p42/mitogen-activated protein kinase as a converging target for different growth factor signaling pathways: use of pertussis toxin as a discrimination factor

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Mitogen-activated protein (MAP) kinase is a 42-kDa serine/threonine-specific protein kinase that requires phosphorylation on both tyrosine and threonine residues for activity. This enzyme is rapidly and transiently activated in quiescent cells after addition of various agonists, including insulin, epidermal growth factor, platelet-derived growth factor, and phorbol esters. We show here that addition of the growth factors thrombin or basic fibroblast growth factor to CCL39 fibroblasts rapidly induces tyrosine phosphorylation of the p42 MAP kinase protein and concomitantly stimulates MAP kinase enzymatic activity. To elucidate the signaling pathways utilized in this activation, we took advantage of the sensitivity of CCL39 cells to the toxin of *Bordetella pertussis*, which ADP-ribosylates two G_i proteins in this cell system. We show that pretreatment of cells with the toxin inhibited thrombin stimulation of MAP kinase by >75% but had no detectable effect on the stimulation induced by basic fibroblast growth factor. We also demonstrate that these two growth factors that synergize for mitogenicity are able to cooperate in activation of MAP kinase and that this synergism is partially sensitive to pertussis toxin. Finally, we describe a 44-kDa protein, the tyrosine phosphorylation of which appears to be coregulated with p42 MAP kinase. We conclude that p42 MAP kinase (and the pp44 protein) are at or are downstream from a point of convergence of two different receptor-induced signaling pathways and might well play a key role in integrating those signals.

Introduction

Tyrosine phosphorylation of proteins in the molecular mass range of 40–45 kDa is character-

istic of quiescent mammalian cells stimulated by various mitogens to re-enter the cell cycle (Cooper *et al.*, 1982; Nakamura *et al.*, 1983; Cooper *et al.*, 1984; Hunter and Cooper, 1984; Kohno, 1985; Kohno and Pouyssegur, 1986), and it is widely suspected that these proteins play an important role in phosphorylation cascades that control the G₀ → G₁ transition. Identifying these proteins, determining their cellular functions, and elucidating the biochemical pathways controlling their phosphorylation are likely to be necessary for understanding how extracellular agonists stimulate cell growth.

The first of these proteins to be identified was pp42, which was first discovered as a tyrosine-phosphorylated 42-kDa protein on 2-D gels of cells treated with diverse mitogens (Cooper *et al.*, 1982). This protein has been shown to correspond to mitogen-activated protein (MAP) kinase (Rossomando *et al.*, 1989), a serine/threonine-specific protein kinase first reported by Ray and Sturgill (1987) in insulin-stimulated 3T3-L1 adipocytes and referred to in this communication as p42^{mapk}. A cDNA encoding p42^{mapk} has been cloned and sequenced (Her *et al.*, 1991) and was found to be 85% identical to another MAP kinase, extracellular signal-regulated kinase 1 (ERK1) (Boulton *et al.*, 1990). Thus there appears to be a family of related serine/threonine protein kinases whose activity is regulated by extracellular agonists (Boulton *et al.*, 1990; Boulton and Cobb, 1991; Rossomando *et al.*, 1991).

p42^{mapk} becomes phosphorylated both on tyrosine and threonine and concomitantly activated as an enzyme, after quiescent cells are stimulated by polypeptide hormones that activate tyrosine kinase receptors (e.g., insulin, epidermal growth factor, platelet-derived growth factor) (Ray and Sturgill, 1988a,b; Hoshi *et al.*, 1989; Rossomando *et al.*, 1989; L'Allemain *et al.*, 1991). Interestingly, phorbol esters, which activate protein kinase C, also stimulate the tyrosine and threonine phosphorylation of p42^{mapk}, even though protein kinase C, the receptor for phorbol esters, has a strict specificity for serine and threonine phosphorylation. This latter result

led us to suggest that p42^{mapk} integrates signals emanating from tyrosine kinase receptors and from protein kinase C (Vila and Weber, 1988; L'Allemain *et al.*, 1991) and to hypothesize the existence of a novel tyrosine kinase that could serve as an intermediate in the protein kinase C signaling pathway (L'Allemain *et al.*, 1991). The integrative role of p42^{mapk} is also supported by the fact that both the tyrosine and the threonine phosphorylations are necessary for this kinase to display enzymatic activity (Anderson *et al.*, 1990). Recently, Ahn *et al.* (1991) have described a "factor" capable of stimulating the phosphorylation of p42^{mapk} on both tyrosine and threonine. It remains to be seen whether this factor is a single protein, what its role is in mitogen-induced signaling, and how its activity is regulated.

Not only is p42^{mapk} regulated by phosphorylation, but at least one of its downstream functions appears to be the regulation of ribosomal S6 kinase by phosphorylation. Two lines of evidence support this view. First, p42^{mapk} in vitro was able to reactivate S6 kinase II that had previously been deactivated by phosphatase treatment (Sturgill *et al.*, 1988; Gregory *et al.*, 1989). Second, two cell lines defective in phorbol ester-induced activation of p42^{mapk} are similarly defective in activation of S6 kinase (L'Allemain *et al.*, 1991; Meier *et al.*, 1991). Thus at least one of the functions of p42^{mapk} appears to be in a kinase cascade that is involved in translational regulation (see Sturgill and Wu, 1991 for a review).

To better understand the regulation of p42^{mapk} activity, we have analyzed the phosphorylation and activation of this enzyme in CCL39 hamster fibroblasts, a cell line whose mitogenic signaling pathways have been extensively studied (see Pouyssegur, 1990 for a review). Previous work (Chambard *et al.*, 1987; Pouyssegur *et al.*, 1991) has shown that pretreatment of these cells with Bordetella pertussis toxin blocks the mitogenic and metabolic effects of thrombin but not those same responses to basic fibroblast growth factor (bFGF). The effects of pertussis toxin in these cells can be accounted for by its ADP-ribosylation of the α_i2 and α_i3 subunits of G_i proteins (Pouyssegur, 1990; P. Giershik, G. L'Allemain, B. Rouot, unpublished observations).

In this communication, we show that the enzymatic activity and tyrosine phosphorylation of p42^{mapk} are stimulated by α -thrombin and basic FGF, two growth factors whose effects on this enzyme had not been previously reported. However, the effects of thrombin, but not the

effects of basic FGF, were found to be largely inhibited by pertussis toxin, indicating the involvement of a G_i protein in the signaling pathway utilized by thrombin. We also present evidence for a pertussis toxin-sensitive synergism between basic FGF and thrombin. Finally, we describe a 44-kDa protein, clearly separable from p42^{mapk} by chromatographic procedures, but whose tyrosine phosphorylation is coregulated with p42^{mapk}. These results extend the correlation between mitogenicity and the phosphorylation and activation of p42^{mapk}, and thus are consistent with an important role for p42^{mapk} in the G₀ → G₁ transition.

Results

Effect of pertussis toxin on thrombin- and bFGF-induced tyrosine phosphorylation of p42^{mapk}

Tyrosine phosphorylation of cellular proteins after treatment of quiescent CCL39 cells with thrombin or bFGF was assessed by separating total cell proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-phosphotyrosine antibodies. Thrombin at 1 unit/ml (10 nM) induced tyrosine phosphorylation of the pp42 protein as rapidly as 1 min after addition (Figure 1, panel A) and the induction peaked at 3 min. With 10 times less thrombin (1 nM), the maximum effect occurred at 10 min. Previous results established that the IC₅₀ value for reinitiation of DNA synthesis induced by thrombin was 3 nM (Chambard *et al.*, 1987), in the same range as that found effective here for pp42 tyrosine phosphorylation.

Figure 1 (panel B, right) shows that the addition of bFGF at 1 ng/ml was ineffective at inducing detectable pp42 tyrosine phosphorylation, as it was for reinitiation of DNA synthesis (Magnaldo *et al.*, 1986). However, 100 ng/ml of bFGF strongly induced both the phosphorylation of the 42-kDa band (Figure 1, panel B, right) and DNA synthesis (Magnaldo *et al.*, 1986). This bFGF effect peaks at 10 min and returns to basal levels by 1–2 h (L'Allemain, Pouyssegur, and Weber, unpublished observations).

To investigate the possible involvement of a G protein in this tyrosine phosphorylation, we took advantage of the sensitivity of the CCL39 fibroblastic cell line to pertussis toxin (Chambard *et al.*, 1987), a toxin known to inhibit by ADP-ribosylation (Ui, 1984) the function of the G_i proteins involved in regulation of adenylate cyclase. Figure 1, (panel B, left) shows the strong inhibitory effect of pertussis toxin on

Figure 1. Immunoblotting of whole cell lysates from CCL39 fibroblasts with anti-phosphotyrosine antibodies. (Panel A) Serum-starved cells were reactivated by 1 or 10 nM thrombin (10 nM = 1 unit/ml) for the indicated times. (Panel B) Parallel dishes were identically arrested, then pretreated (+) or not (–) in vivo with 50 ng/ml pertussis toxin for 4 h before addition of thrombin (units/ml) or bFGF (ng/ml) for 3 and 10 min. Each panel represents a different experiment and 140 µg protein/lane were loaded in each case. The results are characteristic of two independent experiments.

Figure 2. Tyrosine phosphorylation and enzymatic activation of p42^{mapk} in quiescent and thrombin-stimulated CCL39 cells. G₀-arrested cells were stimulated (◆) or not (◇) by thrombin (10 nM, 5 min) and processed for p42^{mapk} purification (see Materials and methods). The p42^{mapk} kinase activity determined on FPLC fractions is displayed in the main panel and the anti-phosphotyrosine immunoblots of the corresponding fractions are shown in the inset. The linearity of the assay was also checked with crude and with purified fractions. The results indicated (data not shown) that whatever the fraction tested was the incorporation of phosphate onto the MBP substrate was linear at least for 16 min rendering reliable the 10-min assay used in this study. The inset also shows the CL3 fraction (see Materials and methods) from activated cells in which the two pp42 and pp44 proteins are both detectable.

pp42 phosphorylation induced by thrombin. By densitometric scanning of the autoradiogram (not shown), we found that the inhibition was complete at 3 min of activation and close to 75% when the tyrosine phosphorylation was maximal. On the other hand, the same toxin treatment was without effect on the tyrosine phosphorylation of pp42 induced by bFGF when added at 100 ng/ml (Figure 1, panel B, right) or at the suboptimal concentration of 5 ng/ml (data not shown).

Stimulation of p42^{mapk} by thrombin

The p42^{mapk} protein was partially purified according to the method previously described (Ray and Sturgill, 1988a,b) by chromatography on DEAE cellulose and phenyl-Superose. P42^{mapk} can be detected by immunoblotting with anti-phosphotyrosine antibodies or enzymatically, with myelin basic protein (MBP) as a substrate (Erickson *et al.*, 1990). The p42^{mapk} protein from CCL39 hamster fibroblasts exhibited the same characteristic chromatographic behavior as did

the enzyme previously isolated from other species and cell types (Ray and Sturgill, 1988a; Rossomando *et al.*, 1989; L'Allemain *et al.*, 1991), eluting from phenyl-Superose at 37% ethylene glycol. Insets in Figure 2 show that the tyrosine-phosphorylated form of the protein is only detected when cells are reactivated by an agonist but not in quiescent cells. Similarly, the kinase activity was found only after stimulation, and not in the G₀-arrested cells (Figure 2, main panel).

To explain the absence of both the p42 phosphoprotein and kinase activity in arrested cells, we considered the possibility that this protein could exist in quiescent cells in a phosphorylated and activated state, but that it eluted at a different position in the chromatography gradient (due, for example, to some difference in post-translational modification). However, we have analyzed the fractions eluted before and after the activated MAP kinase, and did not find any 42-kDa band detectable with our phosphotyrosine antibodies or any phosphorylating ac-

tivity toward the MBP protein (data not shown). We also considered the possibility that this protein that is known to be poorly abundant (Cooper and Hunter, 1985) might be undetectable in resting cells because of the sensitivity of the method used. But overexposure of an immunoblot containing 2.5 times more of transferred proteins also failed to reveal any tyrosine-phosphorylated p42 (unpublished observations). Anti-peptide antibodies that recognize p42^{mapk} confirm that the pp42 protein exists in quiescent cells as a 42 kDa apoprotein, and that it becomes detectable as an active enzyme or with anti-phosphotyrosine antibodies only after agonist stimulation (unpublished observations).

Pertussis toxin strongly inhibits thrombin-induced p42^{mapk} activity

Figure 3 shows that pretreatment of cells with pertussis toxin strongly inhibited the thrombin-induced increase in p42^{mapk} activity, reducing it by 75–85%. Similarly, densitometric scanning of the anti-phosphotyrosine immunoblots of the partially purified p42^{mapk} displayed in Figure 3 indicated that the phosphotyrosine signal from the purified pp42 band was inhibited by ≥80% in the toxin-treated cells. This toxin concentra-

tion is maximally inhibitory because no higher blockade was detected even after a 6-h treatment in the presence of 100 ng/ml of the toxin (data not shown).

Figure 3 also shows the thrombin-induced tyrosine phosphorylation of a 44-kDa protein and the inhibition of this phosphorylation in the presence of pertussis toxin. Recent work from this laboratory (Rossomando *et al.*, 1991) has demonstrated that this 44-kDa protein is also a serine/threonine protein kinase and is one of the 43–45-kDa proteins whose tyrosine phosphorylation has been shown to be mitogen-induced in parallel with pp42 (Cooper *et al.*, 1982; Cooper *et al.*, 1984; Hunter and Cooper, 1984; Kohno, 1985; Kohno and Pouyssegur, 1986). Because the 44-kDa protein is only slightly retained on phenyl-Superose, and thus is not separated from other contaminating proteins, this material was not suitable for assay of enzymatic activity without further purification.

FGF-activated p42^{mapk} activity is not sensitive to pertussis toxin inhibition

MAP kinase was also isolated from bFGF-activated CCL39 cells that were pretreated or not with 100 ng/ml pertussis toxin for 6 h. The re-

Figure 3. Tyrosine phosphorylation of pp42 and pp44 proteins and enzymatic activation of p42^{mapk} in pertussis toxin-treated or untreated CCL39 cells stimulated by thrombin. Main panel, Quiescent cells were treated (◇) or not (◆) with 50 ng/ml of pertussis toxin (PT) for 6 h before thrombin stimulation (10 nM, 10 min), processed as described in the legend to Figure 2, and then assayed for MBP phosphorylating activity. Anti-phosphotyrosine immunoblots from the same FPLC purified material are displayed in insets. Inset A, –PT; inset B, +PT (top, same exposure as inset A; bottom, fivefold overexposure). Arrows indicate positions of p42 and p44 proteins. Note the exact correlation between the phosphotyrosine signal and the level of enzymatic activity.

sults, shown in Figure 4, indicate that the FGF-inducible levels of tyrosine phosphorylation onto p42 and p44 proteins were the same in the presence or in the absence of the toxin (Figure 4, panel B). The corresponding fractions containing the purified p42 protein were assayed for MBP kinase activity and were found not to be inhibited by the toxin as well (Figure 4, panel A).

Synergistic effect of bFGF + thrombin on p42^{mapk} activation is partially sensitive to pertussis toxin

Previous results established the existence of a synergism between thrombin and bFGF with respect both to activation of phospholipase C and induction of DNA synthesis in CCL39 cells (Paris *et al.*, 1988). Here, we looked for such an interconnection taking place with respect to p42^{mapk} enzymatic activity. Figure 5 indicates a submaximal, but clearly detectable, induction of p42^{mapk} activity when quiescent CCL39 cells were stimulated for 10 min with 0.1 nM thrombin. A concentration of 5 ng/ml of bFGF induced even greater activity. The simultaneous addition of these submaximal concentrations of thrombin and bFGF produced a synergistic effect on MAP kinase activity that was twice as much as the additive effect of the two growth factors added alone and that was equivalent to the activity induced by 20 ng/ml of bFGF, a saturating concentration of this mitogen for the p42^{mapk}

activation (data not shown). The fact that this synergy can also be seen in whole cell lysates (unpublished observations) excludes the possibility of an artifact of purification.

Interestingly, pretreatment of cells with 100 ng/ml pertussis toxin for 5 h resulted in a partial inhibition (50%) of the p42^{mapk} activity induced by the combination of 0.1 nM thrombin + 5 ng/ml bFGF (Figure 5). Moreover, such a toxin treatment, which is maximally inhibitory for ADP-ribosylation (Chambard *et al.*, 1987) and for thrombin-induced pp42/MAP kinase (see Figure 3), is responsible for blocking at 65–70% the enzymatic activity with respect to the synergistic effect alone (Figure 5). This suggests that the portion of the thrombin-stimulated activity that is resistant to pertussis toxin is still capable of partially synergizing with the FGF-stimulated pathway.

Discussion

Activation of p42^{mapk} by thrombin or bFGF

In this communication we demonstrate that thrombin and bFGF each are able to induce the tyrosine phosphorylation and enzymatic activation of p42^{mapk}. We also identified a 44-kDa protein whose tyrosine phosphorylation is co-regulated with p42^{mapk} and that appears to be a related protein kinase (Rossomando *et al.*, 1991). This extends the list of mitogenic agonists shown to activate p42^{mapk}. For both thrombin and FGF, this is the first report in

Figure 4. Tyrosine phosphorylation of pp42 and pp44 proteins and enzymatic activation of p42^{mapk} in pertussis-treated or untreated CCL39 cells stimulated by bFGF. Quiescent cells were treated or not with 100 ng/ml pertussis toxin for 6 h before FGF addition (100 ng/ml, 10 min) and then processed as described in the legend of Figure 2. (Panel A) MBP kinase activity in purified fractions from toxin-treated (◆) or untreated (◇) cells. (Panel B) Overnight exposures of anti-phosphotyrosine immunoblots from the corresponding purified fractions. Top panel, -pertussis toxin; lower panel, +pertussis toxin. Note the exact correlation between the signals displayed in both panels.

Figure 5. Synergy between bFGF and thrombin on p42^{mapk} activation. (Panel A) Quiescent CCL39 cells were pretreated (closed symbol) or not (opened symbols) with pertussis toxin for 5 h at 100 ng/ml before a 10-min addition of growth factor: 5 ng/ml bFGF (◇), 0.1 nM thrombin (△), or 5 ng/ml bFGF + 0.1 nM thrombin (O, ●). Pp42-containing fractions were purified from FPLC phenyl-Superose and analyzed for their MBP kinase activity, as described in Materials and methods. (Panel B) Enzymatic activities present in all the fractions of inset A were pooled for each condition of stimulation and the total was expressed as percent (with 100% to be the simple additive activity calculated from the effects of 0.1 nM thrombin + 5 ng/ml bFGF when added alone).

which the identity of a tyrosine-phosphorylated cellular substrate protein has been established. In addition, the dose-dependency of this activation was found to be comparable with the dose-dependency of mitogenesis induced by these agonists (Chambard *et al.*, 1987), strengthening the correlation between mitogenesis and p42^{mapk} activation.

Because the FGF receptor is a tyrosine protein kinase, it is perhaps not surprising to observe the appearance of tyrosine-phosphorylated substrate proteins in cells treated with FGF. But the thrombin receptor is not a tyrosine protein kinase (Rasmussen *et al.*, 1991; Vu *et al.*, 1991) and thus induction of tyrosine phosphorylations by this agonist must occur indirectly. In fact, it is probable that at least a portion of the thrombin-induced activation of p42^{mapk} relies on the ability of thrombin to stimulate phosphatidyl inositol turnover and consequently to activate protein kinase C because phorbol esters, which activate protein kinase C, are also able to induce the phosphorylation and activation of this enzyme. However, pertussis toxin inhibits only 40–50% of phosphatidyl inositol turnover (Paris *et al.*, 1988) but as much as 85% of p42^{mapk} activation and of myc induction (Letterio *et al.*, 1986; Paris *et al.*, 1987). The basis for this quantitative discrepancy remains to be determined; however, it is possible that a pertussis toxin-sensitive G protein is required

for these signaling events, aside from any role in controlling phosphatidyl inositol turnover. In addition, the level of activation of p42^{mapk} correlated closely with the degree of induction of mitogenesis in all these circumstances.

The role p42^{mapk} plays in mitogenesis is not yet established. Both in vitro (Sturgill *et al.*, 1988; Gregory *et al.*, 1989) and in vivo (L'Allemain *et al.*, 1991; Meier *et al.*, 1991) evidence point to a role in translational control. In any event, this enzyme very likely functions in some step common to a variety of signaling pathways, because activation of p42^{mapk} or other members of this family has been observed, not only as a prelude to the G₀ → G₁ transition, but also in cells induced either to differentiate (e.g., nerve growth factor-treated PC12 cells [Gotoh *et al.*, 1990; Miyasaka *et al.*, 1990] and phorbol ester-treated thymocytes [Nel *et al.*, 1990]) or to secrete (e.g., secretagogue-treated adrenal chromaffin cells [Ely *et al.*, 1990]).

Inhibition of thrombin-induced p42^{mapk} activation by pertussis toxin

Pertussis toxin was able to inhibit by 75–85% the thrombin-induced activation of p42^{mapk} and the tyrosine phosphorylation of both p42^{mapk} and the p44 protein but not that induced by FGF, indicating that the two agonists utilize different signaling pathways to induce these phosphor-

ylations and to activate this enzyme. Presumably, $p42^{mapk}$ activation occurs at or downstream from a point of convergence of the two signaling pathways. The effects of pertussis toxin on thrombin-induced signaling can be accounted for by inhibition of two G_i proteins (Chambard *et al.*, 1987). This result is consistent with the recent determination that the thrombin receptor belongs to the superfamily of seven spanning domain receptors (Rasmussen *et al.*, 1991; Vu *et al.*, 1991), a family type found coupled to GTP-binding proteins.

The results presented here constitute the first evidence that a GTP-binding protein plays a role in the activation pathway of MAP kinase. Parallel work (Anderson *et al.*, 1991; G. L'Allemain and M. Nori, unpublished observations) demonstrates that the addition of the fluoroaluminate complex to quiescent cells is also able to induce both tyrosine phosphorylation and enzymatic activation of $p42^{mapk}$. Because fluoroaluminate can function as a nonspecific activator of GTP-binding proteins, this provides further evidence that one or several GTP-binding proteins can have a regulatory function in the control of $p42^{mapk}$.

Synergism between thrombin and bFGF

One of the most interesting aspects of the work reported here is the demonstration that sub-optimal concentrations of thrombin and bFGF known to be synergistic for triggering DNA synthesis also are synergistic for activation of $p42^{mapk}$. This synergy implies that the two agonists act on different steps in the signaling pathway or in different ways on the same step. For example, the thrombin receptor might regulate a G-protein coupled to an activator of $p42^{mapk}$, whereas the FGF receptor might directly phosphorylate the $p42^{mapk}$ activator protein. Alternatively, the FGF receptor could stimulate phosphorylation of $p42^{mapk}$ and the thrombin receptor could inhibit a $p42^{mapk}$ tyrosine phosphatase. Either of these sorts of scenario would permit a synergistic interaction. However, if both thrombin and FGF activated $p42^{mapk}$ by the same mechanism, then the effects of these two agonists would be only additive.

In conclusion, at least two separate converging pathways can activate $p42^{mapk}$ and perhaps other members of the MAP kinase family. One pathway is initiated by a tyrosine kinase receptor and is insensitive to pertussis toxin. The other pathway is initiated by a seven transmembrane domain receptor, is linked to a per-

tussis toxin-sensitive G_i protein, and likely requires protein kinase C. Thus $p42^{mapk}$ may serve as a converging kinase, integrating regulatory signals coming from various transmembrane receptors and funneling these signals into some of the common targets activated during G_0 . Elucidating the biochemical steps in these signaling pathways will be important in understanding how the cell processes diverse extracellular signals.

Materials and methods

Materials

Human plasma α -thrombin (3000 units/mg) and tetradecanoyl phorbol acetate were purchased from Sigma Chemical Co. (St. Louis, MO). The basic form of bFGF was from Collaborative Research Inc. (Bedford, MA). DE52 (DEAE cellulose) was from Whatman Biosystems Ltd., (England) and the phenyl-Superose column from Pharmacia-LKB (Piscataway, NJ). Tween-20 was from Bio-Rad (Richmond, CA). Bovine serum albumin (BSA) fraction V was from Boehringer Mannheim (Indianapolis, IN) and fetal calf serum (FCS) was from GIBCO (Grand Island, NY). The Pertussis toxin (holotoxin form), purified from *Bordetella pertussis*, was a gift from Dr. Eric Hewlett (University of Virginia). ^{125}I -labeled protein A was purchased from Amersham (Arlington, IL) and ^{32}P -ATP (6000 Ci/mmol) from NEN-DuPont Research Products (Wilmington, DE). Specific anti-phosphotyrosine antisera were generated in this laboratory as described (Rossomando *et al.*, 1989).

Cell culture

The chinese hamster lung fibroblast cell line CCL39 (ATCC) was cultured at 37°C in a humidified atmosphere of 7.5% CO_2 with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS until confluence. Then, cells were rendered quiescent by a 20-h treatment in FCS-free DMEM and, whenever it was required, the Pertussis toxin was added directly in the same medium 4–6 h before growth factor activation.

Cell lysis

Growth factor stimulation was rapidly stopped by rinsing the plates twice with ice-cold phosphate-buffered saline and by adding either hot 2× SDS sample buffer for immediate electrophoresis or by initiating the purification procedure as initially described by Ray and Sturgill (1988a,b).

Purification of $p42^{mapk}$

The $p42^{mapk}$ protein was partially purified by sequential DEAE-cellulose and phenyl-Superose chromatography as described (Ray and Sturgill, 1988a,b). Briefly, nuclei-free cell lysates were step-eluted from DE52 at 350 mM NaCl (Cl3 fraction) and loaded onto a phenyl-Superose 5:5 column (4 mg protein per run). From a linear gradient of 0 → 60% ethylene glycol (EG), 250 → 0 mM NaCl, the peak of $p42^{mapk}$ is typically recovered at 37% EG, 100 mM NaCl.

Precipitation of purified material

Whole fractions (0.5–1 ml except 10 μ l saved for assay of the enzymatic activity) eluted through the phenyl-Superose step were precipitated as described (Bensadoun and Wein-

stein, 1976) with deoxycholate/trichloroacetic acid, and then reconstituted with 1× hot SDS sample buffer before SDS-PAGE electrophoresis and phosphotyrosine antibody immunoblotting.

Immunoblotting

Whole cell lysates (100–120 µg protein per lane) or purified material (4 mg protein per fast protein liquid chromatography FPLC run) were electrophoresed on SDS-10% polyacrylamide gels (SDS-PAGE) at 40 mA for 3 h and electroblotted overnight onto nitrocellulose (S&S, BA83) in 25 mM Tris(hydroxymethyl)aminomethane (Tris), 192 mM glycine, 20% methanol. Ponceau S-stained filters were then processed at 37°C. First, they were preblocked in 0.15 M NaCl, 50 mM Tris, pH 7.4, 0.1% Tween-20 (TBS) solution + 3% BSA for 1 h before addition of anti-phosphotyrosine antibodies at 5 µg/ml for 2 h. Filters were then washed twice in TBS solution + 0.5% BSA, probed with ¹²⁵I-protein A (0.5 µCi/ml) in TBS + 3% BSA for 1 h, and washed again three to six times in TBS + 0.5% BSA until no more radioactivity was found in the washes. The blots were then usually exposed overnight, unless otherwise specified, on Kodak X-omat RP film (Kodak, Rochester, NY).

Enzymatic activity measurements

The purified FPLC fractions were assayed for their kinase activity by the *in vitro* phosphorylation of MBP as already described (Erickson *et al.*, 1990). Briefly, the incorporation of ³²P onto MBP (1 mg/ml) was catalyzed for 10 min at 30°C by 5 µl of the FPLC fractions in the presence of ³²P-γ-ATP (50 µM, 5000 cpm/pmol), 10 mM Mg²⁺, 1 mM dithiothreitol in 40 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5. The reaction was stopped by spotting the incubations onto P81 phospho-cellulose paper (Whatman) and immediately rinsing them with 0.2 M phosphoric acid (4 washes, 5 min each) before a rapid wash with 100% ethanol, air-drying, and liquid scintillation counting.

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