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Protein kinase C-independent activation of mitogen-activated protein kinase by epidermal growth factor in skin fibroblasts

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Abstract

In this study, we demonstrated that epidermal growth factor (EGF) stimulated the phosphorylation of myelin basic protein (MBP), a mitogen-activated protein kinase (MAPK) substrate, in crude extracts of human dermal fibroblasts. Moreover, using a selective protein kinase C inhibitor, GF 109203X (3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride), we observed that protein kinase C was partially involved in the total MBP phosphorylation. To determine the role of protein kinase C in the MBP phosphorylation, we separated, using fast protein liquid chromatography, the proteins present in the fibroblast crude extracts; we thus detected two distinct MBP kinase activities. The first one was stimulated by EGF and corresponded to $p42^{mapk}$ and $p44^{mapk}$ isoforms; this stimulation was not modified by GF 109203X. The second MBP kinase activity was not stimulated by EGF and was due to two protein kinase C isoforms reacting with an anti-protein kinase C ζ antibody. These results show that, in human dermal fibroblasts, EGF stimulates $p42^{mapk}$ and $p44^{mapk}$ isoforms in a protein kinase C-independent manner.

Keywords: EGF (epidermal growth factor); MAPK (mitogen-activated protein kinase); Isoforms ($p42^{mapk}$ and $p44^{mapk}$); Protein kinase C; Dermal fibroblast, human

1. Introduction

An initial event in the cellular response to growth factors is the change in the phosphorylation state of a number of proteins. Mitogen-activated protein kinase (MAPK) is a serine/threonine kinase which seems to play a central role in these cellular phosphorylations. This kinase is rapidly activated in response to a wide variety of extracellular signals such as growth factors, hormones, lymphokines, phorbol esters (Hoshi et al., 1988; Miyasaka et al., 1992; Welham et al., 1992). All these signals stimulate diverse transduction pathways which converge to activate MAPK. Two ubiquitous isoforms, $p44^{mapk}$ and $p42^{mapk}$, also named extracellular signal-related kinase 1 and 2 respectively, have been widely studied (Mordret, 1993). These MAPK isoforms regulate a variety of cellular functions and especially cellular proliferation (Kahan et al., 1992; Pagès et al., 1993).

Epidermal growth factor (EGF) is well known to stimulate fibroblast growth (Carpenter and Cohen, 1979; L'Allemain and Pouysségur, 1986). The transduction of EGF effects at the intracellular level is very complex. The EGF signalling pathway requires first tyrosine kinase activation of the EGF receptor which phosphorylates diverse substrate proteins (Dvir et al., 1991). Many studies have also pointed out the involvement of protein kinase C in the EGF transduction pathway (Cochet et al., 1984; Meisenhelder et al., 1989). In addition, using GF 109203X a selective protein kinase C inhibitor (Toullec et al., 1991; Le Panse et al., 1994a), we demonstrated that protein kinase C controls negatively the effects of EGF on human keratinocyte growth (Le Panse et al., 1994b). We have also shown, in contrast, that if protein kinase C participates in the proliferation process of human dermal fibroblasts (Le Panse et al., 1994a), it does not seem to be involved in the stimulation of their growth by EGF (Le Panse et al., 1994b).

As MAPK activation seems necessary for triggering fibroblast proliferation in response to growth factors (Pagès et al., 1993), we analysed the effects of EGF on MAPK

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activity in human dermal fibroblasts and the possible involvement of protein kinase C.

2. Materials and methods

2.1. Reagents

Earle's modified Eagle's medium (EMEM) and fetal calf serum were from Gibco. GF 109203X (3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride) was kindly supplied by Glaxo Laboratories. [γ-^{32}P]ATP (4000 Ci/mmol) was obtained from ICN (USA). All other chemicals were from Sigma.

2.2. Fibroblast cultures

Fibroblast cultures were started from skin explants obtained during breast plastic surgery from 18–45-year-old healthy donors. Fibroblasts were grown in 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. For experiments, fibroblasts from three different cell strains (between passages 4 and 7) were seeded at 1.25×10^4 cells/cm^2 in 60 or 100 mm diameter culture dishes.

Myelin basic protein (MBP) kinase activity assays were carried out with confluent cultures of fibroblasts grown for 7–8 days. Cultures were treated with 20 ng/ml EGF for various periods of time in kinetic experiments and for 15 min in the others. In some cases, cells were also first preincubated for 2 h with 1 μM GF 109203X or for 24 h with 200 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) before being treated with EGF. At the end of the different treatments, cultures were rinsed twice with ice-cold phosphate-buffered saline. Then, fibroblast cultures were immediately frozen in dry ice and stored at −20°C until MBP kinase assays.

2.3. Preparation of cytosolic extracts and MBP kinase activity assays

Scraped monolayer cultures were homogenised in a glass homogeniser 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 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. After centrifugation at $110\,000 \times g$ for 60 min (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 ≈ 1000 cpm/pM). The reaction was started by adding [γ-^{32}P]ATP and was allowed to proceed for 10 min at room temperature. Aliquots, 40 μl, of the 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. They were then 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.

2.4. Chromatographic fractionation of cytosolic extracts

Fast protein liquid chromatography (FPLC) was carried out using a Mono Q HR 5/5 column (Pharmacia LKB Biotechnology). Cytosolic extracts from 100 mm diameter culture dishes (3 mg of proteins) were prepared as described above. After 5-fold dilution and filtration through a 0.22 μm filter (Millipore), cytosolic extracts were loaded at a rate of 0.5 ml/min 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. Protein elution was performed at a rate of 1 ml/min with a 30 ml gradient from 0 to 0.5 M NaCl in buffer B (buffer A + 0.5 M NaCl); 10 μl of a protease/phosphatase inhibitor mixture was added to each fraction with a final concentration similar to that described in Section 2.3. 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/pM) was used. The fractions eluted were also used to test in cell free assays the effects of increasing concentrations of GF 109203X (25–500 nM) on MBP kinase activities, and for immunoblot analysis.

2.5. Immunoblot analysis

Fractions separated by FPLC were electrophoresed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane (0.45 μm, Schleicher and Schuell) using a semi-dry transfer cell (Bio-Rad).

Non-specific binding sites of nitrocellulose membranes were blocked in Tris-buffered saline (Tris-HCl 20 mM (pH 7.6), NaCl 137 mM and Tween 20 0.2%) containing 5% fat-free milk powder for 2 h and exposed to primary

antibody in the blocking solution for 1 h at room temperature. After three washes in Tris-buffered saline, nitrocellulose membranes were exposed to secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:7500) (Promega), in the blocking solution for 1 h at room temperature. The membranes were then washed three times and immunoreactivity was determined using the ECL chemiluminescence reaction (Amersham ECL system).

The primary antibodies used corresponded to a rabbit anti-MAPK antiserum (kindly provided by J. Pouysségur) or a rabbit anti-extracellular signal-related kinase 1 antiserum (Santa Cruz Biotechnology) (1:5000), both recognising $p42^{mapk}$ and $p44^{mapk}$ proteins (Dubois et al., 1994). We also used rabbit anti-protein kinase C ε, δ (1:2000) and anti-protein kinase C ζ (1:500) antibodies (Gibco).

3. Results

3.1. Effects of EGF on MBP phosphorylation in fibroblast crude extracts.

We first studied the effects of 20 ng/ml EGF on MBP phosphorylation in the crude extracts from fibroblast monolayer cultures. In confluent fibroblasts, EGF rapidly stimulated 3.63 ± 0.53 (mean ± S.E.M, $n = 4$ experiments)-fold MBP phosphorylation with a maximal effect between 15 and 30 min. Thereafter, this stimulation declined toward the basal level in two phases; a first rapid decrease within 2 h followed by a sustained progressive decrease of MBP phosphorylation (Fig. 1).

3.2. Effects of protein kinase C inhibition on MBP phosphorylation induced by EGF in fibroblast crude extracts.

Using a highly selective protein kinase C inhibitor, GF 109203X (Toullec et al., 1991; Le Panse et al., 1994a), we analysed the involvement of protein kinase C in the EGF effects on MBP phosphorylation in fibroblast crude extracts. Fibroblast monolayer cultures were preincubated for 2 h with 1 μM GF 109203X (a concentration chosen on the basis of previous experiments (Le Panse et al., 1994a,b)) and thereafter treated for 15 min with 20 ng/ml EGF.

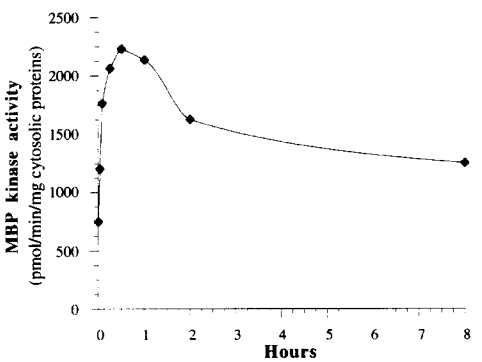

Fig. 1. Time course of MBP phosphorylation in crude extracts of human fibroblasts. Fibroblasts grown in monolayer cultures were treated with 20 ng/ml EGF. Cytosolic extraction and MBP kinase activity assays were done as described in Section 2. Representative results of three independent experiments carried out with different cell strains of human dermal fibroblasts.

Table 1
MBP phosphorylation assays on crude fibroblast extracts

MBP kinase activity (pmol/min/mg cytosolic proteins)		
Control		133.0 ± 14
GF 109203X (μM)	0.1	21.3 ± 4
	0.5	12.8 ± 3
	1	7.2 ± 2.8
	5	4.9 ± 1.5

Representative results of two independent experiments, values are means of ± S.E.M.

Table 2
MBP phosphorylation assays on crude fibroblast extracts

MBP kinase activity (pmol/min/mg cytosolic proteins)	
Control	133.00 ± 17
EGF 20 ng/ml	476.02 ± 34
GF 109203X 1 μM + EGF 20 ng/ml	372.11 ± 25

Representative results of three independent experiments, values are means ± S.E.M.

In preliminary experiments, we observed a marked decrease of MBP phosphorylation in fibroblasts treated with GF 109203X 1 μM alone (Table 1). Nevertheless, the stimulation of MBP phosphorylation by EGF was only partially but significantly inhibited, to about 20–25%, in fibroblast cultures pretreated with GF 109203X (Table 2). Fibroblast cultures were also pretreated with staurosporine (25 nM) which is a widely used protein kinase C inhibitor although it is not very selective. We then observed again inhibition to about 30–35% of the MBP phosphorylation induced by EGF in fibroblast monolayer cultures.

These results with the crude extracts of human dermal fibroblasts suggest that protein kinase C, which seems to be mainly involved in basal MBP phosphorylation in control fibroblasts, was only partially involved in total MBP phosphorylation in EGF-treated fibroblasts.

3.3. FPLC of MBP kinase activities.

To analyse the different protein kinases participating in the phosphorylation of MBP, we separated by FPLC the proteins present in the crude extracts of fibroblast monolayer cultures. We then measured MBP phosphorylation in each fraction eluted. FPLC on Mono Q columns of crude extracts revealed two peaks of MBP kinase activity (Fig. 2).

The first peak (I) was eluted at 0.20 M NaCl and was stimulated about 15.0 ± 4.9 (mean ± S.E.M, $n = 3$ experi-

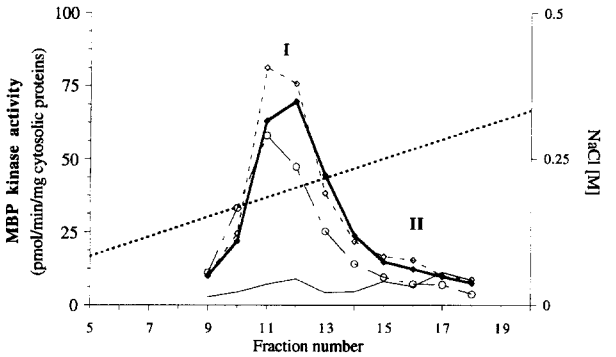

Fig. 2. FPLC of crude extracts from human fibroblasts. Fibroblast monolayer cultures were either treated directly for 15 min with 20 ng/ml EGF (♦) or not (control) (————), or preincubated for 2 h with 1 μM GF 109203X (◇) or for 24 h with 200 ng/ml TPA (○) before being treated for 15 min with 20 ng/ml EGF. Cytosolic extracts were applied to Mono Q columns and MBP kinase activity assays were done with eluted fractions as described in Section 2. Representative results of two independent experiments carried out with different cell strains of human dermal fibroblasts.

ments) fold when fibroblasts were treated for 15 min with 20 ng/ml EGF. We also analysed the effects of EGF on peak I when protein kinase C was inhibited by GF 109203X or depleted by 24 h TPA treatment in fibroblast cultures; in both cases, the stimulation of peak I by EGF was not inhibited (Fig. 2).

The second peak (II) was eluted around 0.25 M NaCl and was not stimulated by the treatment of fibroblast cultures with EGF (Fig. 2).These observations reveal the presence of distinct MBP kinase activities in fibroblast crude extracts. However, only MBP kinase activity from peak I is responsible for the EGF-induced MBP phosphorylation.

3.4. Analysis of peak I and II from FPLC with anti-MAPK antiserum

MBP is considered as a good substrate for in vitro assays of MAPK activity. We thus tested, after SDS-polyacrylamide gels, the FPLC fractions corresponding to peaks I and II with rabbit anti-MAPK antiserum that recognises both $p42^{mapk}$ and $p44^{mapk}$ isoforms. Peaks I and II from either unstimulated cells (control) or cells stimulated for 15 min by 20 ng/ml EGF were analysed.

In peak I from control cells, anti-MAPK antiserum revealed two protein bands corresponding to $p42^{mapk}$ and $p44^{mapk}$ isoforms. When the cells were stimulated with EGF, anti-MAPK antiserum also revealed two protein bands in peak I. However, these two bands showed higher apparent molecular sizes. The activation of MAPK isoforms requires their phosphorylation, which changes their electrophoretic mobility. Consequently, the two protein bands detected in peak I from EGF-stimulated cells most probably corresponded to phosphorylated $p42^{mapk}$ and $p44^{mapk}$ isoforms (p42-P and p44-P) (Fig. 3). In peak II, the anti-MAPK antiserum never revealed any protein band (Fig. 3).

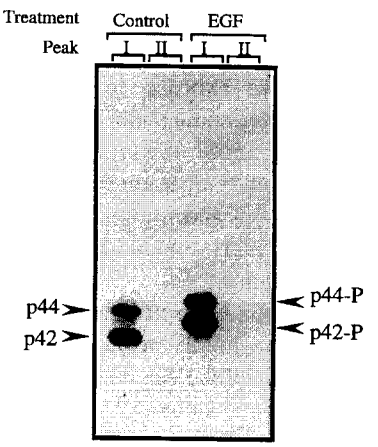

Fig. 3. Analysis of MBP kinase activities present in peak I and II with anti-MAPK antiserum. Fibroblast monolayer cultures were treated for 15 min with 20 ng/ml EGF or not (control). Cytosolic extracts were applied to Mono Q columns and two MBP kinase activities corresponding to peak I and II were eluted. After SDS-polyacrylamide gels with the fractions corresponding to these peaks, immunoblot analysis was done using rabbit anti-MAPK antiserum recognising both $p42^{mapk}$ and $p44^{mapk}$ as described in Section 2. Representative results of two independent experiments carried out with different cell strains of human dermal fibroblasts.

These results demonstrate that, in peak I, the $p42^{mapk}$ and $p44^{mapk}$ isoforms are stimulated by EGF and are most likely responsible for MBP phosphorylation in human fibroblast extracts.

3.5. Analysis of peak I and II from FPLC for protein kinase C activity

MBP can also be a substrate for in vitro assays of protein kinase C activity (Kazanietz et al., 1993). We thus analysed, using cell-free assays, the dose-dependent response for GF 109203X effects (25–500 nM) on MBP phosphorylation in peak I and II eluted from EGF-stimulated cells. In peak I, MBP kinase activity was not affected whereas, in peak II, it was strongly inhibited by GF 109203X in a dose-dependent manner (Table 3). Using a protein kinase C assay kit (Gibco), we also directly con-

Table 3
MBP phosphorylation assays on FPLC peak I and II eluted from EGF-treated fibroblast

GF 109203X (nM)	MBP kinase activity (pmol/min/mg cytosolic proteins)	
	Peak I	Peak II
0	172±18	42.0±9
25	169±19	15.0±3
50	171±18	11.7±1.1
100	174±16	9.4±0.6
500	170±17	7.1±0.5

Representative results of two independent experiments, values are means ±S.E.M.

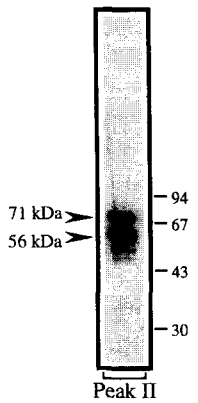

Fig. 4. Analysis of MBP kinase activity present in peak II with anti-protein kinase C ζ antibody. Fibroblast monolayer cultures (controls) were used to analyse MBP kinase activity corresponding to peak II eluted by FPLC. After SDS-polyacrylamide gels with the fractions corresponding to this peak, immunoblot analysis was done using an anti-protein kinase C ζ antibody as described in Section 2. Representative results of two independent experiments carried out with different cell strains of human dermal fibroblasts. Molecular weight standards are shown the right of the figure.

firmed the presence of protein kinase C activity in peak II and not in peak I (data not shown).

The protein kinase C isoforms present in human fibroblasts correspond to protein kinase C α, ε, δ and ζ (Racchi et al., 1994). We thus tested FPLC fractions corresponding to peak II from control cells with anti-protein kinase C ε, δ, ζ antibodies; the Ca^{2+}-dependent protein kinase C isoform (protein kinase C α) elutes up to 160 mM NaCl and could not be detected using MBP kinase assays carried out in the presence of 1.5 mM EGTA. In peak II, anti-protein kinase C ε and δ antibodies did not reveal any protein band (data not shown). In contrast, anti-protein kinase C ζ antibody revealed two proteins with molecular weight of 71 and 56 kDa (Fig. 4).

These results show that, in peak II, protein kinase C isoforms reacting with anti-protein kinase C ζ antibody are involved in the basal MBP phosphorylation.

4. Discussion

Activation of MAPK is an early response to a wide variety of extracellular signals and, in this study, we analysed the involvement of MAPK in the EGF signalling pathway in human dermal fibroblasts. MBP is generally considered as a good substrate for in vitro assays of MAPK activity. We thus studied MBP phosphorylation in response to EGF in crude extract preparations from fibroblast monolayer cultures. In fibroblasts treated with EGF, MBP kinase activity increased rapidly within 15–30 min and declined toward its basal level, first rapidly within 2 h and progressively afterwards. Similar MBP phosphorylation kinetics, in response to EGF, were observed with fibroblasts grown within a collagen matrix. However, in this fibroblast culture model, MBP kinase activity was very much weaker compared to that with fibroblasts from monolayer cultures (data not shown). This low MBP kinase activity could be linked to control of fibroblast behaviour by the surrounding collagen matrix (Sarber et al., 1981; Coulomb and Dubertret, 1992).

The EGF transduction pathway involves, first, tyrosine kinase activation of the EGF receptor (Dvir et al., 1991) and often, subsequently, the activation of serine/threonine kinases (Meisenhelder et al., 1989; Pang et al., 1993; Chao et al., 1994). We analysed the involvement of protein kinase C in the activation of MBP phosphorylation by EGF in human fibroblasts from monolayer cultures using the protein kinase C inhibitor, GF 109203X (Toullec et al., 1991). In previous work with human dermal fibroblasts, we have shown the selectivity of GF 109203X in cell free assays in which it inhibits protein kinase C activity without affecting protein kinase A and membrane tyrosine kinase activies (Le Panse et al., 1994a). Moreover we have shown that GF 109203X is efficient to inhibit protein kinase C activity in intact human dermal fibroblasts (Le Panse et al., 1994a,b). In the present work, GF 109203X decreased only to 20–25% the MBP kinase activity in response to EGF although a stronger decrease of the MBP phosphorylation level was observed in control fibroblasts treated with GF 109203X alone. These results suggest that protein kinase C is partially involved in the MBP kinase activity measured in the fibroblast crude extracts. However, we could not conclude as to its involvement in the MBP kinase activity induced by EGF.

While MBP is considered a good substrate for MAPK and is widely used in in vitro assays, it can nevertheless be phosphorylated by other protein kinases, especially protein kinase C (Kazanietz et al., 1993; Force et al., 1994). Consequently, we investigated whether protein kinase C was involved in EGF-induced MBP phosphorylation, either directly or by a mechanism involving MAPK (Marquardt et al., 1994). Using FPLC on Mono Q columns, we separated proteins of the fibroblast crude extracts to analyse the different protein kinases participating in the MBP phosphorylation. Two MBP kinase activities were then clearly separated by FPLC.

The first kinase activity (peak I), but not the second (peak II), reacted with anti-MAPK antiserum recognising both $p42^{mapk}$ and $p44^{mapk}$ isoforms. The EGF treatment of fibroblasts stimulated strongly the MBP phosphorylation by activated $p42^{mapk}$ and $p44^{mapk}$ isoforms; the activated forms of MAPK were identified on SDS-polyacrylamide gels by a shift of phosphorylated-$p42^{mapk}$ and -$p44^{mapk}$ isoforms.

When protein kinase C activity was inhibited by GF 109203X or when protein kinase C was down-regulated by prolonged TPA treatment, the MBP phosphorylation by $p42^{mapk}$ and $p44^{mapk}$ isoforms was not modified. Therefore, EGF stimulates MAPK activity ($p42^{mapk}$ and $p44^{mapk}$) in human fibroblasts by a protein kinase C-independent pathway. These results are in agreement with

those obtained with different fibroblast cell lines (Pang et al., 1993; Chao et al., 1994). Moreover, we have shown previously that the EGF stimulation of human fibroblast growth is independent of protein kinase C activation (Le Panse et al., 1994b). As growth factor activation of $p42^{mapk}$ and $p44^{mapk}$ is considered as necessary for triggering the proliferative response in chinese hamster lung fibroblasts (Kahan et al., 1992; Pagès et al., 1993), it is conceivable that the EGF effects on human fibroblast growth are mainly mediated by MAPK activation.

The second MBP kinase activity eluted, peak II, did not react with anti-MAPK antiserum. As protein kinase C was involved in the total MBP kinase activity in the human fibroblasts crude extracts, we checked for the possible presence of protein kinase C activity in peaks I and II. We detected protein kinase C activity only in peak II and, in cell-free assays, the effects of this protein kinase C activity on MBP phosphorylation were strongly inhibited by GF 109203X.

The MBP phosphorylation due to protein kinase C activity contained in peak II was not stimulated by EGF. Moreover, it was also not stimulated when fibroblasts were treated for 8 min with TPA (100 ng/ml) (data not shown). This lack of TPA effects suggests that protein kinase C isoforms present in peak II are atypical protein kinase C isoforms which are TPA-insensitive (Zhou et al., 1994).

The protein kinase C isoforms present in human fibroblasts correspond to protein kinase C α, δ, ε and ζ (Racchi et al., 1994). To analyse the protein kinase C activity present in peak II, antibodies against protein kinase C δ, ε and ζ were used; protein kinase C α could not be detected under the MBP kinase assay conditions used. Only anti-protein kinase C ζ antibody revealed two protein bands with an apparent molecular weight of 71 and 56 kDa; the same observation was made by Racchi et al. (1994) in human skin fibroblasts. The definitive identification of these protein kinase C isoforms is difficult, considering that antibodies directed against protein kinase C ζ are known to react with different protein kinase C isoforms also (Batlle et al., 1994; Selbie et al., 1993). Nevertheless, the apparent molecular masses reported for the atypical protein kinase C ζ are 72–76 kDa (Zhou et al., 1994); the protein band of 71 kDa detected in peak II could thus correspond to protein kinase C ζ. In addition, the strong inhibition by GF 109203X of MBP phosphorylation in peak II observed in cell-free assays indicates the presence of another protein kinase C isoform distinct from protein kinase C ζ; indeed, this isoform is known to be resistant to GF 109203X (Martiny-Baron et al., 1993). This other protein kinase C isoform can only be identified as being a Ca^{2+}-independent and TPA-insensitive protein kinase C isoform inhibited by GF 109203X. Consequently, in our experiments, we demonstrated that two protein kinase C isoforms could be involved in the phosphorylation of MBP, but they did not participate in the EGF-induced MBP phosphorylation. The presence of these protein kinase C isoforms explains the decrease of MBP phosphorylation in total crude extracts from fibroblast cultures pretreated with protein kinase C inhibitors.

In this study, we have thus shown that in human dermal fibroblasts, MBP can be phosphorylated by different protein kinases such as MAPK isoforms and protein kinase C isoforms. However, the EGF stimulation of MBP phosphorylation would be due only to marked activation of $p42^{mapk}$ and $p44^{mapk}$ isoforms, which is obtained by a transduction mechanism independent of protein kinase C. In contrast, in previous experiments carried out with human keratinocytes, we have observed that EGF activates $p42^{mapk}$ and $p44^{mapk}$ isoforms through a protein kinase C-dependent transduction pathway (Mitev et al., 1995). These studies show that, depending on cell types, EGF can use different signalling pathways to modulate MAPK activity.

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