Purification of a 12,020-Dalton Protein That Enhances the Activation of Mitogen-activated Protein (MAP) Kinase by MAP Kinase Kinase*

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We have purified 3500-fold from rabbit skeletal muscle a 12,020-Da mitogen-activated protein kinase kinase (MEK)-enhancing factor (MEF) that stimulates both mitogen-activated protein kinase (MAPK) autophosphorylation and the rate (24-fold) at which the enzyme is phosphorylated by MEK in vitro. This was manifest by the finding that in the presence of MEF, molar equivalents of MEK to MAPK were sufficient to produce fully phosphorylated (2.1 ± 0.4 mol/mol; S.D., n = 3) and activated MAPK. This contrasted with the 40:1 molar excess ratio of MEK to MAPK required to produce fully phosphorylated and activated MAPK in the absence of MEF. Phosphoamino acid analysis revealed that in the presence of MEF, phosphorylation of MAPK by MEK was ordered, with Tyr-185 phosphorylation preceding Thr-183 phosphorylation. However, the rate at which Thr-183 was phosphorylated relative to Tyr-185 was greatly increased. The finding that MEF stimulated MAPK autophosphorylation and increased its ability to be phosphorylated by MEK suggests a mechanism of action in which MEF interacts with MAPK to alter its conformation.

The mitogen-activated protein kinases (MAPKs)1 p42MAPK (ERK2) and p44MAPK (ERK1) are thought to mediate the actions of numerous hormones controlling cellular events as diverse as growth, division, and metabolism (for reviews, see Refs. 1–4). p42MAPK and p44MAPK belong to a conserved family of protein kinases that also includes p54MAPK (5, 6) and the stress-activated protein kinases JNKs/SAPKs and p38MAPK/RKs (7–11). All MAPKs in the family are activated uniquely by dual phosphorylation at sites that contain the sequence TXY, seven residues N-terminal to the conserved APE motif found in most protein kinases (5, 7, 8, 12, 13). The MAP kinase kinases (MEKs) also comprise a related gene family and appear to function exclusively to activate their respective MAPKs (7, 9, 14–18). Significantly, many features of the MAPK pathway in higher eukaryotes are conserved in the pheromone and stress response signal transduction pathways of fission and budding yeast (19–22).

Much attention in recent years has focused on delineating the immediate steps from cell-surface receptors to the activation of p42MAPK and p44MAPK. To date, two tentative connections have been made. In one pathway, several laboratories, utilizing a combination of overexpression and antisense techniques, have identified p74raf1 and possibly its homologs A-Raf and B-Raf as activators of MEK in vitro (23–29). Indeed,raf has been demonstrated to phosphorylate MEK on two serine residues, 218 and 222, and mutation of these residues to glutamic or aspartic acid results in constitutive activation (30–35). The mechanism(s) by which p74raf1 is activated by hormone receptors is not completely understood, but appears to involve an interaction with GTP-bound p21ras (29, 36–43). In yeast, an alternative pathway suggests that MEKs are activated by heterotrimeric G protein-coupled receptors via the activation of STE-11 homologs of MEK kinases (MEKKs) (19–22, 44). Other less well characterized activators of MEK have also been described, including c-mos (45–47), a 400-kDa MEK-activating factor in Xenopus (48), and a 50–60-kDa insulin-stimulated MEK kinase (I-MEKK) in isolated adipocytes (49) and NIH 3T3 cells (29). The existence of multiple MEK-activating pathways has led to the hypothesis that MEK acts as a signaling convergence point, explaining how so many diverse cellular agonists can activate MAPKs.

Our laboratory has had a long standing interest in the mechanism by which insulin both activates and then inactivates MAPK in vivo. We recently identified in isolated adipocytes a MEK kinase (I-MEKK) that, like MEK and MAPK, showed acute phasic activity kinetics in response to the hormone (50). Since this initial report, we have made several unsuccessful attempts to purify I-MEKK from insulin-treated adipocytes. Although I-MEKK remains stable over anion-exchange and gel filtration chromatography, further purification results in intermediate loss of all activity. These findings led us to conclude that activation of MEK by MEK kinases in vitro requires additional factors that are lost in subsequent purification steps. Accumulating genetic evidence in yeast may support this hypothesis. In Saccharomyces cerevisiae, the conserved MAPK module, comprising STE-11 (MEKK), STE-7 (MEK), and FUS-3 (MAPK), requires a fourth protein, STE-5, to mediate pheromone responses (Ref. 51; see also Ref. 52). In the module, STE-5 has been proposed to tether these protein kinases together in order to bring about their sequential activation. If an STE-5-like protein exits to activate MEK in higher eukaryotes, this might also explain discrepancies we have observed in the mechanism by which MEK activates MAPK in vitro. We had reported earlier that in the presence of catalytic amounts of purified MEK, the Tyr-185 phosphorylated form of MAPK accumulated. To produce the fully activated and phosphorylated form of MAPK, repeated additions of purified MEK were required (49). From this study, we concluded that phosphorylation of MAPK by MEK was ordered, with Tyr-185 phosphoryl-

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1 The abbreviations used are: MAPKs, mitogen-activated protein kinases; MEKs, mitogen-activated protein kinase kinases; MEKKs, MEK kinases; I-MEK, insulin-stimulated MEK kinase; MEF, MEF-enhancing factor; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.
ation preceding Thr-183 phosphorylation. Recently, Goldsmith and co-workers (53) solved the apo structure of p42MAPK to 2.3-Å resolution. In the apo structure, Thr-183 is exposed on the surface of the enzyme. These findings are paradoxical since the apo structure would indicate that MEK is unlikely to phosphorylate MAPK on Thr-183 first. Taken together, these observations strongly support the hypothesis that additional factors may be required for the activation of MAPK and possibly MEK. In this paper, we report the purification of a 12,020-Da protein that potently enhances the activation of MAPK by MEK. We propose that our findings have general implications for some of the models currently proposed for MEK activation in vivo.

**EXPERIMENTAL PROCEDURES**

Materials

Recombinant p42MAPK was purified from Escherichia coli strain BL21(DE3)pET-MK1 (49). Native MEK was purified from rabbit skeletal muscle as described previously (54). The pGEX-2T plasmids expressed p21, p72MEK-1, S258D/S252MEK1, and KRS2 MAPK were a gift from Dr. Andrew Catlett (Department of Microbiology, University of Virginia).

Methods

Preparation of Recombinant GST-MEK Fusion Proteins—DH5α cells transformed with the respective pGEX-2T-PEL plasmids were streaked onto LB plates containing ampicillin and incubated overnight at 37°C. Six-ml cultures containing LB broth, 50 μg/ml ampicillin, and a single colony of E. coli were grown overnight at 37°C. Six flasks containing 1 liter of LB broth and 50 μg/ml ampicillin were inoculated with the overnight culture and grown at 37°C to an absorbance of 600. Cells were induced with 30 μM isopropyl-β-D-thiogalactoside for 5 h at room temperature and then centrifuged at 3600 × g for 20 min at 4°C. The pellet was stored overnight at −20°C. The thawed pellet was resuspended in 45 ml of lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine), lysed with 2 mg/ml lysozyme, incubated on ice for 15 min, sonicated for 3 min, and then centrifuged at 14,500 × g for 30 min at 4°C. 1.0 ml of glutathione-Sepharose 4B beads at 50% (v/v) was added to the supernatant and rotated overnight at 4°C. The beads were centrifuged at 200 × g and washed four times with 25 ml of lysis buffer. The GST-MEK fusion protein was eluted with 6 ml of 5 mM glutathione, 50 mM Tris, pH 8. The protein was dialyzed overnight against 1 liter of Tris buffer (50 mM Tris, pH 7.3, 1.5 mM EGTA, 1 mM benzamidine) and applied to a Waters Protein-Pak Q AP-1 anion-exchange column equilibrated in buffer A (50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 0.15 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). The column was developed with a 400 mM linear salt gradient (200 mM), and 2.0-ml fractions were assayed for activation of MAPK (54). The peak of activity was pooled and concentrated under a vacuum to a 1-ml volume. Glycerol was added to 50% (v/v), and protein was stored at −20°C.

Preparation of MEF—A fed female New Zealand White rabbit was euthanized by lethal injection with phenobarbital via the marginal ear vein. The back and hind limb skeletal muscle (300 g, wet weight) were rapidly excised, minced, and homogenized at 4°C with 2.5 volumes of buffer B (50 mM β-glycerophosphate pH 7.3, 1.5 mM EGTA, 0.15 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). All subsequent steps were carried out at 4°C. The homogenate was centrifuged at 6000 × g for 45 min, and the supernatant was applied to a FFQ-Sepharose column (20 × 50 cm; Pharmacia Biotech Inc.) equilibrated with buffer B. The column was washed extensively until the absorbance at 280 nm of the eluate was stable. Concentrated MEF was further purified by successive applications to a Waters SW300 gel filtration column equilibrated in buffer C (25 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 10 mM β-mercaptoethanol, and 150 mM NaCl) (see Fig. 3). The most active column fractions were pooled, heated at 90°C for 5 min, and centrifuged at 30,000 × g. Following concentration to 1.0 ml, the extract was applied to a Waters Nova-Pak C18 reverse-phase column (3.9 × 150 mm) equilibrated in 0.1% trifluoroacetic acid. The column was developed as described (see Fig. 4A). For further purification, the most active fractions were pooled and reapplied to a Waters Nova-Pak C18 reverse-phase column (3.9 × 150 mm) equilibrated in 0.1% trifluoroacetic acid, and the column was developed as described (see Fig. 4B).

Assay of MEF Activity—MEF activity was determined by its ability to enhance the activity of MEK toward MAPK. Two types of assay were utilized, either a two-stage robotic assay that measured activation of MAPK or direct phosphorylation of MAPK. In the two-stage robotic assay, 20 μl of column fraction was placed into microtiter plate wells, and reaction was started by the addition of 40 μl of buffer C containing 0.6 μg of p42MAPK, 0.6 μg of MEK, 7.5 mM MgCl₂, and 1 mM microcystin. After 10 min at 22.5°C, the robot removed 20 μl and placed this into a second set of wells containing 5 μg of myelin basic protein, 300 μM [γ-32P]ATP (250 cpm/pmol), and 7.5 mM MgCl₂ in buffer C. The reaction was terminated after 5 min by the addition of 20 μl of 100 mM H₃PO₄. The reactions (40 μl) were spotted onto P-81 paper (Whatman) and washed four times in 100 mM H₃PO₄ and the amount of radioactivity incorporated into myelin basic protein was determined by scintillation counting. To measure endogenous MEK activity, the first reaction was carried out in the absence of exogenous MEK. In the second type of assay, 20 μl of column fraction was placed into microtiter plate wells, and the reaction was started by the addition of 40 μl of buffer C containing 0.6 μg of p42MAPK, 0.6 μg of MEK, 300 μM [γ-32P]ATP (250 cpm/pmol), 7.5 mM MgCl₂, and 1 μM microcystin. After 20 min at 22.5°C, the robot terminated the reaction by the addition of 20 μl of SDS sample buffer (20% glycerol, 62.5 mM Tris-HCl, pH 6.8 [25°C], 2% SDS, and 5% 2-mercaptoethanol). Again, endogenous MEK was measured by carrying out the assay in the absence of exogenous MEK. Following heating (100°C) for 5 min, the phosphorylated proteins were characterized by SDS-PAGE and autoradiography. Quantitiation of phosphorylation was by PhosphorImager analysis. In assays in which the stoichiometry of MAPK phosphorylation was measured, reactions were terminated with 25% trichloroacetic acid, and the radioactivity incorporated into the precipitated proteins was determined by Cerenkov counting.

**RESULTS**

Phosphorylation of MAPK by MEK—The ability of recombinant p42MAPK to be phosphorylated by various concentrations of purified native rabbit skeletal muscle MEK1 was measured (Fig. 1). Fig. 1 shows that in order to achieve a stoichiometry close to 1 mol/mol within a 30-min period, a 3-4-fold (3.6 ± 0.19 S.D., n = 3) molar excess of MEK (−2 μM) over MAPK (0.5 μM) was required. At this time, phosphoamino acid analysis of MAPK revealed phosphothreonine to be incorporated into tyrosine, with the remainder on threonine (Fig. 1, inset). Increasing the concentration of MEK by an order of magnitude (40 μl) achieved a stoichiometry of phosphorylation close to 2 mol/mol within the same time frame. Under these conditions, the phosphothreonine content of MAPK was equal to phosphotyrosine (Fig. 1, inset). When fully phosphorylated, recombinant MAPK displayed a specific activity toward myelin basic protein of 0.32 ± 0.08 μmol/min/mg (S.D., n = 3), approximating values previously estimated for the activated native enzyme (49). Increasing the concentration of MAPK in the assay to 10 μM, with a fixed MEK concentration of 20 μM, resulted in an appreciable reduction in the overall stoichiometry of phosphorylation to 0.2 ± 0.05 mol/mol (S.D., n = 3). Correspondingly, phosphorylation of threonine was considerably reduced relative to tyrosine. These results suggest that the relative poor rate of phosphorylation of MAPK by purified MEK was not due to a suboptimal substrate concentration being utilized in our assays. In previous work, we had also noted that high concentrations of MEK relative to MAPK were required to activate the enzyme completely (49). Also in this earlier study, it was observed that Tyr-183 phosphorylation increased relative to Thr-183, suggesting that phosphorylation of MAPK by MEK was ordered.

Purification of MEF—The finding that in vitro an excess
were assayed in the presence of S218D/S222D MEK1, four distinct peaks of MAPK activity were observed, a broad peak S218D/S222D MEK1. In the absence of the mutant kinase, four their ability to activate MAPK in the presence and absence of profile of muscle extract in which fractions were assayed for MEK-enhancing factors may be similarly activated. during post-mortem dissection (17, 54). Thus, any potential MEK and MAPK are constitutively activated in this tissue skeletal muscle was utilized because of the observation that concentrations of MEK over MAPK is required to achieve full activation of MAPK is some what surprising since both enzymes are postulated to exist in a protein kinase-mediated signal transduction cascade in which MEK activates MAPK (1–4). Indeed, many laboratories have observed a potent and rapid activation of both MEK and MAPK in several cell types in response to various agonists (1–4). To investigate the hypothesis that other factors may be required to bring about a more potent phosphorylation and activation of MAPK, we utilized the recombinant constitutively active MEK mutant S218D/S222D MEK1 mutant, rather than native MEK, excluded the possibility of detecting MEK kinases in our assays (23, 29, 54). Rabbit skeletal muscle was utilized because of the observation that MEK and MAPK are constitutively activated in this tissue during post-mortem dissection (17, 54). Thus, any potential MEK-enhancing factors may be similarly activated.

Fig. 2 shows an anion-exchange chromatography column profile of muscle extract in which fractions were assayed for their ability to activate MAPK in the presence and absence of S218D/S222D MEK1. In the absence of the mutant kinase, four distinct peaks of MAPK activity were observed, a broad peak eluting in the column wash (I) and three other sharper peaks (II–IV) eluting with the salt gradient. When column fractions were assayed in the presence of S218D/S222D MEK1, four peaks were also observed; however, peak I (designated MEF) was more striking. Indeed, the activity of the mutant kinase in this region was 5–6 times greater than that measured in the absence of column fraction or in other regions of the column profile. The enhanced activity in the MEF peak area could not be accounted for by simply adding the constitutive basal activity of S218D/S222D MEK1 to the activity of MAPK measured in the absence of the mutant kinase. Indeed, the data shown in Fig. 2 and subsequently Fig. 3 were derived following subtraction (peaks II and IV) (data not shown). To test whether the MEF peak contained any MEK kinase activity, the anion-exchange column profile was also assayed with wild-type recombinant MEK1. However, in these experiments, no evidence of MEK kinase activity was found in this region (data not shown). In contrast to the MEF peak, peaks II–IV do not change significantly in the presence or absence of S218D/S222D MEK1 once the correction is made for constitutive activity. These later eluting peaks were identified by Western blotting as endogenous MEK (peak II) and MAPK (peaks III and IV) (data not shown).

Further purification of MEF was carried out by gel filtration (Fig. 3). Fig. 3 shows that the majority of MEF activity (>90%) eluted on gel filtration with a putative molecular mass of ∼12 kDa. At its apex, MEF stimulated S218D/S222D MEK1 7–8-fold over its basal constitutive activity. Two minor peaks of MEK activity, of 80 and >150 kDa, respectively, were also recovered from the column. However, because both peaks were less well defined and eluted with the majority of the protein, further purification was not carried out at this stage. All subsequent studies were performed on the 12-kDa MEF fraction. As observed on anion-exchange chromatography, when the gel filtration profile was assayed in the absence of S218D/S222D MEK1, MEF also stimulated MAPK autophosphorylation (see Fig. 6) and activation (Fig. 3). These findings suggest that either MEF is a MEK or it stimulates MAPK autophosphorylation. However, since the molecule is not of sufficient molecular size to accommodate the minimum consensus sequence required for a protein kinase, this suggests that MEF is unlikely to be a MEK (13). Consequently, it is more likely that MEF interacts with MAPK to stimulate autophosphorylation.

Gel filtration fractions were also assayed for their ability to phosphorylate and activate wild-type recombinant MEK1.
However, as observed on anion-exchange chromatography, no phosphorylation or activation of wild-type MEF was observed across the column profile (data not shown), further ruling out the possibility that MEF is a MEK kinase that phosphatase MEF at novel activating sites other than Ser-218 and Ser-222. The effect of MEF on stimulating MAPK autophosphorylation appears to be selective for MAPK since when column fractions were assayed for any other distinct protein kinases, including casein kinase II, p70 ribosomal protein S6 kinase, cyclic AMP-dependent protein kinase, or p60c-src, no effect on the activity of these enzymes was observed (data not shown).

Following gel filtration, MEF was purified to homogeneity over two reverse-phase HPLC columns, C18 and then C3 (Fig. 4, A and B, respectively). Fig. 4A shows that two major peaks of MEF activity eluting at 4 and 57 min, respectively, were recovered from the C18 reverse-phase column. Approximately 50% of the activity eluting at 4 min re-eluted at 57 min when this material was reapplied to the column, suggesting that this was merely unbound MEF. MEF activity eluting at 57 min was pooled and applied to the C3 column. When the column was developed, MEF was eluted at 54 min as a single homogeneous protein as judged by SDS-PAGE and silver staining (Fig. 5). Analysis of the purified protein by time-of-flight laser desorption mass spectrometry gave the molecular mass of MEF as 12,020 Da, consistent with gel filtration (Fig. 3) and SDS-PAGE (Fig. 5) data. Starting with 300 g of rabbit skeletal muscle, MEF was purified to homogeneity 3500-fold (estimated from the FFQ-Sepharose step) with a recovery of 2.7% (Table I). The relatively poor recovery appears to be due to significant loss of activity during the reverse-phase chromatography steps.

Analysis of MAPK Phosphorylation in the Presence of MEF and MEK—To address the question as to the mechanism by which MEF interacts with MAPK and MEK, the kinetics of Tyr-185 and Thr-183 phosphorylation were examined. Fig. 6 (A and B) compares the rates at which MAPK (1 μM) was phosphorylated in the presence of various combinations of MEF and GST-S218D/S222D MEK1 (1 μM). In the absence of MEF or MEK (Fig. 6A, ×), MAPK exhibited a low rate of autophosphorylation, achieving a stoichiometry of phosphorylation of ~0.05 mol/mol by 90 min. When MEF was included in the assay (Fig. 6, A (□) and B (panel i)), the rate of autophosphorylation of MAPK was enhanced ~8-fold to 0.301 mol/mol by 90 min. Phosphoamino acid analysis revealed ~95% of the phosphate to be incorporated into tyrosine, with a trace of phosphothreonine being present (data not shown). These findings are consistent with results observed earlier (Figs. 3 and 4) in which MEF enhanced the activity of MAPK toward myelin basic protein in the absence of MEK. Interestingly, the enhanced rate of autophosphorylation in the presence of MEF was close to the rate of phosphorylation achieved by MEF alone (Fig. 6, A (△) and B (panel iii)). When these experiments were repeated with the kinase-deficient MAPK mutant KR52, the level of autophosphorylation detected in the presence or absence of MEF was ~0.02 ± 0.005 mol/mol (S.D., n = 3). In the presence of GST-S218D/S222D MEK1 alone, KR52 MAPK was phosphorylated to a stoichiometry of 0.26 ± 0.05 mol/mol (S.D., n = 3) by 90 min.

The most striking effects on MAPK phosphorylation were observed when MEF (heat-treated or non-heat-treated) was included in the assay with MEK (Fig. 6, A (○, ○) and B (panel iv)). Within 5 min, MAPK was phosphorylated to a stoichiometry of 0.78 mol/mol, reflecting a 24-fold increase in the rate of phosphorylation measured at this time point in the absence of MEF. By the 20-min time point, MAPK was phosphorylated to close to 2.0 mol/mol (2.1 ± 0.4 mol/mol; S.D., n = 3) and had a specific activity toward myelin basic protein of 0.35 ± 0.01 μmol/min/mg (S.D., n = 3). This compared with the 0.084 mol/mol and a specific activity of 0.01 μmol/min/mg achieved at this same time point in assays containing MEK, but no MEF. MEF also enhanced the ability of MEK to phosphorylate KR52 MAPK, achieving a stoichiometry of 1.86 ± 0.4 mol/mol (S.D., n = 3) by 20 min. This suggests that although KR52 is unable to autophosphorylate, it can interact with MEK and be phosphorylated by MEK to a stoichiometry close to that achieved with wild-type MAPK. Significantly, the data shown in Fig. 6 contrast dramatically with those shown in Fig. 1 in that a molar excess of MEF over MAPK was no longer required to produce fully phosphorylated and activated MAPK. In Fig. 1, a MEK/MAPK molar ratio of 40:1 was required to produce stoichiometrically phosphorylated MAPK within a 30-min period. However, as shown in Fig. 6, in the presence of MEF, molar equivalents of MEK to MAPK were now sufficient to produce fully phosphorylated and activated enzyme within this time frame. Preliminary findings indicate that in the presence of MEF and native MEK, the ratio of MEF to MAPK can be titrated down still further while retaining reasonable rates of MAPK phosphorylation, although at the time of writing, it is not clear whether molar equivalents of MEK to MAPK are required to fully phosphorylate and activate the enzyme in the presence of MEK.²

In assays that contained MAPK, a low level of GST-S218D/S222D MEK1 phosphorylation was also observed (Fig. 6B, panels iii and iv). This finding is consistent with data of others showing phosphorylation of MEK by MAPK at sites other than Ser-218 and Ser-222 (55). However, in our hands, phosphorylation of MAPK by MEK does not appear to affect activity.³ As shown in the autoradiogram in Fig. 6B (panel ii) and also as indicated during column chromatography, purified MEF did not bring about phosphorylation of GST-MEK, further ruling out the possibility that the protein stimulates MEK autophosphorylation or that it is a MEK kinase.

² A. Scott and T. A. J. Haystead, manuscript in preparation.
³ T. A. J. Haystead, unpublished results.
The effect of MEF on MAPK autophosphorylation and phosphorylation by MEK suggests a mechanism of action in which MEF interacts with MAPK to alter its conformation. To explore this hypothesis, the relative rates of Tyr-185 and Thr-183 were measured (Fig. 7). As shown earlier (49), when MAPK is phosphorylated by MEK alone, Tyr-185 phosphorylation proceeds at a significantly greater rate than Thr-183 phosphorylation (Fig. 7A). Quantitation of the two amino acids by PhosphorImager analysis reveals the relative rate of tyrosine phosphorylation to be ~20-fold greater than that of threonine phosphorylation at the 10-min time point and ~5-fold greater by 90 min (Fig. 7A). However, when MAPK is phosphorylated by MEK in the presence of MEF, a significant increase in the rate of threonine phosphorylation relative to tyrosine is observed (Fig. 7B). Within 5 min, threonine phosphorylation was ~45% of that measured for tyrosine, and by 20 min, both residues were equally phosphorylated. These data suggest that in the presence of MEF, phosphorylation of MAPK is still ordered (Tyr-185 precedes Thr-183), but the rate and efficiency of phosphorylation of threonine relative to tyrosine is greatly enhanced.

Data demonstrating that MEF did not cause phosphorylation of MAPK at additional sites other than Tyr-185 and Thr-183 were obtained after tryptic peptide mapping of the 32P-labeled protein by reverse-phase HPLC (Fig. 8). Comparison of the peptide maps obtained from MAPK phosphorylated in the presence or absence of MEF revealed similar patterns. In both cases, 32P-labeled tryptic peptides were recovered eluting at 28.00 and 38.00 min, respectively. Although the amount of radioactivity in the 38.00 min peptide was greater in the MAPK sample phosphorylated by MEK in the presence of MEF (Fig. 8A), phosphoamino acid analysis of the phosphopeptides revealed the 28.00 min peptide to contain phosphotyrosine and the 38.00 min peptide to contain equal proportions of phosphotyrosine and phosphothreonine (Fig. 8A, inset). These findings are consistent with phosphorylation of MAPK by MEK at its activating site, VADPDHDHTGFLTEYVATR (12), and no other.

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In this paper, we have purified to homogeneity a 12,020-Da protein (MEF) that enhances the activation of MAPK by MEK. Three lines of evidence indicate that MEF is not a MEK kinase or a MEK. First, MEF is not of sufficient molecular size to accommodate the minimum consensus sequence required for protein kinases (13). Second, MEF did not phosphorylate wild-type MEK in the absence of active MAPK. Third, MEF did not phosphorylate catalytically inactive KR52 MAPK, but like wild-type MAPK, did enhance the ability of the mutant to be phosphorylated by MEK. Phosphoamino acid analysis revealed that in the presence of MEF, phosphorylation of MAPK by MEK was ordered, with Tyr-185 phosphorylation preceding Thr-183 phosphorylation. However, the rate at which Thr-183 was phosphorylated relative to Tyr-185 was greatly increased. This was manifest by the finding that in the presence of MEF, molar equivalents of MEK to MAPK were now sufficient to produce fully phosphorylated and activated MAPK. This contrasts with the 40:1 molar excess ratio of MEK to MAPK required to produce fully phosphorylated and activated MAPK in the absence of MEF. The finding that MEF stimulated MAPK autophosphorylation and increased its ability to be phosphorylated by MEK by 24-fold suggests a mechanism of action in which MEF interacts with MAPK to alter its conformation (Fig. 9).

**DISCUSSION**

In this paper, we have purified to homogeneity a 12,020-Da protein (MEF) that enhances the activation of MAPK by MEK. Three lines of evidence indicate that MEF is not a MEK kinase or a MEK. First, MEF is not of sufficient molecular size to accommodate the minimum consensus sequence required for protein kinases (13). Second, MEF did not phosphorylate wild-type MEK in the absence of active MAPK. Third, MEF did not phosphorylate catalytically inactive KR52 MAPK, but like wild-type MAPK, did enhance the ability of the mutant to be phosphorylated by MEK. Phosphoamino acid analysis revealed that in the presence of MEF, phosphorylation of MAPK by MEK was ordered, with Tyr-185 phosphorylation preceding Thr-183 phosphorylation. However, the rate at which Thr-183 was phosphorylated relative to Tyr-185 was greatly increased. This was manifest by the finding that in the presence of MEF, molar equivalents of MEK to MAPK were now sufficient to produce fully phosphorylated and activated MAPK. This contrasts with the 40:1 molar excess ratio of MEK to MAPK required to produce fully phosphorylated and activated MAPK in the absence of MEF. The finding that MEF stimulated MAPK autophosphorylation and increased its ability to be phosphorylated by MEK 24-fold suggests a mechanism of action in which MEF interacts with MAPK to alter its conformation (Fig. 9).

**TABLE I**

| Step            | Volume | Activity | Protein | Specific activity | Purified | Yield |
|-----------------|--------|----------|---------|------------------|----------|-------|
| Extract         | 750.0  | NDb      | 9260.00 | ND               | 1        | ND    |
| Wash/FFQ-Sepharose | 1500.0 | 7740.00  | 250.1   | 30.96            | 1.00     | 100.00|
| SW300           | 15.0   | 2202     | 9.2     | 249.1            | 8.00     | 29.6  |
| Heat            | 15.0   | 2201     | 0.15    | 14,673.33        | 489.00   | 28.43 |
| C8              | 0.5    | 658      | 0.02    | 32,900.00        | 1062.00  | 8.5   |
| C18             | 0.5    | 210      | 0.002   | 105,000.00       | 3500.00  | 2.7   |

*1 unit of MEF = amount required to increase the activity of S218D/S222D MEK1 by 50%.

*No data (ND) were derived for units of activity in the initial homogenate due to the background activity from endogenous rabbit skeletal muscle

**FIG. 6.** Effects of MEF on phosphorylation of MAPK. MAPK (1 μM) was incubated at 30 °C in assays (500 μl) containing buffer C, 300 μM ATP (2500 cpm/nmol), 7.5 mM MgCl₂, and the following combinations: ■, without MEF; □, 10 units of MEF; △, 1 μM S218D/S222D MEK1; ○, 1 μM S218D/S222D MEK1 and 10 units of heat-treated MEF. At the indicated times, 50 μl of the reaction mixtures was removed and added to 0.5 ml of ice-cold trichloroacetic acid (25%, w/v). A, stoichiometry of MAPK; B, autoradiograms following SDS-PAGE of the trichloroacetic acid-precipitated proteins phosphorylated with the indicated combinations of MEK, MEF, and MAPK. Experiments were conducted on partially purified MEF isolated from the SW300 column. Similar data were also obtained when purified rabbit skeletal MEK1 was used instead of S218D/S222D MEK1.

**FIG. 7.** Effects of MEF on kinetics of Tyr-185 and Thr-183 phosphorylation by MEK. Phosphoamino acid analysis was carried out on trichloroacetic acid precipitates of MAPK phosphorylated by GST-S218D/S222D MEK1 in the presence (A) or absence (B) of MEF. To detect the presence of phosphothreonine, B was exposed for 48 h compared with a 12-h exposure in A. Quantitation of the 32P-labeled amino acids was carried out on a Molecular Dynamics PhosphorImager.
Identification of MEF is critical for determining its role in the regulation of the MAPK pathway. Preliminary analysis of tryptic fragments of MEF by mass spectrometry indicates that it is unrelated to any known protein in the current data base. MEF may be a fragment of a larger protein; indeed, another less well defined MEF peaks of 50 and ~150 kDa were detected upon gel filtration (Fig. 3). We are currently investigating whether these are complexes of MEF or MEF combined with other proteins, such as MEK or MAPK. We originally hypothesized that MEF might be a mammalian homolog of STE-5 (~100,000 kDa), although its small molecular size and lack of effect on MEK autophosphorylation may suggest otherwise. However, the findings presented in this paper, demonstrating that additional factors exist in the regulation of MAPK, lead one to speculate that proteins with MEF-like function may be necessary for activation of other members of the MAPK pathway, such as MEK. In yeast, STE-5 appears to fulfill such a role. As discussed earlier, STE-5 is thought to tether STE-11, STE-7, and FUS-3 in a large complex that is necessary for the transmission of pheromone responses. In S. cerevisiae, transmission of this signal is thought to begin with activation of the G protein-coupled receptor pheromone that activates the protein kinase STE-20. STE-20 is subsequently thought to activate STE-11 while it is tethered to STE-5. Activation of STE-7 and FUS-3 then ensues. The yeast paradigm has been proposed by several groups to be the model for MAPK activation in mammalian cells responding to hormones that act through G protein-coupled receptors, such as thrombin (21).

Our original motivation of searching for MEK and MAPK activators was driven by the observation that MAPK was a relatively poor substrate for MEK in vitro, an observation that was counter-intuitive to the role of MEK in vivo. The finding that MEF improves the ability of MAPK to be phosphorylated by MEK significantly addresses this discrepancy. However, this finding, coupled with the yeast paradigm, leads one to speculate that similar effectors may also be required to activate MEK. If such factors exist, it will be necessary to re-evaluate c-Raf and c-Mos as MEKKs. In the case of I-MEKK, it has been our experience that inclusion of recombiant or purified native MEK alone in assays is insufficient to isolate the enzyme beyond the crudest of fractionation steps. In this case, it appears that other factors are necessary and that these must be identified before I-MEK can be isolated and studied.

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