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Temperature-dependent tyrosine phosphorylation of microtubule-associated protein kinase in epidermal growth factor-stimulated human fibroblasts

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Treatment of normal human fibroblasts with epidermal growth factor (EGF) results in the rapid (0.5 min) and simultaneous tyrosine phosphorylation of the EGF receptor (EGFr) and several other proteins. An exception to this tyrosine phosphorylation wave was a protein (42 kDa) that became phosphorylated on tyrosine only after a short lag time (5 min). We identified this p42 kDa substrate as the microtubule-associated protein (MAP) kinase using a monoclonal antibody to a peptide corresponding to the C-terminus of the predicted protein (Science *249*, 64–67, 1990). EGF treatment of human fibroblasts at 37°C for 5 min resulted in the tyrosine phosphorylation of 60–70% of MAP kinase as determined by the percent that was immunoprecipitated with antiphosphotyrosine antibodies. Like other tyrosine kinase growth factor receptors, the EGFr is activated and phosphorylated at 4°C but is not internalized. Whereas most other substrates were readily tyrosine phosphorylated at 4°C, MAP kinase was not. When cells were first stimulated with EGF at 4°C and then warmed to 37°C without EGF, tyrosine phosphorylation of MAP kinase was again observed. Treatment of cells with the protein kinase C activator phorbol myristate acetate (PMA) also resulted in the tyrosine phosphorylation of MAP kinase, and again only at 37°C. Tryptic phosphopeptide maps demonstrated that EGF and PMA both induced the phosphorylation of the same peptide on tyrosine and threonine. This temperature and PMA sensitivity distinguishes MAP kinase from most other tyrosine kinase substrates in activated human fibroblasts.

Introduction

Addition of epidermal growth factor (EGF) to responsive cells initiates a series of events that culminates in DNA synthesis, cellular growth, and differentiation (Carpenter and Cohen, 1990). The pathway leading to DNA synthesis is unclear but may initially involve the tyrosine phosphorylation of critical protein substrates on tyrosine by the activated EGF receptor tyrosine kinase. In recent years a number of proteins have been identified that become phosphorylated on tyrosine after EGF stimulation and are excellent candidates to play such a critical role. These include phospholipase Cγ (Margolis *et al.*, 1989), phosphatidylinositol-3-kinase (Skolnik *et al.*, 1991), GTPase activating protein (Ellis *et al.*, 1990), microtubule-associated protein (MAP) kinase (Rossamando *et al.*, 1989; Ahn *et al.*, 1990), among others (see Cantley *et al.*, 1991, for additional references; Carpenter and Cohen, 1990).

MAP kinase is a serine/threonine protein kinase that has been assayed in vitro with the proteins MAP2 (Hoshi *et al.*, 1988; Rossomando *et al.*, 1989; Anderson *et al.*, 1990) and myelin basic protein (Ahn *et al.*, 1990, 1991; Sanghera *et al.*, 1991). MAP kinase has been shown to be activated by both tyrosine and threonine phosphorylation (Anderson *et al.*, 1990) and is apparently identical to the pp42 protein initially found as a tyrosine phosphorylated protein on mitogen stimulation (Nakamura *et al.*, 1983; Cooper and Hunter, 1985; Kohno, 1985; Cooper, 1989; Rossomando *et al.*, 1989). MAP kinase is now known to be phosphorylated on stimulation of cells with EGF, nerve growth factor, insulin, platelet-derived growth factor, serum, phorbol myristate acetate (PMA), and catecholamines (Sturgill, 1987; Rossomando *et al.*, 1989; Ely *et al.*, 1990; Kazlauskas and Cooper, 1988; Erikson, 1991; Miyasaka *et al.*, 1991). More recently, Boulton *et al.* (1990, 1991) purified and cloned a MAP2 kinase (44 kDa) from insulin stimulated rat 1 fibroblasts overexpressing the normal human insulin receptor. Significant homology was observed between MAP kinase (also referred to as extracellular regulated kinase [ERK]) to several other kinases involved in cell cycle regulation of yeast (Boulton *et al.*, 1990).

EGF is a mitogen for normal human fibroblasts (Carpenter and Cohen, 1975) where it binds its surface receptor (Carpenter and Cohen, 1976), activates its tyrosine kinase domain (Ullrich and Schlessinger, 1990), and then becomes internalized and degraded (Stocheck and Carpenter, 1984). Little is known about the cellular substrates that become phosphorylated on tyrosine by the activation of the EGF receptor (EGFr) in human fibroblasts. In our study of substrates for EGFr and receptor internalization, we noted a substrate that was not phosphorylated at 4°C, a condition in which internalization is blocked. This is in contrast to other substrates and the receptor itself that were fully phosphorylated at this temperature. We identify this substrate as MAP kinase and compare the temperature sensitivity and site of phosphorylation to the phosphorylation induced with PMA, a tumor promotor known to result in pp42 phosphorylation in other systems (Kohno, 1985; Hoshi *et al.,* 1988; Vila and Weber, 1988; Cooper, 1989; L'Allemain *et al.,* 1991; Meier *et al.,* 1991). We find that phorbol ester also induced the tyrosine phosphorylation of MAP kinase in human fibroblasts with the same kinetics and temperature-dependence as EGF, resulting in the phosphorylation of the same tryptic peptide on tyrosine and threonine. We suggest that MAP kinase becomes tyrosine phosphorylated by a temperature-dependent kinase(s), which itself becomes activated by at least two different signaling pathways.

Results

Although human fibroblasts respond mitogenically to EGF, they contain a relatively small number of EGF receptors and consequently the tyrosine phosphorylation of substrates is reduced compared with cells overexpressing the receptor (Cooper *et al.,* 1982a). To amplify the tyrosine phosphorylation signal detectable on western blots, we performed immunoprecipitations of cellular lysates with high affinity rabbit polyclonal anti-PTyr antibodies, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to immobilon. The blots were then probed with an ^{125}I-labeled antiphosphotyrosine antibody py20 (Glenney *et al.,* 1988b). Control experiments revealed that the pattern of tyrosine phosphorylated proteins did not change in this two-stage protocol compared with a simple antiphosphotyrosine western blot while the sensitivity was increased at least 10-fold (data not shown). If the immunoprecipitation was performed with py20 before western blot there was selective loss of a 42 kDa p-tyr protein (not shown). On treatment of cells with EGF, proteins with estimated molecular weights of 180, 170, 120, and 76 kDa became rapidly (0.5–1.0 min) phosphorylated on tyrosine. In contrast, a protein of 42 kDa was detected that was not phosphorylated until later times (Figure 1A). Quantitation of the 42-kDa protein phosphorylation revealed that maximal phosphorylation was not achieved until 5–15 min after EGF stimulation as compared with the phosphorylation of the EGFr itself that occurred within 1 min (Figure 1, B and C).

Previous studies have demonstrated the tyrosine phosphorylation of a MAP kinase with a molecular mass of 42–44 kDa in cells stimulated with EGF (see above). To examine the relatedness between the 42-kDa substrate from EGF-stimulated human fibroblasts and MAP kinase, cell lysates from cells treated with EGF were sequentially immunoprecipitated two times with anti-MAP kinase (MK) antibody followed by a third immunoprecipitation with anti-PTyr antibody. Immunoprecipitation with MK antibodies specifically depleted the 42-kDa protein from the cell lysate that was recovered in the immunoprecipitate, thus indicating that the 42-kDa phosphotyrosine substrate is related to MAP kinase (Figure 2A). To further confirm the identity, immunoprecipitations with the MK antibody were performed under nondenaturing conditions and used in a kinase reaction with myelin basic protein (MBP) as substrate. As expected, the immunoprecipitate from EGF-stimulated cells contained MBP kinase activity, whereas that from control cells was undetected (data not shown). Next, we performed a quantitative immunoprecipitation with anti-PTyr to estimate the stoichiometry of the tyrosine phosphorylation of MAP kinase. Equivalent amounts of total cell lysate, nonadsorbed and bound (immunoprecipitated) anti-PTyr fractions, were probed with MK antibody on a western blot (Figure 2B). Quantitation revealed that 60–70% of the total cellular MAP kinase is tyrosine phosphorylated at 5 min after the addition of EGF in normal human fibroblasts. Given the accuracy of this method, this should be considered a lower estimate.

The EGF receptor is known to be activated but not internalized at 4°C, a process that occurs at 37°C (Carpenter and Cohen, 1976). EGF stimulation at 4°C results in tyrosine phosphorylation of the EGFr and other proteins with the exception of MAP kinase even up to 1 h (Figure 3). When cells that were stimulated with EGF at 4°C for 30 min were further incubated

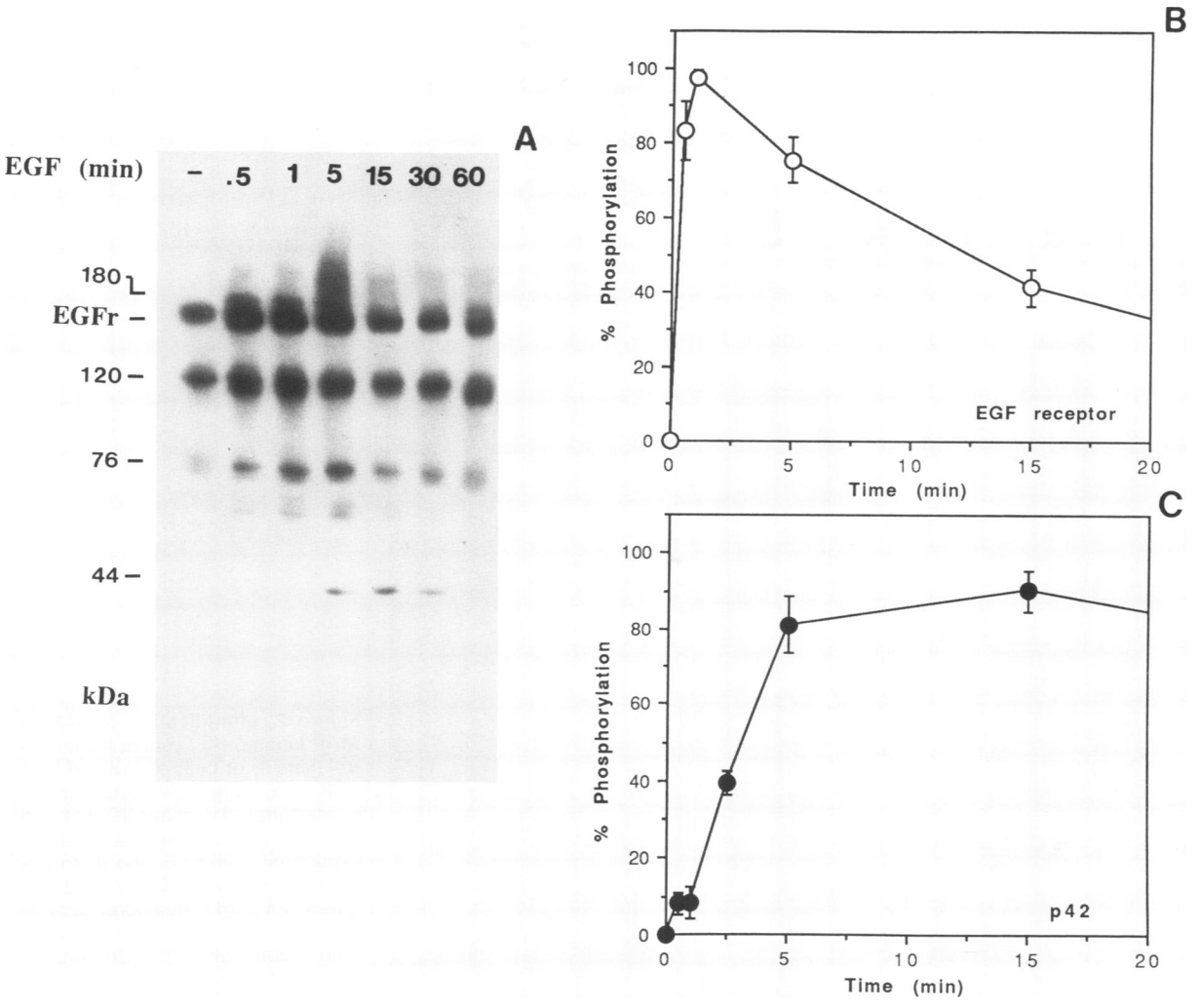

Figure 1. **Time-dependent tyrosine phosphorylation in human fibroblasts.** EGF (25 ng/ml) was added to human fibroblasts for the indicated times before lysis. (A) Cell lysates were immunoprecipitated with rabbit polyclonal anti-PTyr, immunoblotted, and probed with ^{125}I-monoclonal anti-ptyr (PY20). (B) EGFr was immunoprecipitated with anti-receptor antibodies, immunoblotted, and probed with ^{125}I-py20 and quantitated by γ-counting. (C) Tyrosine phosphorylation of p42 from panel A and quantitated as above. Tyrosine phosphorylation of the EGFr and p42 substrate are expressed as the means ± SEM from 6–8 different experiments.

at 37°C, there was a rapid tyrosine dephosphorylation of the EGFr (Figure 3, A and D) concomitantly with the tyrosine phosphorylation of MAP2 kinase (Figure 3D). Control temperature shift (without EGF) also resulted in a small and variable amount of tyrosine phosphorylation of MAP kinase (Figure 3, A and B) that we interpret as a return to the basal phosphotyrosine content in MAP kinase in untreated cells. The tyrosine phosphorylation of MAP kinase was also observed in B82L cells expressing the human EGF receptor (Glenney *et al.*, 1988a) at 37°C but not at 4°C, even up to 8 h (data not shown).

To further examine the temperature-dependence on the tyrosine phosphorylation of MAP kinase, cells were preincubated at different temperatures and EGF added for 5 min (Figure 4). Tyrosine phosphorylation of the EGFr and several cellular substrates was evident in cells incubated at temperatures from 0 to 37°C (Figure 4A). Other substrates, like p76, attained maximal phosphorylation at 16°C. MAP kinase, however, was poorly phosphorylated on tyrosine at temperatures between 0–22°C, intermediate phosphorylation at 30°C, and became maximally phosphorylated at 37°C (Figure 4, A–C). We also noticed two other polypeptides of about 46 and 40 kDa that were tyrosine phosphorylated exclusively at 37°C (Figure 4A). The identity of these two proteins is unknown, but

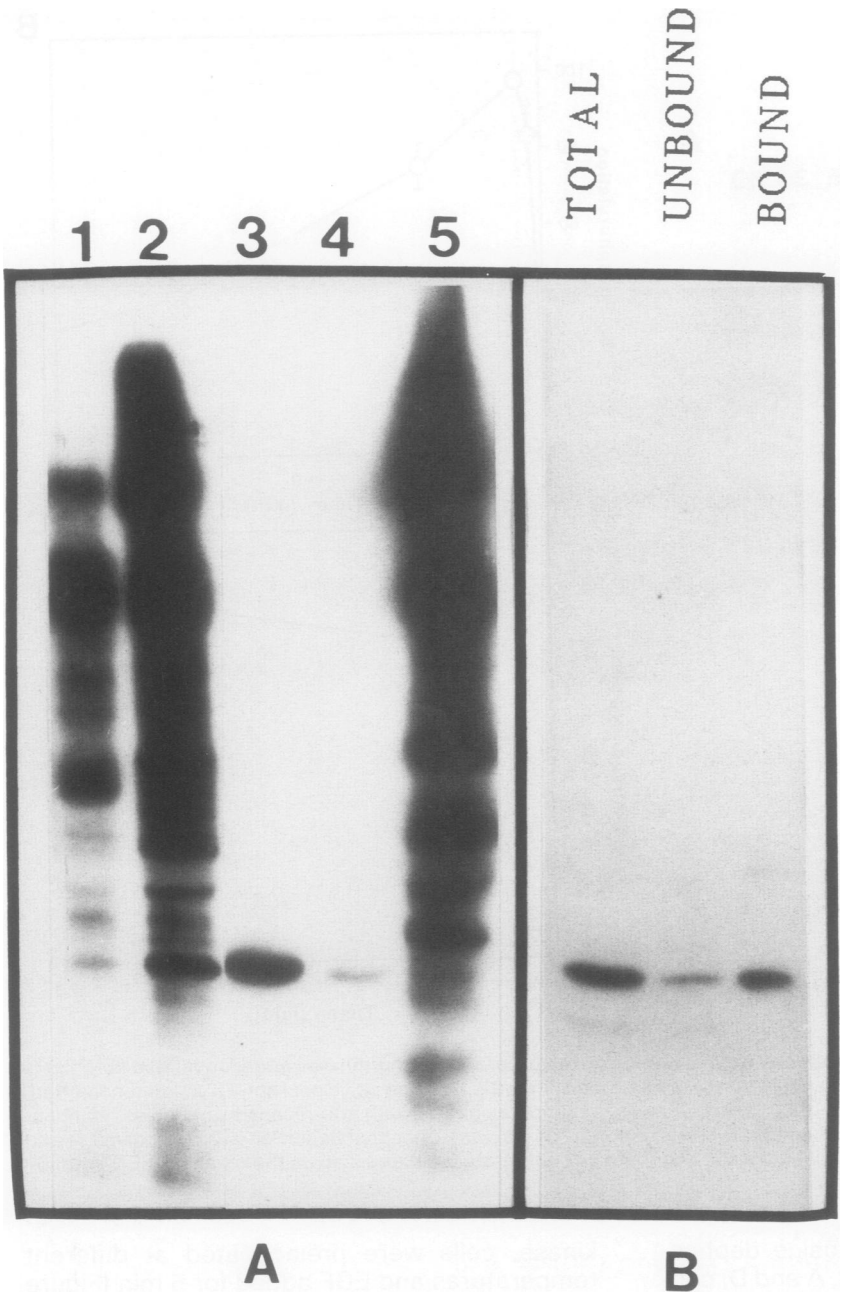

Figure 2. **Identification of p42 as MAP kinase.** (A) Lysates from control (lane 1) or cells stimulated with EGF for 5′ (lanes 2–5) were immunoprecipitated with anti-PTyr (1 and 2) or with anti-MAP kinase two times sequentially (lanes 3 and 4) to deplete the lysate of MAP kinase, followed by a third immunoprecipitation with anti-PTyr (lane 5). All lanes were then probed with ^{125}I-py20 (lanes 1 and 2, 4-h exposure; lanes 3–5, 8-h exposure). (B) Estimation of the extent of tyrosine phosphorylated MAP kinase. Fibroblasts were treated with EGF for 5 min and the lysate was subjected to immunoprecipitation with anti-PTyr antibody. Equivalent amounts of the total cellular proteins, protein unbound or bound to the anti-PTyr antibody, was run on SDS-PAGE and immunoblotted with anti-MAP kinase antibodies and ^{125}I-anti-mouse IgG.

it is possible that they are related to MAP kinase although they do not appear to react with this MK antibody.

Treatment of fibroblasts with the tumor promotor PMA is known to result in the tyrosine phosphorylation of pp42 (Vila and Weber, 1988; Kazlauskas and Cooper, 1988; L'Allemain *et al.*, 1991). PMA also increased the tyrosine phosphorylation of MAP kinase in the human fibroblast system with the same kinetics and temperature dependence, i.e., tyrosine phosphorylation of MAP kinase after PMA treatment was not observed at 4°C but occurred within 5 min on raising the temperature to 37°C (Figure 5). Because PMA did not alter the tyrosine phosphorylation of other protein substrates (data not shown), it suggests that PMA is activating a different tyrosine kinase than the EGFr.

It is possible that tyrosine phosphorylation of MAP kinase occurs on a different site after PMA compared with EGF stimulation. Recent studies

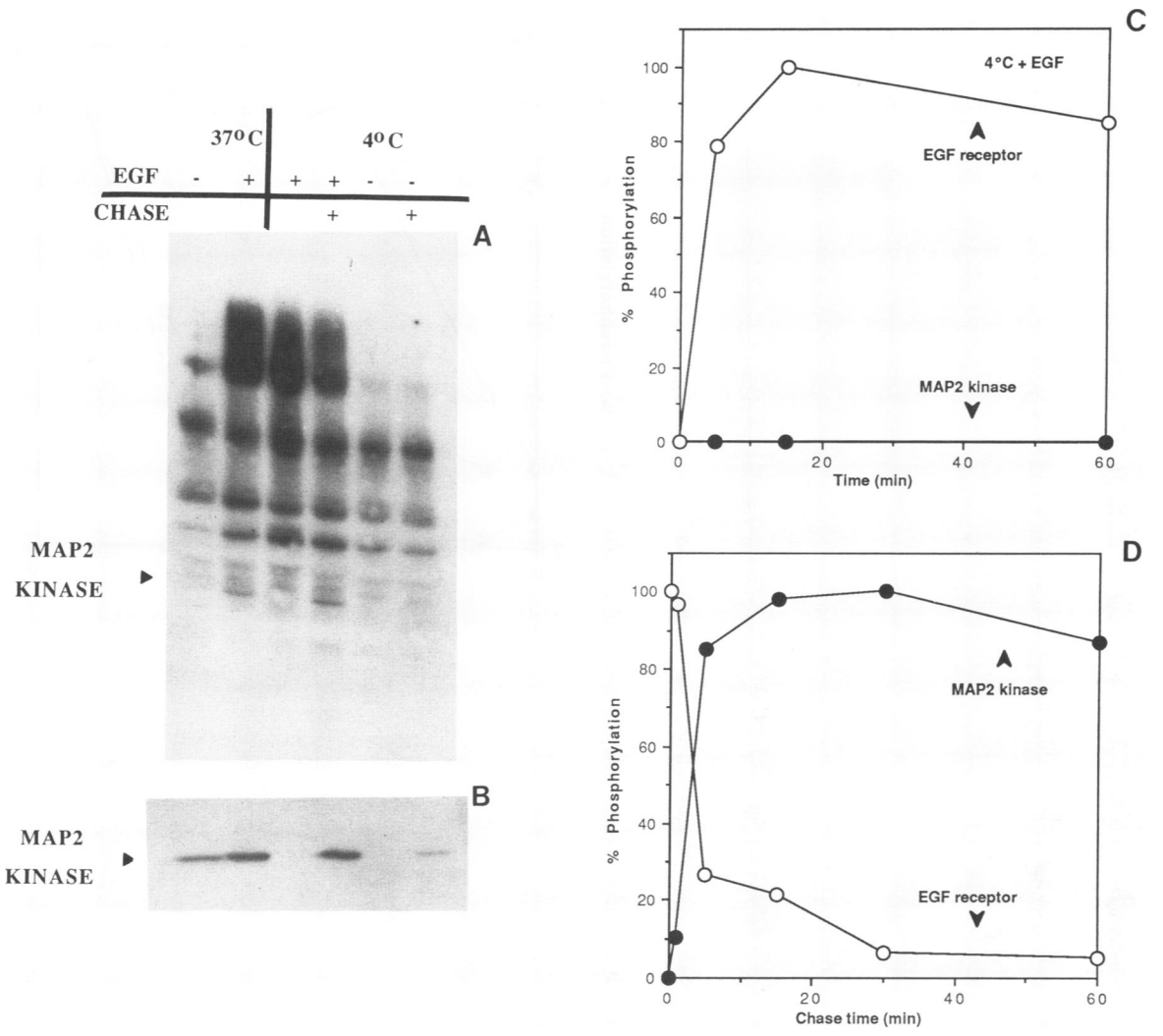

Figure 3. **Tyrosine phosphorylation of MAP kinase at 37°C vs 4°C.** Control cells and those stimulated with EGF at 37 or 4°C were lysed and immunoprecipitated with anti-PTyr antibody. Cells were stimulated with EGF for 5 min at 37°C or 30 min at 4°C. Where indicated, cold-treated cells were chased with a medium change (lacking EGF) and further incubated for the indicated times. (A) Western blot profile of tyrosine phosphorylated proteins immunoprecipitated with anti-PTyr antibody and then probed with ^{125}I-py20. (B) Tyrosine phosphorylation of MAP kinase detected in anti-PTyr immunoprecipitates probed with anti-MAP kinase and ^{125}I-anti-mouse IgG. (C) Quantitation of tyrosine phosphorylation of EGFr and MAP kinase from cells stimulated at 4°C. The receptor was immunoprecipitated with anti-EGFr and probed with ^{125}I-py20. MAP kinase phosphorylation was detected as described for B. (D) Tyrosine phosphorylation of EGFr and MAP kinase after stimulation at 4°C during 30 min followed by a chase with a medium change and 37°C incubation.

have shown that MAP kinase is phosphorylated on both tyrosine and threonine residues in phorbol ester-treated EL4 cells and insulin-stimulated adpocytes (Ray and Sturgill, 1988; Payne *et al.,* 1991). To gain insight into the complexity of MAP kinase phosphorylation, we examined the tryptic phosphopeptides of MAP kinase from cells stimulated under different conditions. EGF stimulation of cells at 37°C resulted in the phosphorylation of one major and several minor peptides (designated a, b, c, and d, Figure 6). Most of the ^{32}P was incorporated into peptide b on both threonine and tyrosine residues (data not shown). The phosphopeptide analysis in cells stimulated at 4°C was similar to control untreated cells (data not shown). Phosphopeptide b was also the major peptide phosphorylated after stimulation of cells at 4°C with EGF or PMA followed by a chase at 37°C (Figure 6). These maps, particularly peptide b, are similar to the published map of the MAP kinase from phorbol ester EL4 cells (Payne *et al.,* 1991).

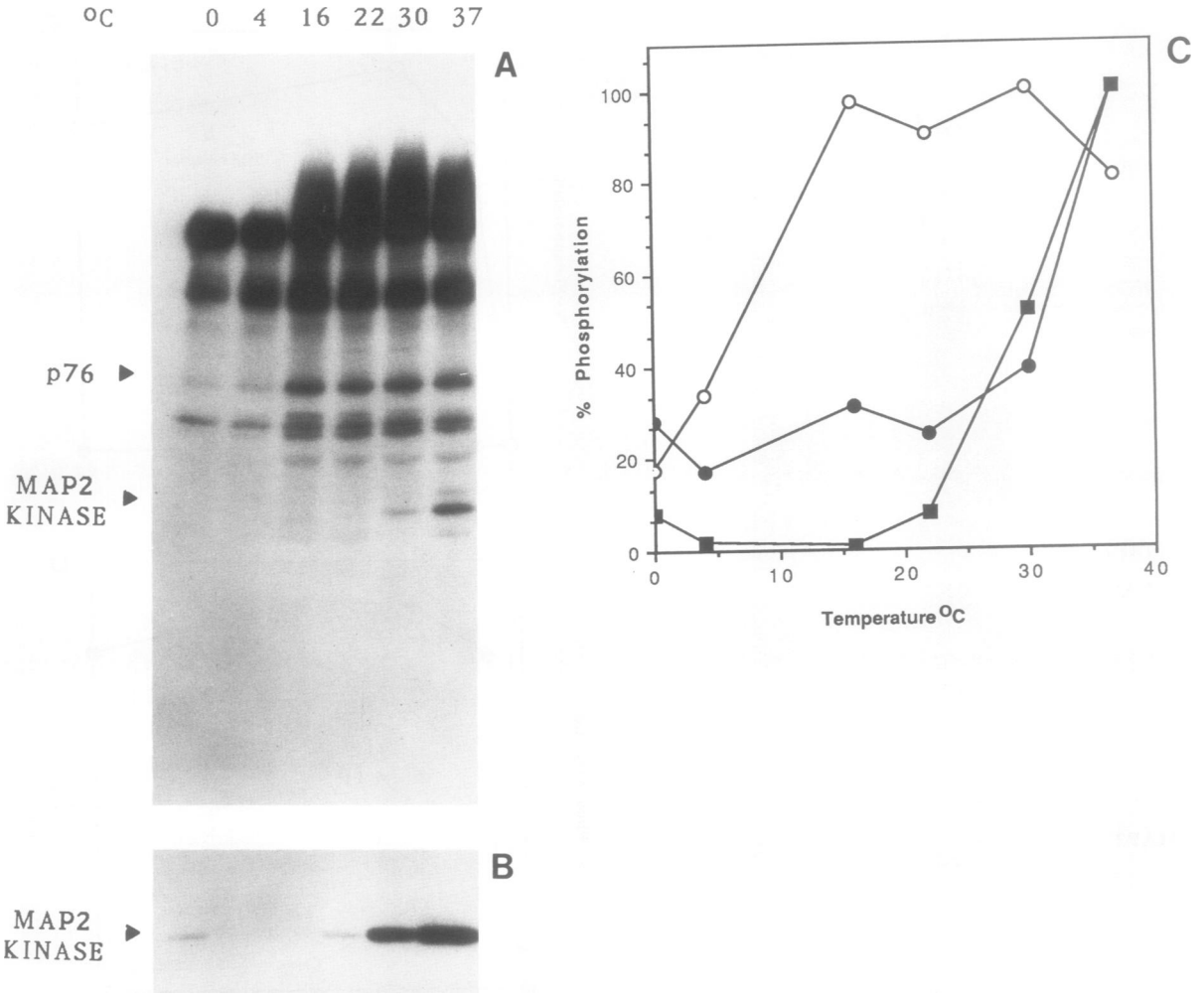

Figure 4. **Temperature-dependent tyrosine phosphorylation of MAP kinase.** Cells were preincubated at the indicated temperature for 10 min and then further stimulated with EGF for 5 min and lysed. (A) Tyrosine phosphorylated proteins were immunoprecipitated with anti-PTyr antibody and then probed with ^{125}I-py20 or (B) anti-MAP kinase antibody and ^{125}I-anti-mouse IgG (only the relevant portion of this western blot is shown). (C) Quantitation of the tyrosine phosphorylation of MAP kinase from the py20 blot (●; see panel A) or anti-MAP kinase immunoprecipitation (■; panel B) and comparison to the phosphorylation of a protein at 76 kDa (○; see panel A).

Discussion

MAP kinase has been proposed to play a role in cell proliferation and during early embryonic development (Sanghera *et al.*, 1990; Ferrell *et al.*, 1991; Gotoh *et al.*, 1991; Posada *et al.*, 1991). Multiple forms of MAP kinase have been detected. Nerve growth factor, for example, induced both the tyrosine phosphorylation of p42 and the activation of MAP2 kinase, yet the two could be distinguished chromatographically (Miyasaka *et al.*, 1991). In other studies at least two distinct isoelectric variants of pp42 were detected in two-dimensional gels (Cooper, 1989). Furthermore, sequence analysis of both Xenopus p42 (Posada *et al.*, 1991) and the phosphorylation site of MAP kinase from 3T3L1 cells (Payne *et al.*, 1991) has confirmed the presence of heterogeneity of this class of proteins. Recently, Boulton *et al.*, (1991a) and Boulton and Cobb (1991) have reported the sequence of ERK2 (p42) and ERK3 (p62) and detected the presence of yet another polypeptide named ERK4 (p45). ERK4 (top), 1 (middle), and 2 (lower) could be resolved by electrophoresis and visualized by immunobloting using rabbit anti-peptide antibodies (Boulton and Cobb, 1991). In the present studies we used an anti-

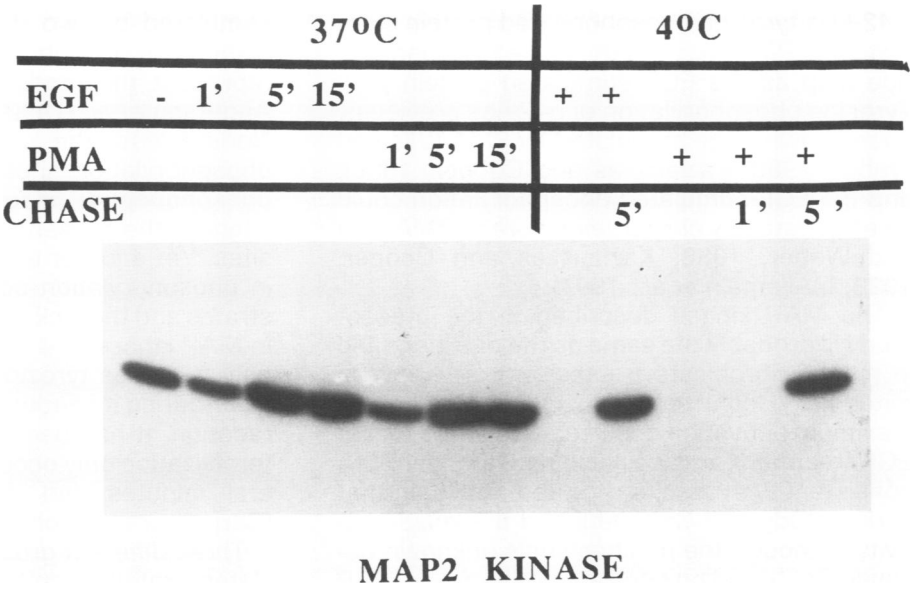

Figure 5. **PMA-dependent tyrosine phosphorylation of MAP kinase.** Cells were stimulated with EGF (25 ng/ml) or PMA (100 ng/ml) for the indicated times before lysis and immunoprecipitation. Tyrosine phosphorylation of MAP kinase was assessed by immunoprecipitation with anti-MAP2 kinase followed by western blot with ^{125}I-py20. Where indicated, cells were treated with EGF or PMA for 30 min at 4°C, followed by a chase with a medium change without these agents and further incubation at 37°C for the times specified.

peptide antibody specific for an ERK sequence close to the C-terminus of ERK-1. Inspection of the amino acid sequence in this region reveals 4 differences (out of 20) between ERK-1 and ERK-2 (Boulton *et al.*, 1991a). We have been unable to detect ERK-2 with this antibody (not shown) even in tissues where it is known to be present at high levels (Boulton and Cobb, 1991). We showed that this antibody immunoprecipitates virtually all of the 42-kDa tyrosine phosphorylated protein from human fibroblasts treated with EGF. Furthermore, it reacted with

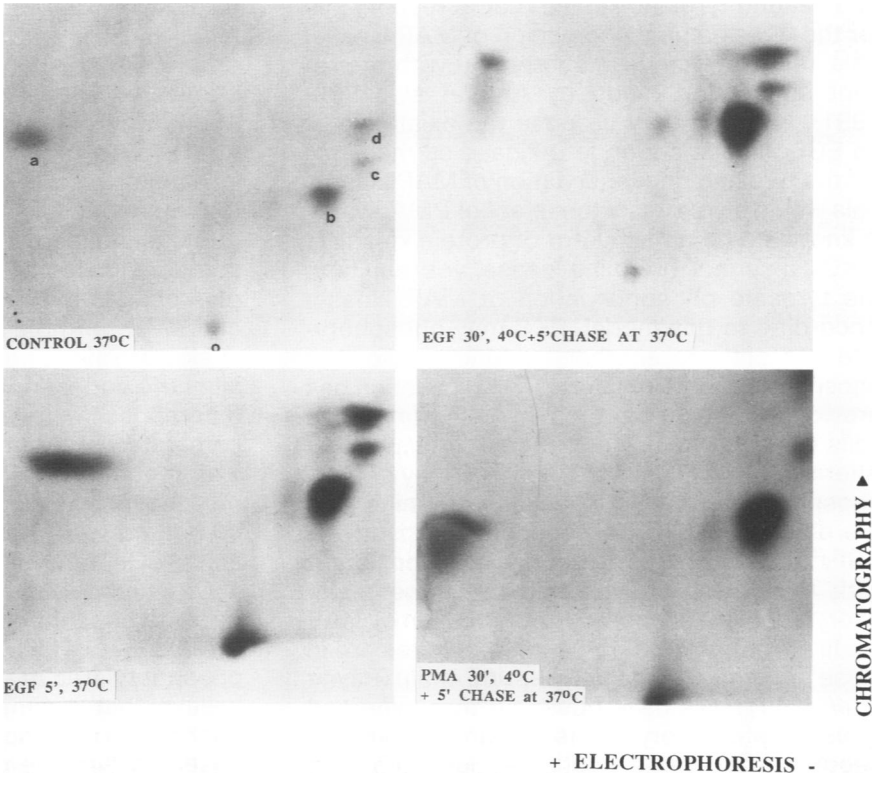

Figure 6. **Tryptic phosphopeptide map of ^{32}P-labeled MAP kinase.** Human fibroblasts were equilibrated with $^{32}PO_4$ for 8 h and then treated without or with EGF 37°C for 5 min. Alternatively, cells were treated with EGF or PMA at 4°C for 30 min followed by a chase at 37°C as described in the legend to Figure 3. MAP kinase was immunoprecipitated, resolved by SDS-PAGE, and the band corresponding to MAP kinase excised, washed, and extensively digested with trypsin. The resulting peptides were resolved by thin-layer electrophoresis followed by ascending chromatography. Peptide b was removed from the plate, and the phosphoamino acid analysis revealed the presence of both phosphothreonine and phosphotyrosine (not shown).

a 42-kDa tyrosine phosphorylated protein stimulated by PMA that has the same phosphopeptide map as the EGF stimulated protein p42. Tyrosine phosphorylation of p42 has previously been shown to be stimulated by PMA (Vila and Weber, 1988; Kazlauskas and Cooper, 1988), and the EGF-stimulated phosphorylation could be attenuated by chronic exposure to PMA (Vila and Weber, 1988; Kazlauskas and Cooper, 1988; L'Allemain *et al.,* 1991).

The MAP kinase described in the present study is probably the same as the one identified in human fibroblasts treated with a variety of mitogens (Hoshi *et al.,* 1988). In that study, maximum activation occurred after 5–15 min of EGF treatment and was also activated by PMA consistent with results reported above. In that earlier study, μM Ca^{++} inhibited the kinase activity, although the mechanism is unknown because Ca^{++}-binding sites are not found on MAP kinase (Boulton *et al.,* 1990). Given the complexity of this gene family uncovered in just the past year (see above), it is difficult to conclude that the Hoshi enzyme is the same as that studied here.

A delay is observed in the tyrosine phosphorylation of MAP kinase compared with other endogenous substrates at 37°C. This may be due to an indirect phosphorylation mechanism whereby the EGFr leads to the phosphorylation of a second tyrosine kinase that is responsible for the tyrosine phosphorylation of MAP kinase. This interpretation is in agreement with the recent suggestion made by Ahn *et al.* (1990, 1991), in which they propose the existence of an EGFr kinase-dependent kinase as mediator for the tyrosine phosphorylation of MAP kinase. This would also explain the effect of PMA, which is known to be an activator of protein kinase C (PKC), a serine/threonine kinase, yet results in the tyrosine phosphorylation of MAP kinase. According to this model, PKC may phosphorylate and activate another tyrosine kinase that phosphorylates MAP kinase. PKC activation has previously been observed in EGF-stimulated cells (Pelech *et al.,* 1986; Farese *et al.,* 1989). Alternatively, it is possible that PKC may directly phosphorylate MAP kinase on threonine and thereby allow it to be phosphorylated by the EGFr (or another tyrosine kinase). Consistent with this, both tyrosine and threonine phosphorylation are necessary for activation of MAP kinase (Anderson *et al.,* 1990), both sites are in close proximity on the polypeptide chain (Payne *et al.,* 1991). We have observed threonine and tyrosine phosphorylations occurring simultaneously in the same tryptic peptide from cells stimulated by two different signaling mechanisms. The phosphorylations on peptide b may represent the modulatory sites proposed by Anderson *et al.* (1990) and Payne *et al.* (1991). Nonetheless, the presence of several other phosphorylation sites detected in the tryptic phosphopeptide maps of MAP kinase would suggest the presence of additional regulatory sites. Yet another explanation for the lag time in phosphorylation compared with other substrates and the lack of tyrosine phosphorylation in MAP kinase at 4°C may be that MAP kinase only becomes tyrosine phosphorylated by the EGFr during its endocytic traveling toward degradation. It has been well established that internalization only occurs after a lag time of several minutes and does not occur at 4°C (Carpenter and Cohen, 1976).

Three different groups of tyrosine phosphorylated proteins can be distinguished in human fibroblasts according to their temperature-dependent tyrosine phosphorylation. The first group, which includes the EGFr, becomes tyrosine phosphorylated at all temperatures from 0–37°C. The second group, represented by p76, reached maximal tyrosine phosphorylation at temperatures of 16°C. MAP kinase and two minor proteins make up the third group, which begins to be phosphorylated at 30°C, and is maximally phosphorylated only at 37°C. The selective tyrosine phosphorylation of some proteins at 37°C but not at low temperatures (0 and 18°C) had been previously observed in EGF-stimulated rat liver cells (McCune and Earp, 1989); However, no identification of these substrates was provided and a 42-kDa substrate was not observed in those cells. In other studies (Sengupta *et al.,* 1988; Wei Li and Stanley, 1991), stimulation of macrophages with colony stimulating factor, resulted in the tyrosine phosphorylation of a set of proteins at both 4 and 37°C, with the lower temperature resulting in slower kinetics of phosphorylation of almost all of the endogenous substrates. In that study, differences in the appearance of tyrosine phosphorylated proteins was observed at 4°C that was thought to reflect a lowering of the rates of phosphorylation and dephosphorylation compared with 37°C. In contrast to these results, EGF treatment of human fibroblasts at 4°C resulted in only a slight delay in all of the tyrosine phosphorylation with the exception of MAP kinase, which was not detectibly tyrosine phosphorylated at this temperature. We do not believe that a simple slowing down of phosphorylation is responsible because we have not observed tyrosine phosphorylation of MAP ki-

nase at 4°C even in cells treated with EGF for up to 8 h. Furthermore, the temperature-dependent phosphorylation of map kinase probably does not involve the phase transition of the lipid bilayer because such processes are not known to occur in complex mammalian cells at these temperatures (Hillman and Schlessinger, 1982).

Although MAP kinase has been shown to effectively use MAP2, S6 kinase, and myelin basic proteins as substrates in in vitro assays, the physiological substrates for this enzyme in vivo may include other proteins. It was reported that a MAP kinase from *Xenopus* altered the dynamics of microtubules in a cell-free system (Gotoh *et al.*, 1991), supporting a possible role for MAP kinase in cytoskeletal functions. MAP kinase can also phosphorylate acetyl-CoA carboxylase in sea star oocytes that correlates with activation of the enzyme and stimulation of fatty acid synthesis (Pelech *et al.*, 1991). Recent studies performed with a purified MAP kinase showed that this enzyme phosphorylates a synthetic peptide corresponding to one of the major phosphorylation sites (Thr-669) of the EGFr (Takishima *et al.*, 1991). Interestingly, other studies have shown that the phosphorylation of Thr-669 of the EGFr is necessary for EGF receptor internalization (Countaway *et al.*, 1989; Heiserman *et al.*, 1990). It is possible that MAP kinase is part of a feedback control system with the tyrosine phosphorylation and activation of MAP kinase leading to the threonine phosphorylation and internalization of the EGFr. The temperature sensitivity of both events leads us to believe that such a causal relationship may exist. This is also consistent with the previous observation showing that microinjection of anti-phosphotyrosine antibody blocked internalization (Glenney *et al.*, 1988a; Campos-González and Glenney, 1991), suggesting that the tyrosine phosphorylation of a crucial substrate is required for internalization. MAP kinase is an excellent candidate for such a substrate.

Materials and methods

Cells and antibodies

The normal human fibroblasts AG1523 were obtained from American Type Culture Collection (Rockville, MD) and used between passages 9 and 25. They were grown in Dulbecco's modified Eagle's medium (DMEM) + 10% calf serum (GIBCO, Grand Island, NY), 50 μg streptomycin, and penicillin/ml at 37°C in an humidified atmosphere of 5% CO_2. Monoclonal anti-phosphotyrosine antibody py20 was used as described (Glenney *et al.*, 1988b; Campos-González and Glenney, 1991). Anti-EGFr mAb c11 and affinity-purified anti-EGFr Rec 1 were as in Campos-González and Glenney (1991). Polyclonal anti-PTyr antibodies were obtained from rabbits immunized with phosphotyrosyl proteins from Rous sarcoma virus-transformed chicken embryo fibroblasts (Glenney and Zokas, 1989) and affinity purified on a column of phosphotyrosine conjugated to Affigel-10 (BioRad, Richmond, CA). Monoclonal antibody (MK12) to MAP kinase was obtained from Zymed Laboratories (San Francisco, CA). This is an anti-peptide antibody raised to a sequence (TDEPVAEEPFTFDMELDDLPK) corresponding to amino acids 325–345 near the C-terminus of ERK-1 (Boulton *et al.*, 1990).

Immunoprecipitation and western blot

Confluent dishes of human fibroblasts were preincubated at the indicated temperature in serum-free DMEM + 25 mM *N*-2-hydroxy ethylpiperazine-*N'*-2-ethane sulfonic acid (pH 7.4) to which mitogenic doses of EGF (25 ng/ml) or PMA (100 ng/ml) (Sigma, St. Louis, MO) were added. Cells were lysed on the culture dish with boiling 1% SDS + 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, boiled further for 5 min, briefly sonicated, and centrifuged for 5 min.

Immunoprecipitations were carried out for 1 h at 4°C in 1.0-ml volume containing buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 0.5% NP-40, 1.0 mM EDTA, 1.0 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.2 mM sodium vanadate, and 0.2 mM phenylmethylsulfonyl fluoride [PMSF]) and diluting the cell lysates to a final concentration of 0.1% SDS. Purified antibodies were used at 2–3 μg/ml. If the immunoprecipitation was done with mAb c11 or anti-MAP kinase mAb (MK 12), the reactions were supplemented for 30 min with 3.0 μg of affinity purified rabbit and anti-mouse IgG (Zymed). Antigen-antibody complexes were precipitated with 50 μl of 10% Pansorbin (Calbiochem, San Diego, CA) for 30 min and collected by centrifugation. Pellets were washed two times with the same buffer and disrupted with 30 μl of twice concentrated electrophoresis sample buffer and boiled for 5 min. Electrophoresis and western blot were done essentially as described previously (Campos-González and Glenney, 1991). Quantitation of the blots was performed by cutting the desired bands and counting in a γ counter.

Peptide maps and phosphoamino acid determination

Immunoprecipitations were performed with cell lysates from ^{32}P-labeled cells as previously described (Campos-González and Glenney, 1991) using mAb MK12 conjugated to Affigel 10 (BioRad). ^{32}P-labeled MAP kinase was extensively digested with trypsin in the gel slice and peptides resolved by thin-layer electrophoresis at pH 1.9 and ascending chromatography on cellulose plates. After autoradiography, phosphopeptides were excised from the plates hydrolyzed with boiling 6M HCl for 1 h, dried, dissolved in a mixture of 1.0 mM phosphothreonine, phosphoserine, and phosphotyrosine, and resolved by thin-layer electrophoresis in two dimensions (Cooper *et al.*, 1982b). Individual phosphoaminoacids were visualized with ninhydrin and the ^{32}P-labeled amino acids detected by autoradiography in Kodak AR film (Rochester, NY) at −70°C.

Miscellaneous

Protein determination were done with the BCA method (Pierce, Rockford, IL). Iodination of antibodies were performed with iodobeads (Pierce) and Na[^{125}I] (ICN, Irvine, CA).

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