Biochemical Characterization of a Family of Serine/Threonine Protein Kinases Regulated by Tyrosine and Serine/Threonine Phosphorylations*

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Mitogen-activated protein kinase (p42mapk) becomes transiently activated after treatment of serum-starved murine Swiss 3T3 cells or EL4 thymocytes with a diversity of mitogens. Similarly, a meiosis-activated protein kinase (p44mapk) becomes stimulated during maturation of sea star oocytes induced by 1-methyladenine. Both p42mapk and p44mapk have been identified as protein-serine/threonine kinases that are activated as a consequence of their phosphorylation. Because homologous protein kinases may play essential roles in both mitogenesis and oogenesis, we have compared in detail the biochemical properties of these two kinases.

We find that these kinases are highly related based on their in vitro substrate specificities, sensitivity to inhibitors, and immunological cross-reactivity. However, they differ in apparent molecular weight and can be separated chromatographically, indicating that the two enzymes are distinct. Furthermore, in the course of this investigation, we have identified a 44-kDa protein kinase in mitogen-stimulated Swiss mouse 3T3 cells and EL4 thymocytes that co-purifies with ~44-42~11, meiosis-activated protein kinase; MAP-2, microtubule-associated protein; MAP-kinase, termed herein mitogen-activated protein kinase (MAP kinase); ~44~11, murine 44 kDa MAP-kinase; p42~11, a serine/threonine protein kinase, with a preference for phosphorylating MAP-2 substrate specificities, sensitivity to inhibitors, and immunological cross-reactivity. However, they differ in apparent molecular weight and can be separated chromatographically, indicating that the two enzymes are distinct. Furthermore, in the course of this investigation, we have identified a 44-kDa protein kinase in mitogen-stimulated Swiss mouse 3T3 cells and EL4 thymocytes that co-purifies with ~44-42~11, meiosis-activated protein kinase; MAP-2, microtubule-associated protein; MAP-kinase, termed herein mitogen-activated protein kinase (MAP kinase); ~44~11, murine 44 kDa MAP-kinase; p42~11, a serine/threonine protein kinase, with a preference for phosphorylating MAP-2.

Re-entry of quiescent cells into the cell cycle from G0 can be stimulated by binding of peptide growth factors to their cognate receptors. Many of these receptors are ligand-stimulated protein-tyrosine kinases whose enzymatic activity is essential for mitogenesis (1, 2). Signals from these receptors are most likely propagated in part by protein phosphorylation cascades. Presumably, the receptor kinase phosphorylates cellular substrates on tyrosine, initiating a cascade of events resulting in cell division. Thus, identifying the in vivo targets for these mitogen-stimulated tyrosine kinases is a recognized goal for understanding the G0/G1 transition.

Two potentially important tyrosine kinase substrates of approximately 42 and 44 kDa were initially identified in murine fibroblasts stimulated with many diverse mitogenic agents (3–7). These proteins become phosphorylated nearly stoichiometrically on tyrosine and also on threonine within 10 min after mitogen stimulation. Furthermore, phorbol esters, which are thought to act exclusively through protein kinase C, a serine/threonine protein kinase, also elicit the phosphorylation of p42 and p44 on tyrosine as well as threonine (8–10). Based on two-dimensional gel electrophoresis, phosphorylated p42 (pp42) was found to exist in two phosphoforms, pp42A and pp42B (11). Rossomando et al. (12) have shown that pp42A corresponds to the activated form of mitogen-activated protein kinase (MAP kinase, termed herein p42mapk); a serine/threonine protein kinase, with a preference for phosphorylating MAP-2 or MBP (13, 14). Activated p42mapk is phosphorylated on both tyrosine and threonine (13), and both phosphorylations are necessary for kinase activity (15).

Tyrosine-phosphorylated proteins of 40–45 kDa have also been identified in Xenopus laevis eggs. Using two-dimensional electrophoresis, Lohka et al. (16) found that a pair of 42-kDa proteins contained phosphotyrosine and phosphothreonine only at M-phase in maturing frog oocytes, and these were rapidly dephosphorylated following fertilization or parthenogenetic activation of Xenopus eggs. Cooper (17) provided further evidence that these phosphoproteins were related to mammalian p42 and p44 by partial phosphopeptide mapping. The activation of MBP kinases in maturing Xenopus oocytes has also been observed by column chromatography (18). Recently, Gotoh et al. (19) purified a tyrosine- and serine-phosphorylated Xp42 to homogeneity from unfertilized Xenopus eggs. The abbreviations used are: p42mapk, 42-kDa mitogen-activated protein kinase (MAP kinase); p44mapk, murine 44 kDa MAP-kinase; p44~11, meiosis-activated protein kinase; MAP-2, microtubule-associated protein-2; MBP, myelin basic protein; ERK 1, extracellular signal-regulated kinase 1.
opus eggs and demonstrated that it possessed many properties in common with an epidermal growth factor-activated MAP-2 kinase from rat 3Y1 cells.

Pelech et al. (20) have previously described the marked stimulation of an MBP kinase near the time of germinal vesicle breakdown in maturing sea star oocytes. Purified sea star MBP kinase (designated pp44<sup>pk</sup> for meiosis-activated protein kinase) migrates as a 44-kDa protein on sodium dodecyl sulfate-polyacrylamide gels (21). The following evidence indicated that murine pp42<sup>pk</sup> and sea star pp44<sup>pk</sup> might be related enzymes: in addition to their similar sizes, both enzymes are cytosolic, tyrosine-phosphorylated, require phosphorylation for activity, and exhibit a preference for MAP-2 or MBP as substrates (13, 15, 21–23).

To determine whether murine pp42<sup>pk</sup> and sea star pp44<sup>pk</sup> are species homologs or related isoforms, we have compared their biochemical properties in detail. Based on these studies, we conclude that the sea star kinase is closely related but not identical to pp42<sup>pk</sup>. Moreover, we have identified a 44-kDa MAP-2/MBP kinase in mammalian cells which appears to be more closely related to the sea star pp44<sup>pk</sup>. This murine pp44<sup>pk</sup> appears to correspond to pp45<sup>k</sup>, a phosphatidylinositol-containing protein first identified on two-dimensional gels by Cooper et al. (4). We also provide evidence that pp42<sup>pk</sup> and pp44<sup>pk</sup> correspond to the epidermal growth factor-activated protein kinases described by Ahn et al. (24). MBP kinase I (peak E3) and MBP kinase II (peak E4), respectively. The data provide biochemical evidence of a gene family for the 42–44-kDa MAP-2/MBP kinases regulated by tyrosine phosphorylation. This conclusion is in agreement with the recent identification by Boulton et al. (25) of at least four potential genes related to ERK 1, a MAP-2 kinase.

**EXPERIMENTAL PROCEDURES AND RESULTS<sup>2</sup>**

**DISCUSSION**

**Identification of the MAP Kinase Family—Evidence for a family of 42-45-kDa tyrosine-phosphorylated proteins was provided by investigators studying proteins that become phosphorylated in response to mitogenic stimulation. The expectation has been that a subset of these proteins would play crucial roles in the transition from Go into the cell cycle (3–7). Two proteins, variously estimated to be 41–42 kDa (pp42) and 44–45 kDa (pp44), were found to be transiently phosphorylated on tyrosine in response to many mitogenic agents, including peptide growth factors and phorbol esters (3–11).**

We previously identified pp42 as mitogen-activated protein kinase (p<sub>AKT</sub>) (12). Since pp42 and pp44 had similar one-dimensional phosphopeptide maps (4, 5, 7), it was reasonable to expect pp44 to be a similarly activated protein kinase. Evidence presented here is consistent with this expectation. We have identified a 44-kDa protein which becomes phosphorylated on tyrosine in response to mitogenic stimulation in both murine fibroblasts and thymocytes. Using two-dimensional gel electrophoresis, this 44-kDa phosphoprotein migrates to a position similar to that observed by Cooper and Kohno for pp44 (4, 5, 7). In addition, the 44-kDa phosphoprotein was found to be a protein kinase, a conclusion based in part on its co-purification with MBP kinase activity over five chromatographic steps. Both kinases phosphorylated MBP at a single site (Thr-97), implying that they possess very similar substrate specificities. Finally, both pp42 and pp44 could be recognized by antisera raised against a sea star 44-kDa protein kinase (pp44<sup>pk</sup>). Thus, pp42 (p<sub>AKT</sub>) and pp44 are structurally (4, 5, 7) and immunologically related serine/threonine protein kinases, with as yet indistinguishable substrate specificities. We therefore will refer to pp44 as pp44<sup>pk</sup>.

Activation of p42<sup>pk</sup> requires the phosphorylation of two closely spaced tyrosine and threonine residues (15) that were found to occur on a single tryptic peptide (27). p44<sup>pk</sup> also contained a tryptic peptide with equal amounts of phosphotyrosine and phosphothreonine, and an electrophoretic mobility similar to that of p42<sup>pk</sup>. Similarly, the kinase activity of p44<sup>pk</sup> is also reduced by both the protein-tyrosine phosphatase CD45 and the protein-serine/threonine phosphatase 2A, thereby demonstrating the importance of both types of phosphorylation in its activation (data not shown).

Although p42<sup>pk</sup> and p44<sup>pk</sup> are highly related, they can be distinguished by electrophoretic mobility and chromatographic procedures, and even though p42<sup>pk</sup> and p44<sup>pk</sup> each contained a tryptic phosphopeptide with similar electrophoretic mobilities and equal amounts of phosphotyrosine and phosphothreonine, the two phosphopeptides can be separated by TLC: the p42<sup>pk</sup> phosphopeptide is less hydrophobic than that of p44<sup>pk</sup>. The recent determination that the tyrosine and threonine phosphorylation sites of p42<sup>pk</sup> occur within the middle of the protein (27) strongly argues against p42<sup>pk</sup> being a proteolytic fragment of p44<sup>pk</sup>. If p42<sup>pk</sup> was a fragment of p44<sup>pk</sup>, then a single phosphopeptide common to both proteins should have been identified.

Sea star p44<sup>pk</sup> also resembled the murine p42<sup>pk</sup> with respect to substrate specificity, but differed in size and chromatographic behavior. In marked contrast, the sea star p44<sup>pk</sup> and the murine p44<sup>pk</sup> were indistinguishable by these criteria. Particularly striking was the co-purification of murine p44<sup>pk</sup> with the sea star enzyme over polylysine-agarose, phosphocellulose, Mono-Q (data not shown), and phenyl-Superose, the latter two being capable of separating p42<sup>pk</sup> from p44<sup>pk</sup>. However, in vivo labeling studies with [32P]orthophosphate revealed that, unlike pp42 and pp44, the activated sea star p44<sup>pk</sup> was not phosphorylated on threonine (data not shown). Thus, although it is clear that the sea star p44<sup>pk</sup> is most closely related to p44<sup>pk</sup>, it is uncertain whether these enzymes are truly homologous.

Ahn et al. (24) have identified two MBP kinases (I and II) in epidermal growth factor-treated murine fibroblasts using Mono-Q chromatography and a shallow salt gradient. Likewise, Gomez et al. (30) have used this ion-exchange resin to resolve two nerve growth factor-activated MBP kinases in rat pheochromocytoma (PC12) cells. Based on several similarities, Ahn et al. (24) proposed that MBP kinase I is identical to p42<sup>pk</sup>. We also find that Mono-Q resolves two MBP kinase peaks, but have observed the best and most consistent resolution of the kinases could be obtained with phenyl-Superose fast protein liquid chromatography. By utilizing phenyl-Superose combined with sequential Mono-Q fast protein liquid chromatography, we have confirmed and extended the work of Ahn et al. (24) by identifying MBP kinase I (peak E3) and MBP kinase II (peak E4) as p42<sup>pk</sup> and p44<sup>pk</sup>, respectively.

Boulton et al. have cloned a rat brain cDNA (ERK 1) encoding a MAP-2 kinase which is a member of the kinase family described here (25). We have cloned and sequenced a murine cDNA corresponding to p42<sup>pk</sup>, and found it to encode a protein with a predicted amino acid sequence 85% identical.
to that of ERK 1 (32). This corresponds to the ERK 2 sequence of Boulton et al. (33). Microprotein sequencing of sea star pp43\(^{map}\) revealed approximately 70% amino acid identity with the predicted sequence of ERK 1, but a different sea star cDNA has been obtained which corresponds more closely to the sequence of ERK 1. Thus, ERK 1 and pp44\(^{map}\) appear to be different members of this kinase family. It is not clear at present how many members the MAP kinase family has, but the best current estimate is a lower bound of four, based on the analyses of Boulton et al. (25, 33).

**Regulation of the MAP Kinase Family**—Some evidence exists that p42\(^{map}\) and p44\(^{map}\) are differentially regulated. Acute treatment of fibroblasts with mitogens such as epidermal growth factor of phorbol esters stimulates a greater increase in p42\(^{map}\) kinase activity than of p44\(^{map}\) kinase activity. Similarly, we have found the relative amount of pp42 phosphorylation to be 4-6-fold greater than that of pp44, as determined by immunoblotting with antiphosphotyrosine antibodies, even though blotting with the anti-GLAYIGE-GAYGMV peptide antibody indicated that there were equal amounts of each protein in the sample (data not shown). Further work will be required to determine the circumstances and significance of differential activation of these highly related enzymes.

Although most of our work has concerned cells stimulated to enter the cell cycle from G0, p42\(^{map}\) and p44\(^{map}\) are likely to have distinct functions in terminally differentiated cells. For example, nicotine treatment of adrenal chromaffin cells stimulates secretion rather than mitogenesis. Nevertheless, p42\(^{map}\) is robustly activated by this treatment (31). The demonstration by Gomez et al. (30) that nerve growth factor treatment of PC12 cells, which induces neurite outgrowth, also induces activation of MBP kinases, supports the suggestion that these enzymes serve a wider role in cellular regulation than in controlling the G1-G0 transition.

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Experimental Procedures

Materials. PCD and bovine MBP were purchased from Sigma (St. Louis, MO). EGF was procured from Collaborative Research. The sources of all other reagents have been given elsewhere (12, 13, 15). EL4-l-2 thyomaya cells and Swiss mouse 3T3 cells were grown using culture conditions reported elsewhere (12, 14). Chromatographic media were obtained from Sigma (polyvinyl-agarose), Worthington (DE52 cellulose), and all others from Pharmacia LKB (Mono Q, phenyl-Sepharose, and Superose 12).

Chromatography. Cells were mitogen-treated and disrupted in the same manner as described elsewhere using identical buffers and inhibitors (12). The cytoplasmic fractions were chromatographed on DE-52 cellulose prior to phenyl-Sepharose chromatography as previously reported (12).

Polylysine-agarose chromatography was performed as follows: 1 mg/ml of polylysine-agarose (Biorad) packed into a 1-ml Pharmacia (5/30) PCCL column. The column was equilibrated with 10 ml of buffer A (25 mM Tris-HCl, pH 7.5 [at 5°C], 25 mM NaCl, 2 mM EGTA, 1 mM Dithiothreitol, 40 mM p-nitrophenyl phosphate). Samples, diluted to 25 mM NaCl using buffer B without NaCl, were applied at a flow rate of 1 ml/min using a Pharmacia PCCL system. The matrix was then washed with 6 ml of buffer A followed by protein elution with a 21 ml linear gradient (25-700 mM NaCl) in buffer A. One ml fractions were collected into tubes containing ethylene glycol and Tween-20 (10% and 0.05%, final concentrations, respectively), added to minimize loss of kinase activity, and either assayed immediately or stored at -70°C.

Mono Q chromatography was performed essentially as outlined by Ahn et al. (24). Superose 12 gel filtration chromatography was carried out as described (26), except all fractions were also collected into tubes containing ethylene glycol and Tween-20 (as above).

Sample preparation and SDS gel electrophoresis. Cell lysates (100 µg/cell) were prepared for 1-dimensional SDS-PAGE by the addition of hot SDS sample buffer (3% SDS, 10 mM Dithiothreitol, 1 mM EDTA, 20 mM Tris-HCl, pH 8.3) directly to cells washed with Heps-buffered saline. When it was necessary to use labeled homogenate, 3 x 10⁶ EL4 lymphocytes were labeled with 50 nCi [3H]orthophosphate (NE) using conditions identical to those previously stated (27). All SDS-polyacrylamide gel electrophoresis gels were 10% acrylamide in some cases, proteins were precipitated with TCA/DOC prior to addition of SDS sample buffer (12).

Other procedures. Antibodies used in this study (polyclonal rabbit anti-phospho-protein antisera and anti-p44) and conditions for 2-dimensional electrophoresis, mapping of tryptic phosphopeptides, phosphoamino acid analysis, and immunoblotting procedures have been described in detail in other communications (12,22,27). In some experiments, use was made of an affinity-purified rabbit polyclonal antibody that was used in the synthetic peptide GLAYIGEGAYGMV, which corresponds to the ATP-binding site of sea star p44 (p44*).

Murine mitogen-activated protein kinase and sea star meiosis-activated myelin basic protein kinase display similar substrate specificities and inhibitor sensitivities. To explore the possibility that murine p42 and sea star p44* have species homologies or related isoforms, we initially compared the relative abilities of these kinases to phosphorylate various known proteins in vitro (Table I). Both enzymes phosphorylated the tested proteins with similar or identical efficiencies, with MBP and MAP-2 being the best substrates (12). Furthermore, both p42* and p44* were purified and used in parallel experiments. In the same way in MBP (Thr 97), and phosphorylation of MAP-2 by either enzyme yielded extremely complex, but indistinguishable phosphopeptide maps (data not shown).

Table I. Substrate specificity comparisons of murine p42* and sea star p44*.

| SUBSTRATE | murine p42* | sea star p44* |
|-----------|-------------|--------------|
| MBP       | 100.00      | 100.00       |
| Casein    | 0.80        | 0.70         |
| Histone H1 | 2.60       | 3.00         |
| Histone H2A | 0.90       | 0.70         |
| Proline    | 0.10        | 0.00         |
| α-Tubulin  | 0.30        | 0.30         |
| Acetyl-CoA carboxylyase | 1.90 | 1.10 |
| Neurofilament protein 68 | 0.05 | 0.08 |
| MAP-2*     | 122.00      | 99.00        |
| Proteins   | 0.10        | 0.10         |

Results

Murine mitogen-activated protein kinase and sea star meiosis-activated myelin basic protein kinase display similar substrate specificities and inhibitor sensitivities. To explore the possibility that murine p42 and sea star p44* have species homologies or related isoforms, we initially compared the relative abilities of these kinases to phosphorylate various known proteins in vitro (Table I). Both enzymes phosphorylated the tested proteins with similar or identical efficiencies, with MBP and MAP-2 being the best substrates (12). Furthermore, both p42* and p44* were purified and used in parallel experiments. In the same way in MBP (Thr 97), and phosphorylation of MAP-2 by either enzyme yielded extremely complex, but indistinguishable phosphopeptide maps (data not shown).

The sensitivity of p42* and p44* to numerous inhibitor compounds was also evaluated. Both enzymes exhibited very similar or identical sensitivities to the inhibitors tested (Fig. 4A). Therefore, based on the criteria of substrate specificity and inhibitor sensitivity, the two kinases were virtually indistinguishable.

Table II. Inhibitor sensitivity comparisons of murine p42 and sea star p44*.
The ability of different compounds to inhibit p42* or p44* activity was measured. Each value represents the mean of the inhibitor concentration (IC50) at which 50% of the MBP kinase activity is inhibited (IC50).

| INHIBITOR | IC50 (µM) |
|-----------|-----------|
| 0.4 mM EGTA| 60        |
| NaCl       | 1000      |
| NaF        | 50        |
| MnCl2      | 3         |
| CaCl2      | 10        |
| Heparin    | >100      |
| Spermine   | >10       |
| Dithiothreitol | >50   |

Materials and methods used have been previously reported by Sanghera et al. (1996).

Immunoblotting with anti-phospho-protein and a sea star p44* antibody. Enzymically active p42* and p44* have been shown to be phosphorylated on tyrosine (13, 22) and both enzymes are detectable by immunoblotting with an anti-phospho-protein antibody (Fig. 1, lanes 2 and 3, respectively). The nearly homogeneous sea star kinase migrated on SDS-PAGE gels at a slightly higher Mr than that of phenyl-Sepharose-purified p44* (Fig. 1, arrow). Affinity-purified p44* antibody (anti-p44*), generated against the native low Mr star protein (22), also immunoblotted murine p42* and p44* (Fig. 1, lanes 5 and 6, respectively). Further evidence that these enzymes were serologically related was provided by use of an peptide antibody (anti-GLAYIGEGAYGMV), which recognizes the kinase ATP-binding site. This antibody also cross-reacted with both kinases (data not shown). Thus, p42* and p44* were immunologically related, but differed in size.

Figure 1. Immunoblotting of p42* and p44* with an anti-phosphorysine antibody of an affinity-purified anti-p44* antibody.

Figure 2. Chromatographic separations of a murine 44 kDa protein from a murine 44 kDa protein from the sea star 44 kDa and the murine 44 kDa. Both enzymes were purified from PCD-stimulated EL4 cells. The 44 kDa phosphorylated (pp44), which migrated with p44* was detected against anti-phospho-protein antibodies in the photoblotester-treated cells (upper arrow) but not in the unstimulated control cells (data not shown). Thus, as with p42* and sea star p44*, pp44 became tyrosine phosphorylated in response to agonist stimulation. To compare the properties of the murine pp44 to sea star p44* and pp44*, we examined the behavior of these proteins on phenyl-Sepharose chromatography, a procedure we have found to provide the best resolution of p44* from other contaminating kinases and phosphatases. Homogenate from PCD-treated EL4 cells was subjected to sequential chromatography on DE52-cellulose and phenyl-Sepharose, and the resulting fractions were assayed for phosphorylase activity with MBP as a substrate. pp44 eluted at -37% ethylene glycol and 100 mM NaCl (Fig. 2A, peak 3a), in agreement with previously published results (12). In addition, a broad peak of MBP kinase activity was detected in the isocratic wash fractions (Fig. 2A, peak 2). The two fractions were pooled separately and immunoblotted with the anti-phospho-protein antisera. The peak fraction contained the 44 kDa pp44* phosphoprotein and the wash peak fraction contained the 44 kDa pp44* phosphoprotein (lanes 1 and 3, respectively, inset to Fig. 2A).

P44* from sea star, partially purified on hydroxyapatite, was also chromatographed on phenyl-Sepharose to determine whether this enzyme co-eluted with the p44* or p44*. Each column fraction was assayed for phosphorylase activity (Fig. 2B), and the pooled kinase peak was immunoblotted with the anti-phospho-protein antibody, Fig. 2B, inset). pp44* activity eluted in the same wash fractions as the murine 44 kDa phosphorylated and no kinase activity was detected in the gradient, where p44* could not be eluted. Thus pp44* did not co-migrate with p42* on phenyl-Sepharose, but rather behaved like murine p44.*

To exclude the possibility that p44* did not bind to the phenyl-Sepharose matrix because of interfering factors within the hydroxyapatite eluate containing the sea star kinase, the eluate was mixed with phenyl-Sepharose-purified p44* and applied to phenyl-Sepharose. All of the p44* eluted in the gradient at its expected position, while p44* was found in the isocratic wash fractions (Fig. 2B, Fig. 2C). This confirmed the anti-phospho-protein immunoblotting of each kinase peak (Fig. 2C, inset). Therefore, the distinct chromatographic behavior of these proteins reflects their intrinsic properties.

S. Pelech, unpublished data.
MAP-2/MBP Kinases Activated in the Cell Cycle

Phosphorylation of Thr-97 in MBP by the murine 44 kDa kinase. Recently, our groups have independently identified Thr-97 as the major site of phosphorylation of MBP by murine p42\(^{mm}\) (14) and sea star p44\(^{mm}\) (2B). Accordingly, we have compared the HVE/LTC tryptic phosphopeptide maps of MBP phosphorylated by the murine 44 kDa kinase with MBP phosphorylated by these kinases (Fig. 4). As with p42\(^{mm}\) (Fig. 4A) and p44\(^{mm}\) (Fig. 4B), the murine 44 kDa kinase (Fig. 4C) phosphorylated MBP specifically on the tryptic peptide that contained Thr-97. To confirm this, MBP was phosphorylated by the 44 kDa kinase or by the sea star p44\(^{mm}\) The digests were mixed, and subjected to HVE/LTC; the major phosphopeptide identified in both Figure 4B and 4C corresponded (Fig. 4D). Therefore, the 44 kDa kinase has a similar phosphorylation site specificity as does murine p42\(^{mm}\) and sea star p44\(^{mm}\).

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Co-purification of the murine 44 kDa phosphoprotein with MBP kinase activity. To determine if the 44 kDa phosphoprotein from PDB-treated EL4 cells was responsible for the phosphotransferase activity detected in the phenyl-Superose wash, the partially purified p44\(^{mm}\) was further subjected to sequential column chromatography on polylysine-agarose, Mono Q and Superose 12. Phosphotransferase activity monitored during each of these purification steps with MBP as substrate, eluted in a single peak from polylysine-agarose and Mono Q (data not shown). The 44 kDa phosphoprotein was identified in each of these kinase peaks by immunoblotting with the anti-phosphoseryl protein antiserum (Fig. 3A). Furthermore, MBP kinase activity detected in each Superose 12 gel filtration fraction correlated exactly with p44 (Fig. 3B).

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in a single tryptic peptide (27). To determine whether this is also true for pp44, pp42aa, and pp44 were isolated from [33]P-phosphate-labeled cells, and were subjected to proteolytic cleavage with trypsin followed by 2-dimensional HVEI TLC. As previously reported (27), pp44 contained a single tryptic phosphopeptide (arrow in Fig. 6A). Murine pp44 also contained a single tryptic phosphopeptide with an electrophoretic mobility nearly identical to that of the pp42aa phosphopeptide. The phosphopeptides from both enzymes contained similar amounts of phosphotyrosine and phosphothreonine (data not shown). In contrast to the phosphopeptide from pp42aa, however, the pp44 phosphopeptide was slightly more hydrophilic as indicated by its higher mobility in the solvent chromatography direction (arrow in Fig. 6B). A mixing experiment (Fig. 6C) confirmed their similar but non-identical properties (see Discussion).

A. p42 MAP K. B. p44 MAP K. C. Mix

Figure 6. Phosphotryptic peptide maps of murine [33]Ppp42aa and [33]Ppp44. [33]Ppp42aa and [33]Ppp44 were proteolyzed with trypsin and subjected to HVEI TLC. Origin (●), cathode (○), anode (△). p42aa (panel A), pp44 (panel B), mixture (panel C).

Two-dimensional gel electrophoresis of EL4 p42aa and pp44. The 42 kDa phosphoprotein was originally identified by 1- or 2-dimensional gel electrophoresis as a ubiquitous protein-tyrosine kinase substrate (4, 7) and was subsequently shown to be identical to p42aa (12). Other tyrosine-phosphorylated substrates of 44-45 kDa have also been identified on 2-D gels. Therefore, we compared the relative migration of murine pp44 to that of p42aa using this procedure. [33]Ppp42aa was partially purified by phenyl-Superose chromatography and subjected to 2-dimensional gel electrophoresis (arrow, Fig. 7A). [33]Ppp44 was partially purified as for Fig. 5 and was similarly electrophoresed. Fig. 7B). pp44 migrated at a higher molecular weight and exhibited a more acidic pI than p42aa, and this was confirmed by a mixing experiment (Fig. 7C). The relative positions of pp44 and of p42aa on these 2-D gels, and the fact that the phosphorylation of pp44 is mitogen-induced, suggest this kinase may be pp45, one of the 42-45 kDa tyrosine kinase substrates first identified by Cooper et al. (4) (see Discussion).

Panel A. [33]P-labeled p44aa purified by phenyl-Superose chromatography was electrophoresed on a 2-D polyacrylamide gel, dried and exposed to film. The acidic portion of the gel is on the left. pp42aa, the active form of p42aa, is identified by an arrow. Panel B. [33]P-labeled pp44, sequentially purified through phenyl-Superose, polylysine-agarose, and Mono Q. was also subjected to 2-D gel electrophoresis (panel B, arrow). Panel C. Mixture of equal Cerenkov cpm of material shown in A and B.

Figure 7. Two-dimensional gel electrophoresis of murine [33]Pp42aa and [33]Ppp44.

Chromatographic comparisons of p42aa and pp44 from EGFS-stimulated murine 3T3 fibroblasts. Murine pp44 was also identified in EGFS-treated 3T3 cells by phenyl-Superose chromatography, using MAP-2 as a substrate. The cells were stimulated for 10 min with EGFS, followed by sequential chromatography on DE52-cellulose and phenyl-Superose. A peak of MAP-2 phosphorylating activity eluted at the predicted position of p42aa at 37% ethylene glycol and 100 mM NaCl (peak I, Fig. 8) (12). The presence of p42aa was verified by immunoblotting this peak with the anti-phosphotyrosine antibody (lane I, inset Fig. 8). In addition to the p42aa peak, a broad peak of MAP-2 phosphorylating activity eluted in the phenyl-Superose wash fractions (peak II, Fig. 8). These fractions were pooled and pp44 was detected by immunoblotting with the same antibody (lane II, inset Fig. 8). Unstimulated cell extract, put through the same purification procedures, had no detectable MAP-2 phosphorylating activity (Fig. 8) nor was any phosphoprotein detected (data not shown). Therefore, pp44 is present in EGFS-stimulated murine 3T3 cells, and it phosphorylates MAP-2 like p42aa, but it elutes in the phenyl-Superose wash as does pp44 from PDB-treated EL4 cells.

Figure 8. Phenyl-Superose chromatography of murine Swiss 3T3 fibroblasts stimulated with EGFS.

EGFS-stimulated Swiss 3T3 homogenate was applied to sequential DE52-cellulose and phenyl-Superose chromatography. Each phenyl-Superose fraction was monitored for phosphotransferase activity using MAP-2 as a substrate (a). Two peaks of kinase activity were identified (peaks I and II). Peak I contained p42aa and the broad wash fraction peak contained the 44 kDa kinase, as shown by immunoblotting with the anti-phosphotyrosine antiserum (labeled arrow). Unstimulated cell extract was also subjected to the same fractionation procedures and no MAP kinase activity was identified (b) nor was any phosphoprotein detected (data not shown).

Krebs and co-workers utilized Mono Q chromatography to identify two peaks of MBP phosphorylating activity in EGFS-stimulated Swiss 3T3 fibroblasts and phorbol ester-treated EL4 cells (24, 29). To compare the kinases described by these workers to the kinases reported here, p42aa and pp44 were isolated from PDB-treated EL4 cells, mixed together and then separated by Mono Q chromatography (Fig. 9). Two peaks of MAP kinase activity were identified, as reported by Ann et al. (24). The first kinase peak (I) eluted at 80 mM NaCl and contained p42aa, as determined by anti-phosphotyrosine immunoblotting, the second peak at 100 mM NaCl (II) contained pp44 (inset, Fig. 9).