTH FACTOR STIMULATES MITOGEN-ACTIVATED PROTEIN KINASE BY A PKC-DEPENDENT PATHWAY IN HUMAN KERATINOCYTES

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Epidermal growth factor (EGF),20 ng/ml,stimulated myelin basic protein (MBP) phosphorylation in crude extracts from human keratinocyte primary cultures. In order to identify the involved kinases, we separated by fast protein liquid chromatography proteins participating in MBP phosphorylation.
We detected three MBP kinase activities in the keratinocyte crude extracts. The first MBP kinase activity was the only one stimulated by EGF and reacted with anti-mitogen-activated protein kinase (MAPK) antiserum recognising $p42^{mapk}$ and $p44^{mapk}$ isoforms. However, when protein kinase C (PKC) was either inhibited by the PKC inhibitor GF 109203X or depleted by a prolonged TPA treatment, the stimulation of MBP phosphorylation by EGF was strongly inhibited. The second MBP kinase activity eluted was due to a PKC isoform reacting with an anti-PKC ζ antibody, and the third was not identified.
With this work, we have thus shown that, in human keratinocytes, EGF activates MAPK activity by a PKC-dependent pathway.

In the skin, the epidermis is a stratified squamous epithelium mainly composed of keratinocytes. Keratinocyte behaviour can be modulated by diverse factors which can favour either keratinocyte proliferation or keratinocyte differentiation. Epidermal growth factor (EGF) is well known to stimulate normal keratinocyte proliferation (1, 2) and migration (3). Therefore, EGF seems to play an important role to maintain skin homeostasis.

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The intracellular mechanism by which EGF modulates cellular functions is complex. It requires first tyrosine kinase activation of the EGF receptor (4). Moreover, many studies have also pointed out the involvement of serine/threonine kinases in the EGF signalling pathway such as protein kinase C (PKC) (5, 6) and mitogen-activated protein kinase (MAPK) (7, 8, 9).
MAPK is known to regulate various cellular functions, in particular cellular proliferation (10) but also cellular differentiation (8). This kinase is activated in response to a wide variety of extracellular signals such as growth factors, hormones, phorbol esters, ... (11). All these signals stimulate diverse transduction pathways which converge to activate MAPK; it is stimulated by dual phosphorylation on threonine and tyrosine residues by a specific upstream activator, MAPK kinase (12).

In human keratinocytes, PKC plays an important role in the modulation of EGF effects on cellular functions, such as keratinocyte growth (13). While activated PKC is able to stimulate MAPK, its involvement in the EGF transduction pathway can vary from cell type to cell type (14, 15). In the present study, we analysed the EGF effects on MAPK activity and the possible involvement of PKC in these effects.

MATERIALS AND METHODS

Reagents

Serum-free medium (SFM), Earle's modified Eagle's medium (EMEM) and fetal calf serum were from Gibco. GF 109203X was kindly supplied by Glaxo laboratories. [γ-^{32}P]ATP (4,000 Ci/mmol) was obtained from ICN (USA). All other chemicals were from Sigma.

Keratinocyte primary cultures

Keratinocytes were isolated from skin explants obtained during breast plastic surgery from 18-45-year-old healthy donors, as previously described (16). Keratinocytes from three different donors were seeded at 2 .10^5 cells/cm^2 in 60- or 100-mm diameter culture dishes, using EMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2.5 mg/ml amphotericin B in 5% CO_2-95% air at 37°C. After keratinocyte adhesion (3 hours), the medium was replaced by SFM which was renewed every three days.

Myelin basic protein (MBP) kinase activity assays were carried out from confluent primary keratinocytes grown for 9-12 days in monolayer. Cells were treated by 20 ng/ml EGF for various periods of time in kinetic experiments and for 15 minutes in the others. In some cases, cells were also first preincubated either for 2 hours with 1 µM GF 109203X or for 24 hours with 200 ng/ml TPA before being treated by EGF. At the end of the different treatments, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS). Then, Petri dishes were immediately frozen in dry ice and stored at -20°C until MBP kinase assays.

Preparation of cytosolic extracts and MBP kinase activity assays

Cells from 60-mm diameter culture dishes were scraped in 0.5 ml of ice-cold extraction buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM NaF, 10 mM p-nitrophenylphosphate, 50 mM β-glycerophosphate, 10 µM ammonium molybdate, 1 mM Na_3VO_4, 5 mM $MgCl_2$, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 150 mM NaCl, 0.5% Triton X-100 and a protease/phosphatase inhibitor mixture containing 2 µg/ml aprotinin, 0.7 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 0.5 µg/ml chymostatin, 0.5 µg/ml antipain, 1 mM benzamidine and 100 nM microcystin-LR. The mixtures were homogenised in a glass homogeniser and centrifugated at 110,000 g for 60 minutes (4°C). The supernatants were used to measure protein concentrations by the Bradford method (Bio-Rad) and were tested for MBP kinase activity.

Aliquots (10 µl containing 5-10 µg of proteins) of cytosolic extracts were used in a final incubation volume of 50 µl. The kinase incubation medium contained 50 mM Tris-HCl (pH 7.5),

0.5 mg/ml MBP, 10 mM $MgCl_2$, 1.5 mM EGTA, 2 μM protein kinase A inhibitor peptide (Sigma), 10 μM calmidazolium and 100 μM [γ-^{32}P]ATP (specific activity ≈ 1,000 cpm/pmol). The reaction was started by adding [γ-^{32}P]ATP and was allowed to proceed for 10 minutes at room temperature. 40 μl aliquots of reaction mixtures were spotted on 2.5 cm^2 P-81 phosphocellulose filter papers (Whatman). The papers were washed four times with 1% H_3PO_4 and rinsed twice with ethanol. Then, they were dried and the radioactivity was counted in the presence of a scintillation liquid. The MBP kinase activities measured were the difference between samples with or without MBP. All samples were run in duplicate.

Chromatographic fractionation of cytosolic extracts

Fast protein liquid chromatography (FPLC) was carried out using a Mono Q HR 5/5 column (pharmacia LKB Biotechnology Inc). Cytosolic extracts from 100-mm diameter culture dishes (10 mg of proteins) were prepared as described above. After a 5-fold dilution and a filtration through 0.22 μm filter (Millipore S.A.), the cytosolic extracts were loaded at a rate of 0.5 ml/minute in buffer A, containing 20 mM Tris-HCl (pH 7.5), 10 mM NaF, 20 mM β-glycerophosphate, 10 μM ammonium molybdate, 0.2 mM Na_3VO_4, 2 mM EGTA, 2 μg/ml aprotinin and 2% glycerol. The protein elution was performed at a rate of 1 ml/minute with a 30 ml gradient from 0 to 0.5 M NaCl in a buffer B (buffer A + 0.5 M NaCl). 10 μl of a protease/phosphatase inhibitor mixture were added to each fraction with a final concentration similar to this described in the previous paragraph. All chromatographic steps were performed at 4°C.

Mono Q column fractions (15 μl) were assayed for MBP kinase activities as described above, except that 50 μM [γ-^{32}P]ATP (specific activity ≈ 500 cpm/pmol) were used. Mono Q column fractions were also used for immunoblot analysis.

Immunoblot analysis

Fractions separated by FPLC were electrophoresed on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels. Proteins were transferred to nitrocellulose membrane (0.45 μm, Schleicher & Schuell) using a semi-dry transfer cell (Bio-Rad).
Nonspecific binding sites of nitrocellulose membranes were blocked in Tris-buffered saline (TBS: Tris-HCl 20 mM (pH 7.6), NaCl 137 mM and Tween-20 0.2%) containing 5% fat-free milk powder for 2 hours and exposed to primary antibodies in the blocking solution for 1 hours at room temperature. After three washes in TBS, nitrocellulose membranes were exposed to secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG [1:7,500] (Promega) in the blocking solution for 1 hour at room temperature. Then, membranes were washed three times and immunoreactivity was determined using ECL chemiluminescence reaction (Amersham ECL system).

The primary antibodies used correspond to a rabbit anti-MAPK antiserum (kindly provided by Dr. Pouysségur) or a rabbit anti-ERK1 antiserum (Santa Cruz Biotechnology) [1:5,000], both recognising $p42^{mapk}$ and $p44^{mapk}$ proteins (17). We also used rabbit anti-PKC ε, δ [1:2,000] and anti-PKC ζ [1:500] antibodies (Gibco).

RESULTS

EGF effects on MBP phosphorylation in keratinocyte crude extracts

We first studied the effects of 20 ng/ml EGF on MBP phosphorylation in the crude extracts from keratinocyte primary cultures. EGF weakly stimulated 1.98 ± 0.32 (mean ± SE, n=4) -fold MBP phosphorylation. This stimulation was rapid with a maximal effect between 5 and 10 minutes, and declined toward basal level within 30 minutes (figure 1).

FPLC of MBP kinase activities

MBP is generally considered as a good substrate for in vitro assays of MAPK activity, but it can also be a substrate for other protein kinases (18, 19). FPLC of the crude extracts from

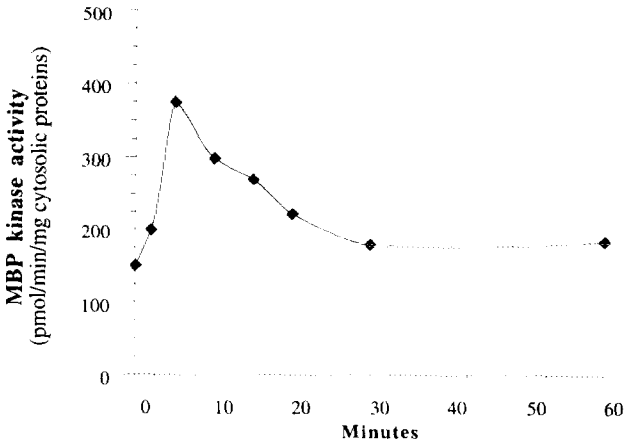

Figure 1 . Time course of MBP phosphorylation in keratinocyte crude extracts.
Keratinocyte cultures were treated by 20 ng/ml EGF. Cytosolic extraction and MBP kinase activity assays were realised as described in section "Materials and Methods". Representative results of three independent experiments carried out with different cell strains of human keratinocytes.

keratinocyte primary cultures revealed two main peaks of MBP kinase activities and a third much weaker (figure 2).

The first peak (peak I around fraction 11) was eluted at 0.20 M NaCl and, according to keratinocyte strains, was stimulated 2- to 10-fold by 20 ng/ml EGF; in each experiment, this stimulation was proportional to the one observed in the crude extract from keratinocyte cultures.

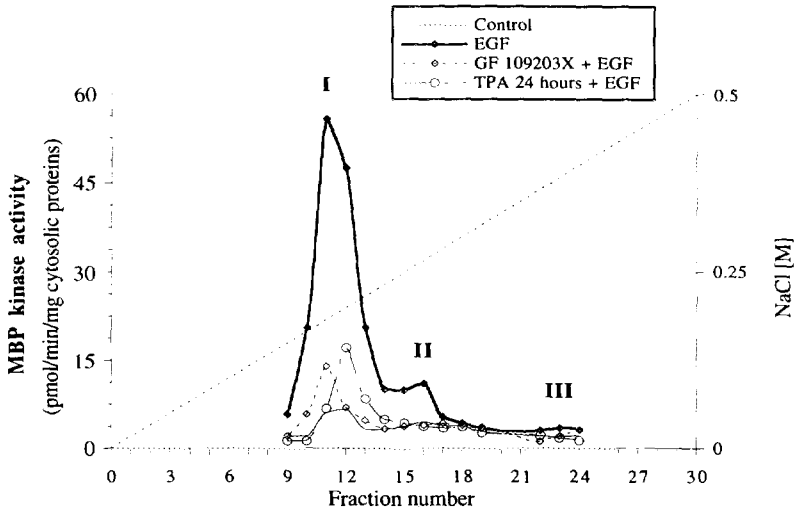

Figure 2 . FPLC of keratinocyte crude extracts.
Keratinocyte cultures were either directly treated for 15 minutes by 20 ng/ml EGF or not (control), or preincubated for two hours with 1 μM GF 109203X or for 24 hours with 200 ng/ml TPA before being treated for 15 minutes by 20 ng/ml EGF. Cytosolic extracts were applied to Mono Q column and MBP kinase activity assays were realised on eluted fractions as described in section "Materials and Methods". Representative results of two independent experiments carried out with different cell strains of human keratinocytes.

When, in keratinocyte cultures, PKC was either inhibited by the selective PKC inhibitor GF 109203X or depleted by 24 hours-TPA treatment, the stimulation of peak I by EGF was strongly inhibited (figure 2).
The second peak (peak II around fraction 16) was eluted at about 0.25 M NaCl. It was weakly but not significantly stimulated by EGF treatment of keratinocytes (figure 2).
A third peak (peak III around fraction 23) was eluted at 0.4 M NaCl. It was much weaker than the others and was never modified regardless of the different treatments (figure 2).

Analysis of the different peaks from FPLC with anti-MAPK antiserum

We tested the FPLC fractions corresponding to peaks I, II and III after SDS-polyacrylamide gels with rabbit anti-MAPK antiserum recognising both p42^{mapk} and p44^{mapk} isoforms. In the fractions around peak I (fractions 10, 11, 12) from control cells, anti-MAPK antiserum revealed two protein bands corresponding to p42^{mapk} and p44^{mapk} isoforms (figure 3).
The FPLC fractions corresponding to peak II and III were also tested with anti-MAPK antiserum which never revealed any protein band (data not shown).

These results show that, in peak I, the p42^{mapk} and p44^{mapk} isoforms are most likely responsible for MBP phosphorylation in human keratinocyte extracts.

Analysis of the different peaks from FPLC for PKC activity

MBP can also be a substrate for in vitro assays of PKC activity (18). The PKC isoenzymes present in human keratinocytes correspond to PKC α, ε, δ, η and ζ (20). We thus tested fractions 11, 16 and 23 with anti-PKC ε, δ and ζ antibodies. The Ca^{2+}-dependent PKC isoform (PKC α) elutes up to 160 nM NaCl and could not be detected using the MBP kinase assays carried out in the presence of 1.5 mM EGTA. In the three peaks eluted from FPLC, anti-PKC ε and δ antibodies did not reveal any protein band (data not shown). In contrast, anti-PKC ζ antibody revealed one protein in fraction 16 corresponding to peak II but not in fractions 11 and 23, respectively peak I and III. This protein had a molecular weight of 71 kDa (figure 4).

These results strongly suggest that, in peak II, a PKC isoenzyme, reacting with anti-PKC ζ antibody, participates in MBP phosphorylation.

DISCUSSION

Activation of MAPK is an early response to a wide variety of extracellular signals. In the present study, we analysed the involvement of MAPK in the EGF signalling pathway of human keratinocytes. We first analysed kinetic of MBP phosphorylation in response to EGF in crude extracts from keratinocyte primary cultures. MBP phosphorylation was weakly stimulated by EGF within 5-10 minutes and decreased toward basal level within 30 minutes.
MBP is considered as a good substrate for MAPK. However, it can also be phosphorylated by other protein kinases (18, 19). In order to identify more precisely the involved kinases, we separated by FPLC protein participating in MBP phosphorylation in keratinocyte crude extracts. We detected two major and a minor MBP kinase activities. The first one, described as peak I, was

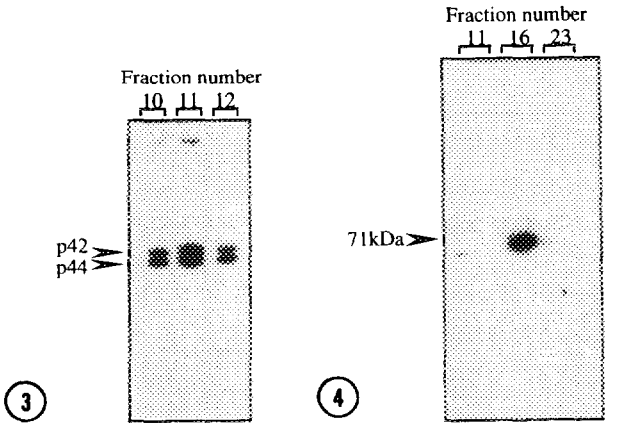

Figure 3 . Analysis of MBP kinase activity present in peak I with anti-MAPK antiserum.
Cytosolic extracts from keratinocyte cultures (control) were applied to Mono Q column and MBP kinase activities were eluted. After SDS-polyacrylamide gels, immunoblot analyses were realised on fractions 10, 11 and 12 corresponding to peak I with anti-MAPK antiserum recognising both $p42^{mapk}$ and $p44^{mapk}$ as described in section "Materials and Methods". Representative results of two independent experiments carried out with different cell strains of human keratinocytes.

Figure 4 . Analysis of MBP kinase activities present in peaks I, II and III with anti-PKC ζ antibody.
Cytosolic extracts from keratinocyte cultures (control) were applied to Mono Q column and MBP kinase activities were eluted. After SDS-polyacrylamide gels, immunoblot analyses were realised on fractions 11, 16 and 23, corresponding to peaks I, II and III with an anti-PKCζ antibody as described in section "Materials and Methods". Representative results of two independent experiments carried out with different cell strains of human keratinocytes.

the only to be clearly stimulated by EGF. In the crude extract from keratinocyte cultures, MBP was only weakly stimulated by EGF; this fact was not surprising since these extracts contain a high basal MBP kinase activity independent of the EGF stimulation.

The MBP kinase activity present in peak I reacted with anti-MAPK antiserum recognising $p42^{mapk}$ and $p44^{mapk}$ isoforms. In addition, we observed that the EGF stimulation of MBP kinase activity in peak I was strongly decreased in GF 109203X pretreated keratinocytes and in prolonged-TPA pretreated keratinocytes. These results demonstrate that EGF stimulates MAPK activity, due most probably to $p42^{mapk}$ and $p44^{mapk}$ isoforms, by a PKC-dependent pathway. Moreover, we observed that a short treatment (8 minutes) of keratinocyte cultures with TPA 100 ng/ml also stimulated MBP phosphorylation in peak I (data not shown).

As growth factor activation of $p42^{mapk}$ and $p44^{mapk}$ isoforms is considered to be necessary for triggering a proliferative response (10), it is reasonable to envisage that the EGF stimulation of keratinocyte growth (1, 2) is mediated by MAPK activation. However, MAPK activation is not always linked to cellular proliferation, and for example while EGF inhibits the growth of human epithelial cells A431 (21), it also stimulates MAPK activity in these cells (22, 23).

The signalling pathway by which EGF activates MAPK is very complex and the involvement of PKC is controversial (7, 9). Burgering *et al.* have proposed that according to the cell type, EGF can use different pathways to activate $p42^{mapk}$, such as $p21^{ras}$-, PKC- and calcium-dependent

pathways (7). In human keratinocytes, activation of $p42^{mapk}$ has been previously shown to be mediated by $p21^{ras}$ (24). Here, we observed that $p42^{mapk}$ and $p44^{mapk}$ activation can also be mediated by PKC in human keratinocytes.

The MBP kinase activities eluted from FPLC with control cells were analysed for PKC activity since this protein kinase can phosphorylate MBP (18). PKC isoforms present in human keratinocytes are PKC α, δ, ε, η and ζ (20). After SDS-polyacrylamide gels, only anti-PKC ζ antibody revealed a protein band with an apparent molecular weight of 71 kDa. The reaction of anti-PKC ζ antibody and the molecular weight of this protein, suggest that it corresponds to the PKC ζ isoform (25). However, it is important to note that antibodies directed against PKC ζ have been shown to also react with other PKC isoforms than PKC ζ (26, 27).
The third weak peak (peak III) phosphorylating MBP did not react with anti-MAPK antiserum or anti-PKC antibodies. It thus corresponds to another protein kinase distinct from MAPK, PKC, and from protein kinase A and Ca^{2+}/calmodulin-dependent kinases which both were inhibited during the MBP kinase activity assays.

In a previous work, we observed that PKC negatively controls EGF effects on keratinocyte growth. This observation, along with the present results, underlines the complexity of the EGF signalling pathway in human keratinocytes. These cells contain different PKC isoforms (20) which have distinct properties concerning for example their regulation, and their substrates (28, 29). Moreover, in human keratinocytes, PKC isoforms participate in a different manner to total PKC activity (16). Consequently, it is possible that the various intracellular effects of EGF are mediated by different PKC isoforms. For example, some PKC isoforms might negatively control EGF effects on keratinocyte growth (13), while others might transduce EGF effects on MAPK activity. Indeed, we have shown that, in human keratinocyte primary cultures, the EGF stimulation of MBP phosphorylation is accompanied by a marked activation of MAPK activity, most probably involving $p42^{mapk}$ and $p44^{mapk}$ isoforms; this stimulation is obtained by a transduction mechanism dependent of PKC activity. This is a clear contrast with human dermal fibroblasts in which EGF activates MAP kinase activity through a PKC-independent mechanism (Le Panse *et al.*, unpublished data).

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