MAP Kinase Abnormalities in Hyperproliferative Cultured Fibroblasts from Psoriatic Skin

Stéphanie Dimon-Gadal, Françoise Raynaud, Danièle Evain-Brion, and Guy Keryer
Unité INSERM 427, Faculté des Sciences Pharmaceutiques et Biologiques, Paris, France

Several studies indicate that dermal fibroblasts have a specific role in the pathophysiology of psoriasis. We have previously found that cultured fibroblasts from psoriatic patients are hyperproliferative and have low cyclic AMP-dependent protein kinase activity. In this study, we observed that these cells are also larger than normal. Given the key role of mitogen-activated protein kinases (MAPK) in the regulation of cell proliferation and cytoskeleton function, we characterized MAPK in psoriatic fibroblasts and in normal fibroblasts. Serum and platelet-derived growth factor treatment of serum-deprived fibroblasts led to a larger increase in MAPK activity in psoriatic cells than in normal cells. We then purified MAPK by ion-exchange chromatography. MAPK activity was again found to be significantly higher in psoriatic fibroblasts than in normal cells, both when deprived of serum (p < 0.01) and when stimulated with serum (p < 0.05). Interestingly, 8-bromo-cAMP treatment inhibited serum-stimulated MAPK phosphorylation in normal fibroblasts but had no effect in psoriatic fibroblasts. We observed a temporal variation in nuclear localization of phosphorylated MAPK in cultured fibroblasts stimulated by either serum or platelet-derived growth factor. No difference in the localization of phosphorylated MAPK in normal and psoriatic skins was found. Psoriatic fibroblasts are the first example of a MAPK pathway abnormality in large human benign hyperproliferative cells. Key words: cyclic AMP-dependent protein kinase/mitogen-activated protein kinases/psoriasis. J Invest Dermatol 110:872–879, 1998

Mitogen-activated protein kinases [MAPK, also known as Erks (extracellular signal-regulated kinases)] are key intermediates in the transduction of extracellular signals to the nucleus (Brunet et al., 1994; Graves et al., 1995; Seger and Krebs, 1995), and therefore in the regulation of cell proliferation. The best characterized mammalian examples of these kinases are Erk1 (also called p42

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MAPK by dephosphorylating tyrosine and threonine residues (for review see Zolnierowicz and Hemmings, 1996). cAMP may be involved in the negative regulation of the MAPK pathway in several cell types, including fibroblasts (Burgering et al., 1993b; Cook and McCormick, 1993; Hordijk et al., 1994). Presumably cAMP-dependent
protein kinase A (PKA) mediate this effect, as no inhibition was observed in cells expressing a mutant regulatory subunit of PKA (Sevetson et al., 1993). Raf-1 is phosphorylated by PKA at a consensus site within the Raf-1 regulatory domain. This diminishes Raf-1 affinity for Ras and explains the inhibition of Raf-1 activation and, consequently, that of the MAPK cascade downstream from Raf-1 (Wu et al., 1993). Moreover, PKA is able to phosphorylate the active domain of Raf-1, which would inhibit Raf-1 activity (Hafner et al., 1994). There seems to be synergy, however, between cAMP and the MAPK pathway effect in some cells; e.g., in PC12 pheochromocytoma cells, growth factor and cAMP have a synergistic effect on neurite formation (Zimmerman et al., 1981). Simultaneously, McKenzie and Pouyssegur (1996) showed that PKA-mediated growth inhibition is independent of the MAPK pathway in rat fibroblasts, PKA simply delaying MAPK activation by mitogens.

MAPK in human dermal fibroblasts is activated by each PDGF AA, PDGF BB (Lubinus et al., 1994), and epidermal growth factor in a protein kinase C-independent manner (Le Panse et al., 1996).

Psoriasis is a chronic human skin disease that affects 2–3% of the population. It is characterized by epidermal abnormalities including keratinocyte hyperproliferation and abnormal differentiation. It also involves abnormal dermoepidermal microcirculation and infiltration of the epidermis and the dermis by inflammatory cells. Psoriasis appears to be a multigenic disease (Elder et al., 1994), although its precise etiology is unclear. On the basis of the clinical response to immunomodulatory agents such as cyclosporin (Gottlieb et al., 1992), and the massive T cell infiltration, psoriasis was recently described as a T cell-mediated autoimmune disease (Valdimarsson et al., 1994), a point of view that is supported by our study.

Fibroblasts were isolated from healthy adults and untreated psoriatic adults (psoriasis vulgaris in flare-up for 2–3 months) and nonlesional tissue, in which fibroblasts also bear the PKA defect (Raynaud et al., 1994; 1995). The calcium ionophore A23187, 0.5 µM collagenase per ml for 3 h at 37°C. Isolated cells were recovered by centrifugation at 400 × g for 10 min, resuspended in 5 ml of DMEM containing 0.5 µM collagenase per ml for 3 h at 37°C. After centrifugation, they were incubated in DMEM, containing 0.5 µM collagenase per ml for 3 h at 37°C. Isolated cells were recovered by centrifugation at 400 × g for 10 min, resuspended in 5 ml of DMEM containing 10% fetal calf serum, penicillin, and streptomycin, and plated in 100 mm diameter cell culture dishes. The cells were grown in humidified 5% CO2/95% air at 37°C and used between passages 4 and 9.

Prior to experiments, subconfluent cells were rinsed twice with 5 ml of warm phosphate buffered saline (PBS) and incubated in 10 ml of serum-free culture medium for 72 h. These serum-deprived cells were incubated in DMEM containing 10% fetal calf serum or 1 ng human recombinant PDGF AB per ml; untreated cells served as controls.

8-bromo-cAMP treatment Serum-deprived fibroblasts were incubated in DMEM in the presence of 0.1 mM 8-bromo-cAMP for 1 h at 37°C, and fetal calf serum was then added to a final concentration of 10% to enable cells to incubated for a further 10 min.

Cell extracts Cells were washed twice with 5 ml of ice-cold PBS. They were lysed and scraped on ice in 50 mM β-glycerophosphate, 10 mM KH2PO4, 1 mM ethylenediamine tetraacetic acid, 5 mM ethylenglycol-bis-(β-aminooethyl ether)-N,N,N’‘,N’ -tetracetic acid, 10 mM MgCl2, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 4 mM p-nitrophenyl phosphate, 2 µg aprotinin per ml, 0.5 µg leupeptin per ml, 1% (vol/vol) NP-40, pH 7.4 (250 µl per dish). The cell lysate was centrifuged at 13,000 × g for 15 min at 4°C, and the supernatant was collected and immediately used for ion-exchange chromatography or for protein kinase assays.

Protein concentrations in the supernatants were determined by fluorometric assay using bovine serum albumin (BSA) as standard (Bohlen et al., 1973).

Ion-exchange chromatography Cytosolic extract (10 µg) was filtered through a 0.2 µm filter (Millipore, Saint-Quentin en Yvelines, France) and loaded onto an anion-exchange Mono Q column (Pharmacia Biotech, Orsay, France) equilibrated in 50 mM β-glycerophosphate, 1 mM ethylenglycol-bis-(β-aminooethyl ether)-N,N,N’‘,N’ -tetracetic acid, 1 mM phenylmethylsulfonyl fluoride, 0.2 µg aprotinin per ml, and 0.01% (vol/vol) β-mercaptoethanol, pH 7.4 at a flow rate of 0.8 ml per min at 4°C. After a washing step with equilibration buffer for 15 min, the sample was eluted with 25 ml of a linear gradient (0–250 mM) of NaCl in equilibration buffer at 4°C (flow rate 1 ml per min) (Gomez and Cohen, 1991; Trigon and Morange, 1995). Fractions of 1 ml were collected and stored at 4°C until analysis.

Antibodies and immunoblotting Anti-Erk1 sc93 was from Santa Cruz Biotechnology, TEBU (Le Perray en Yvelines, France). Anti-rat MAP kinase R2 was from UBI (Euromedex, Souffel Weyersheim, France) and loaded onto an anion-exchange Mono Q column (Pharmacia Biotech, Orsay, France) equilibrated in 50 mM β-glycerophosphate, 1 mM ethylenglycol-bis-(β-aminooethyl ether)-N,N,N’‘,N’ -tetracetic acid, 1 mM phenylmethylsulfonyl fluoride, 0.2 µg aprotinin per ml, and 0.01% (vol/vol) β-mercaptoethanol, pH 7.4 at a flow rate of 0.8 ml per min at 4°C. After a washing step with equilibration buffer for 15 min, the sample was eluted with 25 ml of a linear gradient (0–250 mM) of NaCl in equilibration buffer at 4°C (flow rate 1 ml per min) (Gomez and Cohen, 1991; Trigon and Morange, 1995). Fractions of 1 ml were collected and stored at 4°C until analysis.

Materials and methods Materials Dulbecco’s modified Eagle medium (DMEM) and fetal calf serum were from Seromed (Poly Labo Paul Block et Cie, Paris, France). Streptomycin and penicillin were from Biological Industries (Kibbutz Beit Haemek, Israel). PDGF AB was from Collaborative Research (Lexington, MA). 8-bromo-cAMP, myelin basic protein (MBP), trypsin, and collagenase were from Sigma-Aldrich Chimie SARL (Saint-Quentin Fallavier, France). Anti-rat MAP kinase R2 was kindly provided by Dr. W.B. Anderson (Laboratory of Cellular Oncology, National Cancer Institute, NIH, Bethesda, MA). Anti-PKA catalytic subunit antibody was a generous gift from Dr. S. Lohmann (Labor für Röntgenische Biochemie, Medizinische Universitätsklinik, Würzburg, Germany).

Ten micrograms of cell extract or 1 µg of protein obtained after fast liquid chromatography purification was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophotographically transferred to 0.45 µm pore size nitrocellulose membranes, in 50 mM Tris, 39 mM glycine, 20% ethanol (vol/vol), and 0.04% sodium dodecyl sulfate (vol/vol) at pH 8.5 and 23 V for 50 min. The membrane was incubated with anti-Erk1 sc93, anti-PKA, or anti-PKC antibody (1:1,000), and then with a peroxidase-coupled goat anti-rabbit antibody (1:10,000) (Amersham), and was developed using the ECL method (Amersham). Anti-rat MAP kinase R2 was used according to the manufacturer's recommendations.

Protein kinase assays One microgram of protein obtained after FPLC purification was incubated in the presence of 0.25 µCi[32P]adenosine triphosphate and 6 mM Mg2+ in 20 mM HEPES (pH 7.4), 25 mM MgCl2, 40 µM adenosine triphosphate, in a final volume of 50 µl at 30°C for 30 min. Twenty microliters of the mixture was spotted onto 3 cm2 P81 phosphocellulose strips (Whatman, Poly Labo Paul Block et Cie). The strips were washed four times in 10 ml of 0.5% orthophosphoric acid (10 ml per paper strip per wash) at 200 rpm (2 min each wash), dried, and counted by scintillation. Data were corrected for values obtained with samples incubated in the absence of MBP.

To determine MAPK activity in cytosolic extracts (10 µg protein), the reaction mixture contained 5 µM H-89 (Seikagaku, Paris, France) and 2 µM GF 109203X (3-[1-[3(diethylamino)propyl]-1 H-indol-3-yl]-1 H-indol-3-yl H-glutamate) (Kumada et al., 1991), a generous gift from Dr. J. Kiriakov (Laboratoire de Biologie, Glaxo Research Center, Les Ulis, France). The PKA inhibitor PKI and the PKC inhibitor GF 109203X were added to the phosphorylation mixture to prevent phosphorylation of MBP by PKA and PKC as described by Whisler et al. (1995). The calcium chelators ethylenglycol-bis-(β-aminooethyl ether)-N,N,N’‘,N’ -tetracetic acid and ethylenediamine tetracetic acid included in the lys buffer were also
In the reaction mixture at 0.72 mM and 135 µM, respectively, to inhibit the phosphorylation of MBP by Ca²⁺/calmodulin kinases.

Confocal microscopy Cells were fixed with 4% paraformaldehyde in PBS for 20 min at 37°C, then rinsed twice in PBS and incubated for 10 min with PBS containing 50 mM ammonium chloride. They were then permeabilized with 0.1% Triton X-100 in PBS containing 0.2% BSA. Incubation with an antibody raised against a cell surface marker (N-aminopeptidase) was as previously described.¹

We used a Sarastro 2000 (Molecular Dynamics, Palo Alto, CA) confocal microscope equipped with an argon ion laser (488–514 nm wavelength) and mounted on either an Optiphot II Nikon microscope (X60, NA, 1.40, Plan objective) or a Zeiss Axiopt microscope (X63 plan Neofar objective). In double-staining experiments, both fluorescein and rhodamine secondary antibodies were used. Ten sections, each 0.25 µm thick (averaging on five full frames of the same section) were scanned, and stacks of optical sections for each data set were compiled by Voxel View software on a Silicon Graphics IRIS 4D-70 GT workstation.

Flow cytometric analysis Cells were plated at 3 × 10⁵ per 100 mm dish in DMEM containing 10% fetal calf serum and collected 4 d later by trypsinization. They were fixed with 4% paraformaldehyde and 40% acetone at −20°C for 8 min. After six washes with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), the fixed cells were incubated with 1.5% normal goat serum to inhibit the phosphorylation of MBP by Ca²⁺/calmodulin kinases.

Maximal activity in both normal and psoriatic fibroblasts was obtained 3 h after stimulation, peaking after 15 min and collected 4 and 6 d later by trypsinization. They were then permeabilized with 1.5% normal goat serum in TBST for 1 h at room temperature and then incubated with the phospho-specific MAPK antibody (New England Biolabs, Beverly, MA) in TBS containing 3% BSA (TBS/BSA) (1:100) for 90 min at room temperature. This antibody binds to phosphorylated tyrosine in Erk1 and Erk2. The cells were washed six times with TBST and incubated in TBS/BSA with biotinylated anti-rabbit IgG antibody for 1 h (1:200; Amersham). After six washes with TBST, cells were then incubated in TBS/BSA with Texas Red-streptavidin for 1 h (1:500; Jackson ImmunoResearch, Interchim, Annèrèes, France). After a last washing step, cells were fixed in absolute ethanol for 1 min and covered with mounting medium with 4,6-diamidino-2-phenylindol (Vectorshied, Vector Laboratories, Burlingame, CA). The cells were examined with a fluorescence microscope (Olympus, Rungis, France) under epifluorescent-illumination with excitation-emission filters for rhodamine and for 4,6-diamidino-2-phenylindol. Incubation with biotinylated anti-rabbit IgG antibody and Texas Red-streptavidin alone did not reveal any significant fluorescent signal (data not shown).

Immunocytochemistry Fibroblasts were cultured on glass slides, deprived of serum, and then stimulated with serum or PDGF. Samples were fixed as follows: 15, 30, 60 min, and each 3 h later until 12 h of stimulation. Fibroblasts were washed with PBS and fixed in 60% methanol and 40% acetone at −20°C for 8 min. After six washes with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), the fixed cells were incubated with 1.5% normal goat serum in TBST for 1 h at room temperature and then incubated with the phospho-specific MAPK antibody (New England Biolabs, Beverly, MA) in TBS containing 3% BSA (TBS/BSA) (1:100) for 90 min at room temperature. This antibody binds to phosphorylated tyrosine in Erk1 and Erk2. The cells were washed six times with TBST and incubated in TBS/BSA with biotinylated anti-rabbit IgG antibody for 1 h (1:200; Amersham). After six washes with TBST, cells were then incubated in TBS/BSA with Texas Red-streptavidin for 1 h (1:500; Jackson ImmunoResearch, Interchim, Annèrèes, France). After a last washing step, cells were fixed in absolute ethanol for 1 min and covered with mounting medium with 4,6-diamidino-2-phenylindol (Vectorshied, Vector Laboratories, Burlingame, CA). The cells were examined with a fluorescence microscope (Olympus, Rungis, France) under epifluorescent-illumination with excitation-emission filters for rhodamine and for 4,6-diamidino-2-phenylindol. Incubation with biotinylated anti-rabbit IgG antibody and Texas Red-streptavidin alone did not reveal any significant fluorescent signal (data not shown).

Immunohistochemistry Biopsies of the buttock and abdominal skin were obtained from psoriatic patients and from healthy control subjects. They were quick frozen in isopentane cooled in liquid nitrogen and stored at −80°C. Tissue sections (6 µm) were obtained with a cryostat set at −25°C and were transferred to slides pre-washed with 10% v/v methanol in PBS, dried at room temperature, and stored at −80°C. The sections were sequentially rehydrated and mounted on either an Optiphot II Nikon microscope (X60, NA, 1.40, Plan objective) or a Zeiss Axiopt microscope (X63 plan Neofar objective).

Flow cytometric analysis Cells were plated at 3 × 10⁵ per 100 mm dish in DMEM containing 10% fetal calf serum and collected 4 d later by trypsinization. They were fixed with 4% paraformaldehyde and 40% acetone at −20°C for 8 min. After six washes with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), the fixed cells were incubated with 1.5% normal goat serum in TBST for 1 h at room temperature and then incubated with the phospho-specific MAPK antibody (New England Biolabs, Beverly, MA) in TBS containing 3% BSA (TBS/BSA) (1:100) for 90 min at room temperature. This antibody binds to phosphorylated tyrosine in Erk1 and Erk2. The cells were washed six times with TBST and incubated in TBS/BSA with biotinylated anti-rabbit IgG antibody for 1 h (1:200; Amersham). After six washes with TBST, cells were then incubated in TBS/BSA with Texas Red-streptavidin for 1 h (1:500; Jackson ImmunoResearch, Interchim, Annèrèes, France). After a last washing step, cells were fixed in absolute ethanol for 1 min and covered with mounting medium with 4,6-diamidino-2-phenylindol (Vectorshied, Vector Laboratories, Burlingame, CA). The cells were examined with a fluorescence microscope (Olympus, Rungis, France) under epifluorescent-illumination with excitation-emission filters for rhodamine and for 4,6-diamidino-2-phenylindol. Incubation with biotinylated anti-rabbit IgG antibody and Texas Red-streptavidin alone did not reveal any significant fluorescent signal (data not shown).

Disease score The severity of psoriasis affecting each patient was scored using the Psoriasis Area and Severity Index (PASI) (Fredriksson and Pettersson, 1978).


Figure 1. Morphologic aspect of cultured fibroblasts from normal subjects (A, C) and psoriatic patients (B, D). (A, B) Optical microscopy; scale bar, 20 µm. (C, D) Confocal microscopy; cells were labeled with an antibody against a cell surface antigen (N-aminopeptidase); scale bar, 4 µm.

Statistical analysis All data are representative of at least three experiments performed with different fibroblast cultures. Statistical significance was assessed using by Student’s t test, with a threshold of p < 0.05.

RESULTS

Fibroblasts from psoriatic patients are larger than fibroblasts from normal subjects As previously described (Prieulley and Adams, 1983; Raynaud et al, 1991), fibroblasts from psoriatic patients were hyperproliferative in our experimental conditions of culture. Cultured normal and psoriatic fibroblasts were examined and found to be larger by both standard microscopy (Fig 1A, B, respectively, for normal and psoriatic fibroblasts) and confocal microscopy (Fig 1C, D, respectively).

Several normal and psoriatic fibroblast cultures were trypsinized and the average cell size was measured by flow cytometry. Psoriatic fibroblasts were significantly larger than normal fibroblasts (18.1 ± 0.8 vs 14.8 ± 0.9 µm; p < 0.02).

Time course of MAPK activation by serum and PDGF in normal and psoriatic fibroblasts We studied and compared MAPK activity in normal and psoriatic fibroblasts. The time course of the stimulatory effect of serum (Fig 2A) and PDGF (Fig 2B), a growth factor known to stimulate fibroblast proliferation (Gu et al, 1988), was studied using MBP as the substrate to MAPK in the presence of PKA, PKC, and Ca²⁺/calmodulin kinase inhibitors. Serum and PDGF AB treatment of serum-deprived fibroblasts led to a larger increase in MAPK activity in psoriatic cells than in normal cells, at all time points. Maximal activity in both normal and psoriatic fibroblasts was obtained after 15 min of stimulation with each factor. In both normal and psoriatic cells, MAPK activity remained elevated up to 6 h after serum stimulation. In contrast, after 1 h of stimulation by PDGF, the MAPK activity in normal fibroblasts was similar to that in unstimulated cells, whereas it remained significantly elevated in psoriatic cells.

MAPK phosphorylation was visualized by gel shift assays and western blotting with the polyclonal anti-Erk1 antibody (anti-rat MAP kinase R2) (Fig 2D) or monoclonal or polyclonal antibodies specific for phospho–MAPK (New England Biolabs) (data not shown). The amount of the phosphorylated form of Erk1 and Erk2 in psoriatic fibroblast extracts increased after PDGF stimulation, peaking after 15 min stimulation and declining thereafter with time of stimulation to controls (serum–deprived cell lysate) after 3 h (Fig 2D).

As normal and psoriatic fibroblasts deprived of serum for 72 h
contains the same amounts of MAPK protein (Fig 2C), we conclude that the difference in MAPK activity observed between normal and psoriatic fibroblasts was not due to a difference in MAPK protein level but to a different degree of activation.

High purified MAPK activity in psoriatic fibroblasts To determine MAPK activity more specifically, we purified these kinases from primary cultures from five normal subjects and five psoriatic patients by anion-exchange chromatography as described by Gomez and Cohen (1991). We verified by immunodetection that normal and psoriatic fibroblast extracts contained the same amount of Erks (as illustrated in Fig 2C). MAPK was then purified from serum-deprived (− serum) and serum-stimulated (+ serum) extracts of normal and psoriatic fibroblasts (Fig 3). MAPK kinase activity was measured in the anion-exchange chromatography fractions (FPLC) (Fig 3A, D). The activity was assayed in the presence of phosphatase inhibitors and in the absence of kinase inhibitors. The activity was detected in fractions 41–45 (NaCl concentrations between 160 and 200 mM) for serum-deprived fibroblasts (Fig 3A) and in fractions 41–47 for stimulated fibroblasts (Fig 3D). As shown in Figs 3A and 3D, MAPK activity was higher in the psoriatic cell extracts than in normal cells. The total MAPK activity was estimated by summing MAPK activity of the fractions containing Erk1 and Erk2 immunoreactivity. Results are expressed as picomoles of inorganic phosphorus incorporated per 30 min per 10 mg protein loaded onto the column.

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<th>Table I. Total MAPK activity in Mono Q-purified cell extracts*</th>
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*Normal and psoriatic fibroblast cytoplasmic extracts prepared from serum-deprived cells and from serum-stimulated cells were chromatographed and analyzed in the conditions described for Fig 3. MAPK activity was obtained by summing the MBP phosphorylation activity of the fractions containing Erk1 and Erk2 immunoreactivity. Results are expressed as picomoles of inorganic phosphorus incorporated per 30 min per 10 mg protein loaded onto the column.

**Serum-deprived psoriatic fibroblasts versus normal fibroblasts, p < 0.01.

**Serum-stimulated psoriatic fibroblasts versus normal fibroblasts, p < 0.05.

Figure 2. Time course of stimulation by serum or by PDGF. Subconfluent normal and psoriatic fibroblasts were growth-arrested by 72 h of serum depletion, and then stimulated with 10% fetal calf serum (A) or with 1 ng PDGF per ml (B) for the periods indicated. Cells were rinsed twice with cold PBS, and extracts were obtained as described in Materials and Methods. MAPK activity was measured using MBP in the presence of PKA, PKC, and Ca²⁺/calmodulin kinase inhibitors. MAPK activity is expressed in picomoles of inorganic phosphorus incorporated per 30 min per mg protein. Immunoblots of various cell extracts obtained during PDGF stimulation for the psoriatic fibroblasts (D) were prepared using anti-MAPK antibody (anti-rat MAPK R2).
deprived psoriatic fibroblast extracts correlated ($R^2 = 0.6$) with the severity of cutaneous manifestations (PASI score).

FPLC fractions were analyzed by immunoblotting using anti-Erk1/Erk2 antibody. Erks were immunodetected in fractions 39–47 in serum-deprived fibroblasts (Fig 3B) and in fractions 41–47 in serum-stimulated fibroblasts (Fig 3E). We confirmed that the phosphorylated active forms of Erks were eluted after the inactive forms as demonstrated by Gomez and Cohen (1991). As assessed by mobility shift of Erk1 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the phosphorylated form of Erk1 was immunodetected in fractions 41–44 in serum-deprived fibroblasts (Fig 3B) and in fractions 42–47 in serum-stimulated fibroblasts (Fig 3E).

MBP is a substrate for several kinases including PKA, PKC, and Ca$^{2+}$/calmodulin kinases. In our experimental conditions (see Materials and Methods), Ca$^{2+}$/calmodulin kinases were always inhibited. We checked by immunoblot analysis with antibodies against PKA and PKC that the PKA catalytic subunit eluted in the flow through (data not shown) and PKC was immunodetected in fractions 38, 39, and 40 (NaCl concentrations between 130 mM and 150 mM) (Fig 3C).

8-bromo-cAMP inhibits MAPK phosphorylation in normal but not in psoriatic serum-stimulated fibroblasts PKA may indirectly regulate MAPK via Raf (Wu et al., 1993; Hafner et al., 1994) and psoriatic fibroblasts present a PKA abnormality (Raynaud et al., 1989).

The stimulatory effect of serum on MAPK phosphorylation was therefore compared in normal and psoriatic fibroblasts incubated in the presence or absence of 8-bromo-cAMP. The Erk pattern was studied by immunoblotting with an anti-MAPK antibody (Fig 4A). In normal and psoriatic fibroblasts treated with serum, three bands were detected; the two major bands corresponded to the activated phosphorylated forms of Erk1 and Erk2, and the minor band to nonphosphorylated Erk1. In psoriatic cells, preincubation with 8-bromo-cAMP did not modify the Erk pattern. In contrast, in normal cells, incubation with 8-bromo-cAMP increased the amount of nonphosphorylated Erk1 and resulted in the presence of a fourth band. Using a serum-deprived fibroblast lysate, we demonstrated that this additional fourth band corresponded to the nonphosphorylated form of Erk2. Scanning the immunoblots for normal fibroblast lysates revealed a diminution of phosphorylated forms of Erk1 and Erk2 after pretreatment with 8-bromo-cAMP (Fig 4B) and an increase in the amount of nonphosphorylated forms. For psoriatic fibroblast extracts, 8-bromo-cAMP pretreatment did not provoke any significant change in Erk pattern (Fig 4C). 8-bromo-cAMP pretreatment inhibited serum-stimulated MAPK activity by around 30% and 5%, respectively, in normal and psoriatic fibroblast extracts.

**Variation in nuclear localization of phosphorylated MAPK** Activated MAPK translocate into the nucleus (Chen et al., 1992; Lenormand et al., 1993). We followed the effect of either serum or PDGF AB stimulation on the translocation of MAPK into the nucleus by immunofluorescence microscopy using an antibody specific for the phosphorylated forms of Erk1 and Erk2 (Figs 5 and 6). After 72 h serum depletion, no or very few normal (Figs 5A and 6A) or psoriatic (Figs 5B and 6B) cells showed cytoplasmic staining. Perinuclear staining of phosphorylated MAPK was observed in both psoriatic and normal fibroblasts. There was no significant difference in the immunostaining profile (intensity or number of stained cells) between normal and psoriatic serum-deprived fibroblasts. After a short stimulation with serum (15 min or 30 min), we observed an increase in phosphorylated Erks in the cytoplasm surrounding the nucleus in both normal cells and psoriatic fibroblasts (data not shown). After 1 h of serum stimulation, phosphorylated Erks were detected in the nuclei of the majority of normal fibroblasts (Fig 5C), whereas no nuclear staining was visible in the psoriatic fibroblasts (Fig 5D). After 3 h serum stimulation, most phosphorylated Erk staining was observed inside the nuclei in normal fibroblasts as well as in psoriatic fibroblasts (Fig 5E, F). During the studied time frame, no phosphorylated MAPK was observed in the nuclei of psoriatic fibroblasts before 3 h of serum stimulation in three independent experiments. The phosphorylated Erks were cytoplasmic in normal and psoriatic fibroblasts after 6 h of serum stimulation (data not shown).

The findings for PDGF stimulation were dissimilar to those for serum stimulation. Phosphorylated forms of Erks were apparent in the nuclei of psoriatic fibroblasts within 30 min and after 1 h and 3 h of PDGF stimulation (Fig 6D, F, H). In normal cells, phosphorylated Erks were observed in the nucleus only after 1 h of PDGF stimulation (Fig 6E), and were in the cytoplasm surrounding the nucleus after 30 min and 3 h of PDGF stimulation (Fig 6C, G).

We investigated the subcellular localization of phosphorylated Erks in skin samples from healthy (Fig 7A) and psoriatic (Fig 7B) subjects by immunostaining with the antibody recognizing the phosphorylated forms of Erk1 and Erk2 (three normal and four psoriatic skins were analyzed). Nuclear phosphorylated Erks appeared as bright dots in the nuclei of cells. The nuclear localization was confirmed by DAPI staining. Nuclear staining was observed in keratinocytes and in dermal cells in both normal and psoriatic skin samples. We observed no significant difference in the staining pattern with the phospho-specific Erk antibody between normal and nonlesional psoriatic skins.

**DISCUSSION**

Dermal fibroblasts may play a primary role in psoriasis (Priestley, 1991), and psoriatic fibroblasts are hyperproliferative. Here, we found that...
Figure 5. Immunolocalization of phosphorylated Erk1 and Erk2 after serum stimulation. Normal (A, C, E) and psoriatic (B, D, F) fibroblasts were cultured on glass slides and then subject to serum depletion for 72 h. Cells were not treated (A, B) or were stimulated with serum for 1 h (C, D) or for 3 h (E, F) and fixed in 60% methanol and 40% acetone at –20°C. They were incubated with the phospho-specific MAPK antibody, then with a biotinylated anti-rabbit IgG antibody and finally with Texas Red-streptavidin. The samples were examined with a fluorescence microscope under epifluorescent illumination with excitation-emission filters for rhodamine; scale bar, 10 µm.

Psoriatic fibroblasts in culture were significantly larger than normal fibroblasts. Psoriatic fibroblasts display a PKA abnormality (Raynaud et al., 1989) and we show that they also have high MAPK activity.

We found significantly and reproducibly higher MAPK activity in serum-deprived fibroblasts and in serum- or PDGF-stimulated fibroblasts from psoriatic patients than in fibroblasts from normal subjects. This higher activity was evidenced by MAPK purification from cell extracts by anion-exchange chromatography. The chromatographic behavior of MAPK in normal and psoriatic cell extracts was identical in our experimental conditions, and was consistent with previous reports (Gomez and Cohen, 1991; Trigon and Morange, 1995). A recent study showed an increased MAPK activity associated with an increased protein level in human breast cancer cells (Sivaraman et al., 1997). In our study, MAPK activity was higher in psoriatic fibroblasts than in normal cells but the amounts of MAPK protein level were the same.

The high MAPK activity in psoriatic fibroblasts may have been due to low phosphatase activity, as phosphatases inhibit these kinases. Most dual-specificity phosphatases are immediate-early mitogen inducible phosphatases (Zolnierowicz and Hemmings, 1996). Thus, a diminished activity of these phosphatases would not explain the increase in MAPK activity observed in serum-deprived psoriatic fibroblasts; however, Pyst1, a MAPK dual specificity phosphatase, is expressed constitutively.
The altered PKA activity in psoriatic fibroblasts is associated with serum-induced activation of MAPK in normal dermal fibroblasts, as previously shown (Raynaud et al., 1986; Raynaud et al., 1989). Therefore, studies of MAPK in both normal and psoriatic fibroblasts in culture. Indeed, MAPK is not only involved in the G0/G1 transition of normal cells (Espinoza et al., 1994), but also has two phases of activation during the cell cycle: during the G1 and G2/M phases (Tamemoto et al., 1992). A recent study confirmed the hyperproliferative state of psoriatic fibroblasts and revealed significantly longer S and G2/M phases in psoriatic fibroblasts cultures than in normal cells (Espinoza et al., 1994). Therefore, studies of MAPK in nonsynchronized serum-stimulated cells are difficult to interpret. In our experiments, after 72 h of serum deprivation, 90% of both normal and psoriatic fibroblasts were in the G0/G1 phase (FACscan analysis, data not shown).

The size of psoriatic fibroblasts in culture may be due to a modified expression of proto-oncogenes such as c-nes and c-fos (Basset-Seguin et al., 1991), which are MAPK substrates (Pelch and Sanghera, 1992; Gille et al., 1995). The high nuclear MAPK activity may thus be responsible for the ability of psoriatic cells to hyperproliferate, as well as for the abnormalities in cell size and shape. As MAPK, like PKA, interacts with microtubules (Reszka et al., 1995) and other cytoskeleton proteins (Han and Rubin, 1996), the cytoskeletal abnormalities in psoriatic cells may be due to the modified MAPK activity.

Interestingly, the kinetics of MAPK activation by serum or by PDGF were not the same in normal and psoriatic fibroblasts: the effects were larger and more durable in psoriatic cells. We investigated whether this phenomenon was associated with an abnormal MAPK subcellular localization. We reproducibly detected small amounts of phosphorylated Erks in the cytoplasm of normal and psoriatic serum-deprived fibroblasts. We did not observe any nuclear translocation of Erks after brief serum-stimulation as described by Lenormand et al. (1993) in transformed Chinese hamster lung fibroblasts. The different time course obtained might be due to the use of a transformed cell line (CCL39) in the previous study and fibroblast primary cell cultures in ours. In addition, the localization of phosphorylated forms of MAPK was different in normal and psoriatic fibroblasts: phosphorylated active Erks were nuclear after 30 min, 1 h, and 3 h of PDGF stimulation in psoriatic fibroblasts, but were found in the nuclei of normal fibroblasts only after 1 h of PDGF stimulation. The translocation of phosphorylated and active MAPK in stimulated PDGF psoriatic fibroblasts is more rapid and more sustained than in normal cells. Therefore the mitogenic and chemotactic effects of PDGF are larger in psoriatic fibroblasts that present an increased number of PDGF receptors than in normal cells (Krane et al., 1991). In contrast, serum-stimulated MAPK translocated into the nuclei of psoriatic fibroblasts later than in normal cells. The nuclear translocation in psoriatic fibroblasts was transient, phosphorylated MAPK staying in the nuclei less than 1 h. These observations are consistent with the previous demonstration that the nuclear translocation of MAPK is independent of their phosphorylation and their phosphotransferase activity (Lenormand et al., 1993). The mechanism of MAPK translocation into the nucleus remains unclear, and no nuclear localization sequence can be identified in MAPK. Unlike PDGF, serum contains numerous factors including growth factors and proteases. After serum stimulation of psoriatic fibroblasts, one or more of these factors may delay the translocation of phosphorylated MAPK into the nuclei.

No difference between normal and nonlesional psoriatic skins was observed by immunohistochemistry with an antibody against phosphorylated MAPK. Some cells in the epidermis and the dermis of both skin presented a nuclear immunostaining. The discrepancy between this result and the immunocytochemical findings could be associated with the cells in skin being in different stages of the cell cycle. We also failed to find any difference in nonsynchronized normal and psoriatic fibroblasts in culture.

In conclusion, we found that MAPK activity was higher in cultured psoriatic fibroblasts than in normal fibroblasts. This is the first example of a MAPK pathway abnormality in a human benign hyperproliferative disease. An abnormality of PKA might induce alteration in another major signal transduction pathway due to the close interrelationships between the different cell signal transductions. Elucidation of the mechanisms by which elevated activated MAPK alters cell functions may help to explain the pathogenesis of psoriasis.

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REFERENCES


Figure 7. Immunohistochemical analysis of human skin. Thin sections (6 µm) of either normal skin (A) or nonlesional psoriatic skin (B) fixed at –20°C in 60% methanol and 40% acetone were examined by fluorescence microscopy following staining with the phospho-specific MAPK antibody. White arrows indicate positive staining in nucleus of epidermic and dermic cells; scale bar, 50 µm.


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