# Regulation of Recombinant MEK1 and MEK2b Expressed in *Escherichia coli*†

Marijane Russell,* Carol A. Lange-Carter, and Gary L. Johnson

*Division of Basic Sciences, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206, and Department of Pharmacology, University of Colorado Medical School, Denver, Colorado 80262*

*Received December 19, 1994; Revised Manuscript Received March 13, 1995*⊗

ABSTRACT: Mitogen-activated protein kinase (MAPK) activation is an important signal involved in regulating cellular proliferation and/or differentiation. The immediate upstream kinase MAPK kinase, referred to as MEK, activates MAPK by phosphorylation on specific tyrosine and threonine residues. To date, two MEK's have been cloned and characterized, MEK1 and MEK2. Here we report the cloning of MEK2b from mouse pituitary cDNA. Rat and mouse MEK2 amino acid sequences vary by only three amino acids; the three changes are conserved in the MEK1 sequence. Analysis of recombinant MEK2b and MEK1 demonstrated similar activation by upstream kinases and phosphotransferase activity toward MAPK, while they differed in autophosphorylation and the ability to serve as a substrate for MAPK. The findings demonstrate significant differences in potential regulatory mechanisms of MEK1 and MEK2/2b but not in their activation by upstream regulators.

Signal transduction through both G protein coupled receptors and tyrosine kinase growth factor receptors has been shown to lead to activation of mitogen-activated protein kinase (MAPK). MAPK activation is an important signal in control of cellular proliferation and/or differentiation depending on the cell type (Blenis, 1993; Johnson et al., 1994). The immediate upstream activator of MAPK is the dual specificity threonine/tyrosine kinase MAP kinase kinase (MKK), also known as *M*AP kinase/*E*RK *k*inase (MEK) (Crews et al., 1992). To date, two MEK isoforms have been cloned, MEK1 and MEK2 (Wu et al., 1993; Zheng & Guan, 1993a). Although they differ significantly in primary sequence, both MEK1 and MEK2 can phosphorylate and activate p42 and p44 MAPKs (Zheng & Guan, 1993b). One of the upstream activators of MEK is the serine/threonine kinase Raf-1 (Kyriakis et al., 1992). v-Raf has been shown to phosphorylate both MEK1 and MEK2 (Wu et al., 1993). In this study, we have PCR-cloned a MEK2 isoform of MEK from mouse pituitary, designated MEK2b. Mouse MEK2b is nearly identical to rat MEK2 with three amino acid substitutions. Herein, we have characterized the ability of multiple upstream activators from the Raf and MEKK families to phosphorylate and activate MEK2b relative to MEK1 *in vitro*. Significant differences exist in the regulation of the two MEK proteins.

## MATERIALS AND METHODS

*PCR Cloning of MEK2b.* Specific oligonucleotides (sense: 5′-TCCGAGCTCGAGACTGGCCCGGAGGAAGC-CGGTGTT-3′; antisense: 5′-AGAGGCGAATTCTCATCA-CACTGCAGTACGCGTGG-3′) were designed based on the cDNA sequence of rat MEK2. A PCR product encoding MEK2 was generated using mouse pituitary cDNA as a template and subcloned into the *Xho*I and *Eco*RI sites of pRSETB for subsequent sequencing and protein expression. We refer to mouse MEK2 as MEK2b throughout the paper. Kinase-inactive MEK2b was generated by mutating Lys101 to Met using a specific oligonucleotide and the Promega Altered Sites Mutagenesis kit (Madison, WI).

*Recombinant Protein Preparations.* All of our contructs in this paper utilize the pRSET system which encodes an N-terminal hexahistidine tag. The constructs were expressed in bacteria, and soluble enzyme was purified on $Ni^{2+}$−NTA−agarose (Gardner et al., 1994a). Purified preparations of WTMEK1, WTMEK2b, KMMEK1, and KMMEK2b were all purified on the same day and concentrations normalized by Coomassie staining, so equal amounts of protein were used in all experiments. Approximate concentration was also estimated by Coomassie staining, using BSA as a standard curve. We have generally found in our laboratory that His-fusion proteins are very stable and consistently reliable kinase subtrates while GST-fusions are less reliable in our hands.

*Autophosphorylation Assay.* The ability of wild type MEK1 or -2b to autophosphorylate was determined by an *in vitro* kinase assay containing PAN (10 mM PIPES, pH 7.0, 100 mM NaCl, and 0.2 unit/mL aprotinin), 10× universal kinase buffer (10 mM $MnCl_2$, 0.9 unit/mL aprotinin, and 200 mM PIPES, pH 7.0), recombinant wild-type MEK1 or -2b (100 ng), and [$\gamma$-$^{32}$P]ATP (10 $\mu$Ci). The kinase assays were incubated at 30 °C and quenched at indicated times by the addition of Laemmli SDS buffer and boiled for 5 min. Proteins were separated on a 10% SDS−polyacrylamide gel, and MEK phosphorylation was visualized on an autoradiogram.

*Raf and MEKK Immunoprecipitation and in Vitro Kinase Assays.* Raf-1, B-Raf, and MEKK activity was measured using recombinant kinase-inactive MEK1 or -2b as substrate (Crews et al., 1993; Gardner et al., 1993; Kyriakis et al., 1992). Stimulated or control cells were lysed in 1% Triton X-100, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% BSA, 0.2 unit/mL aprotinin, 1 mM PMSF, and 2 mM $Na_3VO_4$ (EB). Nuclei were removed by centrifugation at maximum speed for 10 min in a microfuge

† This work was supported by NIH Grants DK37871, GM30324, and CA58157 to G.L.J., Grant GM15843 to C.A.L.-C., and Grant CA313-03 to M.R.

* To whom correspondence should be addressed at the National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St., Denver, CO 80206. Phone: (303) 398-1772. Fax: (303) 398-1225.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1995.

0006-2960/95/0434-6611$09.00/0 

and the supernatants incubated with anti-Raf-1 or anti-B-Raf antibody (Santa Cruz Biotechnology) or MEKK antibody (polyclonal rabbit sera raised against an amino-terminal fusion protein) for 1.5 h at 4 °C (Lange-Carter et al., 1994; Vaillancourt et al., 1994). Protein A Insoluble (Sigma) was added, and the mixture was then added to EB containing 10% sucrose and centrifuged for 20 min at 2500 rpm. The pellet was successively washed twice in RIPA−EDTA, twice in PAN + 0.5% NP-40, and finally twice in PAN. The final pellet was resuspended in PAN and assayed for Raf-1, B-Raf, or MEKK activity by the addition of 10× universal kinase buffer, recombinant kinase-inactive MEK1 or -2b (25−50 ng), and [$\gamma$-$^{32}$P]ATP (10 $\mu$Ci). After a 15 min incubation at 30 °C (20 min for MEKK assay), the kinase assays were quenched by the addition of Laemmli SDS buffer, boiled for 5 min, and centrifuged for 2 min in a microfuge. Proteins were separated on a 10% SDS−polyacrylamide gel, and MEK phosphorylation was visualized on an autoradiogram.

*MEK1/2b−MAPK Coupled Assay.* The ability of either Raf-1, B-Raf, or MEKK immunoprecipitated from stimulated or control cells to activate wild-type MEK1 or -2b as measured by phosphorylation of catalytically inactive MAPK was assayed using an *in vitro* kinase-coupled assay system (Gardner et al., 1994b; Lange-Catrter et al., 1993). Immunoprecipitates of each kinase (as described above) were resuspended in PAN, and incubated with 10× universal kinase buffer, recombinant wild-type MEK1 or MEK2b (100 ng), kinase-inactive MAPK (300 ng), and [$\gamma$-$^{32}$P]ATP (10 $\mu$Ci) in a final volume of 50 $\mu$L. After 15 min at 30 °C, the kinase assay was quenched by the addition of Laemmli SDS buffer, and boiled for 5 min. Proteins were separated on a 10% SDS−polyacrylamide gel, and MEK and MAPK phosphorylation was visualized by autoradiography.

*Phosphorylation of Wild-Type MEK1 and -2b by Activated MAPK.* Isolation of partially purified activated MAPK from EGF-stimulated (30 ng/mL, 5 min) wild-type PC12 cells was done as previously described (Gardner et al., 1994b). Recombinant wild-type MEK1 (1) or MEK2b (2b) (100 ng of each) and 10X universal kinase buffer were preincubated in the absence or presence of activated MAPK (20 $\mu$L of a Mono Q FPLC fraction) for 20 min. After preincubation, [$\gamma$-$^{32}$P]ATP was added, aliquots were removed at the indicated times, and the assays were quenched by the addition of Laemmli SDS buffer. Proteins were separated on a 10% SDS−polyacrylamide gel, and MEK phosphorylation was visualized by autoradiography.

## RESULTS

To examine the substrate specificity of kinases from both the Raf and MEKK families for additional isoforms of MEK, a PCR product encoding mouse MEK2 was generated using specific oligonucleotides based on the rat MEK2 sequence and mouse pituitary cDNA as a template. We refer to mouse MEK2 as MEK2b. Amino acid alignment of rat MEK1, rat MEK2, and mouse pituitary MEK2b is shown in Figure 1. MEK2b shares 99% amino acid identity with rat MEK2. It is interesting to note that the only three amino acids which vary in MEK2 and MEK2b are actually identical between MEK2b and MEK1; conservation of these residues may be important for MEK function and indicate that the MEK1 and MEK2/2b genes most likely evolved from a common

```
MEK2    MLARRKPVLPALTINPTIAEGPSPTSEGASEAHLVDLQKKLEELDLD  47
MEK2b   ..............................N.............  47
MEK1     MPKK..TPIQ.NPA.***D.SAVNGTSSA.TN.EA.......E..  43

MEK2    EQQRKRLEAFLTQKAKVGELKDDDFERISELGAGNGGVVTKARHRPS  94
MEK2b   ................................................  94
MEK1    .............Q..........K...........F.VS.K..     90

MEK2    GLIMARKLIHLEIKPAVRNQIIRELQVLHECNSPYIVGFYGAFYSDG 140
MEK2b   ................................................ 139
MEK1    ..V............I.............................     136

MEK2    EISICMEHMDGGSLDQVLKEAKRIPEDILGKVSIAVLRGLAYLREKH 188
MEK2b   ................................................ 187
MEK1    ................K.G....Q........IK..T......      184

MEK2    QIMHRDVKPSNILVNSRGEIKLCDFGVSGQLIDSMANSFVGTRSYMS 235
MEK2b   ................................................ 234
MEK1    K............................................... 231

MEK2    PERLQGTHYSVQSDIWSMGLSLVELAIGRYPIPPPDAKELEASFGRP 282
MEK2b   ................................................ 281
MEK1    ....................M.V.............LL..CQ        278

MEK2    VVDGADGEPHSVSPRPRPPGRPISGHGMDSRPAMAIFELLDYIVNEP 329
MEK2b   ................................................ 328
MEK1    .**EG.AA**ETP....T....L.SY......P.............  321

MEK2    PPKLPSGVFSSDFQEFVNKCLIKNPAERADLKLLTNHAFIKRSEGED 376
MEK2b   ..............................M...........E      375
MEK1    ..........LE..D..............Q.MV.......DA.E     368

MEK2    VDFAGWLCRTLRLKQPSTPTRTAV*                        400
MEK2b   ......................*                          400
MEK1    ........S.IG.N......HA.SI                        393
```

FIGURE 1: Amino acid sequence comparison of rat MEK1, rat MEK2 and mouse pituitary MEK2b. Dots indicate identical amino acids, and asterisks indicate gaps introduced for alignment.

ancestral gene. The three amino acid differences between rat MEK2 and mouse MEK2b were found in multiple PCR reactions sequenced and are changes due to the template used (mouse pituitary cDNA). MEK1 and MEK2/2b are approximately 80% identical. The primary sequences of MEK1 and MEK2/2b are most divergent at their extreme $NH_2$-termini (Figure 1). Additional differences lie within a region located in the COOH-terminus following the kinase domain and at the extreme COOH-terminus (Figure 1).

Figure 2 demonstrates that both MEK2b and MEK1 are functional MAPK kinases (MEKs) with similar activation properties but distinct autophosphorylation characteristics. Figure 2B shows that MEK2 and MEK1 are both activated by Raf-1. MAPK phosphorylation is a function of the recombinant MEK2b and MEK1 proteins because the kinase-inactive recombinant MEK2b and MEK1 proteins do not catalyze this reaction even though they are phosphorylated by Raf-1 (Figure 2C). A distinction between the recombinant MEK2b and MEK1 is apparent from these studies. MEK1 readily undergoes autophosphorylation whereas MEK2b does not (Figure 2A,B). Furthermore, the autophosphorylation of MEK1 leads to its activation as observed in Figure 2B, where it has MAPK phosphorylation activity in the absence of Raf-1. Incubation of MEK1 under the same reaction conditions in the absence of ATP does not result in MEK1 activation (Gardner et al., 1994b). The difference in autophosphorylation between MEK2b and MEK1 was found in numerous independent preparations of the recombinant

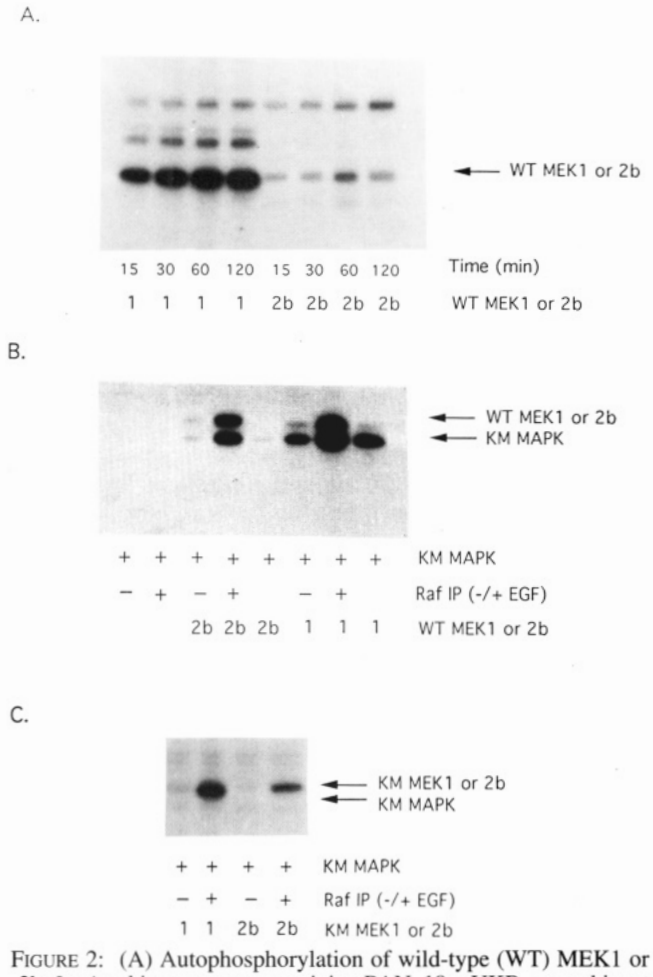


FIGURE 2: (A) Autophosphorylation of wild-type (WT) MEK1 or -2b. *In vitro* kinase assays containing PAN, 10× UKB, recombinant WT MEK1 or MEK2b (100 ng), and [$\gamma$-$^{32}$P]ATP (10 $\mu$Ci) were performed for the indicated times. The reactions were stopped with Laemmli SDS sample buffer and the proteins resolved by SDS−PAGE (10% acrylamide). Three independent experiments gave similar results. (B) Phosphorylation of MAPK by activated WT MEK1 or -2b. Raf-1 was immunoprecipitated from control cells (−) or cells stimulated with 30 ng/mL EGF for 3 min (+) as described under Materials and Methods. *In vitro* kinase assays containing the immunoprecipitated Raf-1, 10× UKB, recombinant kinase-inactive MAPK (300 ng), and [$\gamma$-$^{32}$P]ATP (10 $\mu$Ci) were incubated in the presence (+) or absence (−) of recombinant WT MEK1 or -2b (100 ng) for 15 min. The reactions were stopped with Laemmli SDS sample buffer and the proteins resolved by SDS−PAGE (10% acrylamide). Three independent experiments gave similar results. (C) Kinase-inactive MEK1 or -2b does not phosphorylate MAPK. *In vitro* kinase assays were as described in (B) except instead of WT MEK1 or -2b, kinase-inactive MEK1 or -2b proteins were used. Two independent experiments gave similar results.

proteins. This contrasts with similar Raf-1-stimulated MEK2b and MEK1 kinase activities toward MAPK (Figure 2B), indicating the differences in autophosphorylation are not related to the relative denaturation of one kinase versus the other. Both purified kinases are in native, active forms when expressed and purified in *Escherichia coli*.

MEK2b and MEK1 are both phosphorylated and activated by Raf-1 (Figure 2B and Figure 3). Raf-1 immunoprecipitated from cells stimulated by growth factor tyrosine kinase receptors (EGFR, PDGFR) and G protein-coupled receptors (m2R) (Winitz et al., 1993) phosphorylated both MEK1 and MEK2b. Assaying equal protein concentrations indicated that MEK-1 is a somewhat better substrate than MEK2b (Figure 3). This finding was reproducible in multiple

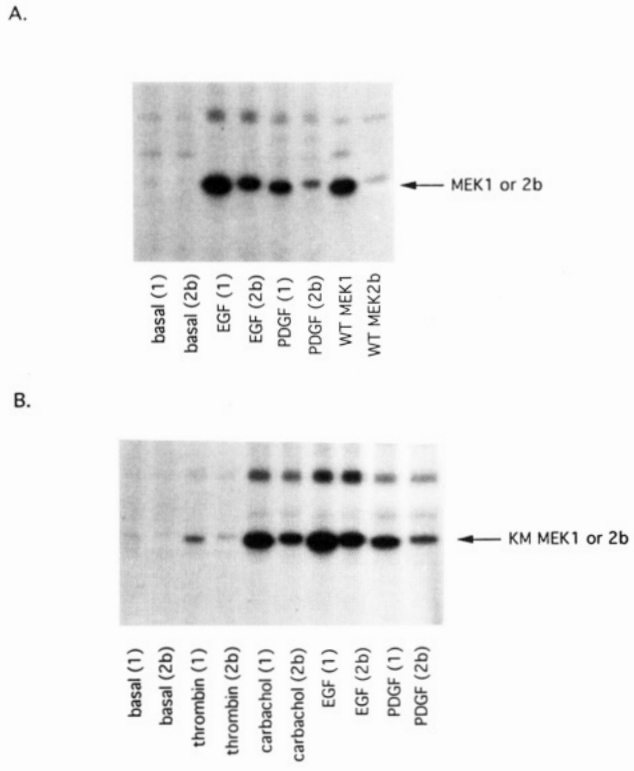


FIGURE 3: (A) Phosphorylation of MEK1 and MEK2b by Raf-1 in Swiss 3T3 cells. Cells were serum-starved overnight in media containing 0.1% bovine serum albumin and stimulated with buffer (basal), 30 ng/mL EGF, or 10 ng/mL PDGF for 3 min. Cell lysates were prepared, and Raf-1 was immunoprecipitated as described under Materials and Methods. Purified recombinant kinase-inactive KM MEK1 (1) or KM MEK2b (2b) was used as a Raf substrate in an *in vitro* kinase assay in the presence of [$\gamma$-$^{32}$P]ATP (10 $\mu$Ci). After incubation for 15 min the reaction was stopped with Laemmli SDS sample buffer, and the proteins were resolved by SDS−PAGE (10% acrylamide). Purified recombinant wild-type MEK1 or -2b was autophosphorylated for 15 min and used as a standard. Three independent experiments gave similar results. (B) Phosphorylation of MEK1 and MEK2b by Raf-1 in Rat 1a cells. A rat 1a cell clone expressing $1.4 \times 10^5$ m2R per cell was serum-starved overnight in media containing 0.1% bovine serum albumin (Winitz et al., 1993). Cells were stimulated with 0.1 unit/mL thrombin for 1 min, 100 $\mu$M carbachol for 3 min, 30 ng/mL EGF for 3 min, or 10 ng/mL PDGF for 3 min. Cell lysates were prepared, Raf-1 was immunoprecipitated, and *in vitro* kinase assays were performed as described above. Four independent experiments gave similar results.

preparations of recombinant MEK2b and MEK1 and independent Raf immunoprecipitations. Similar results were observed with Raf immunoprecipitations from both Swiss 3T3 and Rat 1a cells, indicating that this was a general property of Raf, MEK1, and MEK2b and not peculiar to immunoprecipitates from a specific cell type. Furthermore, the 1.5−3-fold greater Raf-1 phosphorylation of MEK1 relative to MEK2b was observed with both wild-type and kinase-inactive forms of MEK1 and MEK2b (Figures 2B and 3A). This result indicated that the active site K→M mutation did not influence the recognition of MEK1 or MEK2b by Raf-1, an important observation because the kinase-inactive MEK1 protein has been used as an inhibitory mutant postulated to compete with wild-type MEK1 for regulation by Raf (Seger et al., 1994). Our results indicate both kinase-active and -inactive MEK proteins are similarly recognized by Raf-1.

Additional upstream activators of MEK1 which have been identified are the serine/threonine protein kinase B-Raf and

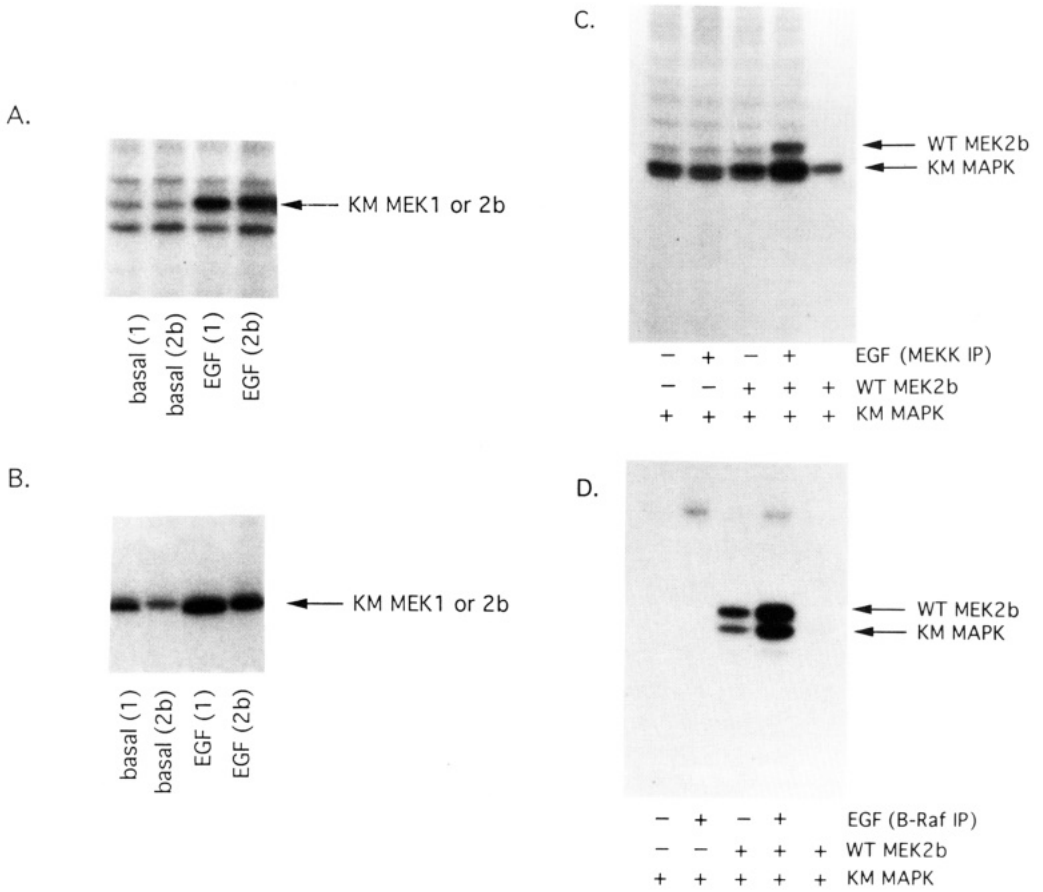


FIGURE 4: (A) Phosphorylation of MEK1 and MEK2b by MEKK. MEKK was immunoprecipitated from EGF-stimulated (30 ng/mL; 5 min) or unstimulated (basal) PC12 cells as described (Lange-Carter & Johnson, 1994) using an antisera raised against the $NH_2$-terminus of MEKK. MEKK activity in immunoprecipitates was assayed using either kinase-inactive (KM) MEK1 (100 ng) or MEK2b (100 ng) as a substrate for *in vitro* kinase assays as described under Materials and Methods. Three independent experiments gave similar results. (B) Phosphorylation of MEK1 and MEK2b by B-Raf. B-Raf was immunoprecipitated from the same PC12 cell lysates as in (A) using an antisera raised against the COOH-terminus of B-Raf (Santa Cruz) and placed in an *in vitro* kinase assay with either KM MEK1 (1) or KM MEK2b (2b) as described under Materials and Methods. Three independent experiments gave similar results. (C) Coupled assay showing activation of MEK2b by activated MEKK. MEKK immunoprecipitated from unstimulated (−) or EGF-stimulated (+) PC12 cells was incubated with wild-type (WT) MEK2b (50 ng) and catalytically inactive (KM) MAPK (150 ng) as described under Materials and Methods. MEKK from EGF-stimulated PC12 cells phosphorylated and activated MEK2b, leading to MAPK phosphorylation. A small amount of background MAPK phosphorylation occurred in the reaction buffer (no IP) due to the weak autophosphorylation and autoactivation of WT MEK2b. Two independent experiments gave similar results. (D) Coupled assay showing activation of MEK2b by activated B-Raf. B-Raf immunoprecipitated from unstimulated (−) or EGF-stimulated (+) PC12 cells was incubated with wild-type (WT) MEK2b (50 ng) and catalytically inactive (KM) MAPK (150 ng) as in (C). Although a modest basal level of B-Raf activity was detected by the assay, activated B-Raf phosphorylated and activated MEK2b, as measured by its ability to phosphorylate KM MAPK. Two independent experiments gave similar results.

the mouse homologue of the yeast byr2 and STE11 protein kinases, MEK kinase (MEKK) (Lange-Carter et al., 1993; Vaillancourt et al., 1994). We investigated the ability of B-Raf and MEKK isolated from PC12 cells to recognize MEK2b as a substrate (Lange-Carter et al., 1994; Vaillancourt et al., 1994). Similar to the results seen with Raf-1, MEKK or B-Raf immunoprecipitated from EGF-stimulated PC12 cells was each capable of phosphorylating both KM MEK1 and KM MEK2b (Figure 4A,B). MEKK and B-Raf phosphorylated and activated WT MEK2b as measured by its ability to phosphorylate kinase-inactive MAPK, indicating that both MEKK and B-Raf regulate MEK2b activity similar to Raf-1 (Figure 4C,D). It has been prevoiusly reported that both MEKK and B-Raf phosphorylate and activate WT MEK1 in a coupled assay with kinase-inactive MAPK (Lange-Carter et al., 1994; Vaillancourt et al., 1994).

On an equal protein basis, MAPK phosphorylates MEK1 to a significantly greater extent than MEK2b (Figure 5). MEK2b is, however, clearly phosphorylated by MAPK. This contrasts with the results of Wu et al., who claimed MEK2 was not a substrate for MAPK. Zheng and Guan (1993b) demonstrated that both MEK1 and MEK2 were phosphorylated by MAPK but could not determine the relative recognition of MEK1 versus MEK2 because of their experimental protocol. MEK1 is phosphorylated primarily at Thr292 and to lesser extent at Thr386 by MAPK (Gardner et al., 1994b). MEK2b has a proline at residue 300 which corresponds to Thr292 in MEK1 (Figure 1). Substitution of proline at residue 300 in MEK2b for threonine at residue 292 in MEK1 appears to account for the difference in phosphorylation of the two kinases by MAPK.

## DISCUSSION

Raf-1 and MEKK phosphorylate similar sites on MEK1 *in vitro*, and these sites are phosphorylated *in vivo* following growth factor stimulation of cells (Gardner et al., 1994b). Additionally, we have found that recombinant MEK2b and MEK1 have similar *in vitro* properties in relation to specific

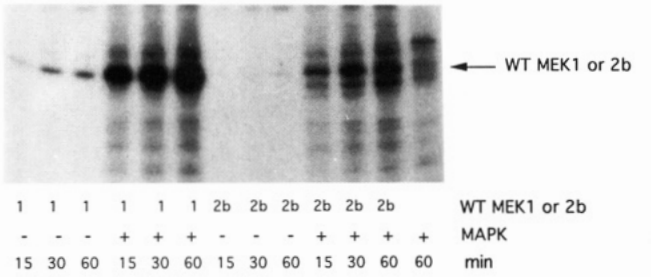


FIGURE 5: Phosphorylation of wild-type MEK1 and -2b by activated MAPK. Recombinant wild-type MEK1 (1) or MEK2b (2b) (100 ng of each) was preincubated in the absence (−) or presence (+) of activated MAPK partially purified by Mono Q FPLC for 20 min. After preincubation, [$\gamma$-$^{32}$P] ATP was added, aliquots were removed at the indicated times, and the assays were quenched by the addition of Laemmli SDS buffer. Samples were electrophoresed on a 10% SDS−polyacrylamide gel, and MEK phosphorylation was visualized by autoradiography. Three independent experiments gave similar results.

upstream kinases (i.e., Raf-1, B-Raf, MEKK) and their ability to phosphorylate MAPK. On a comparative basis of equal protein and MAPK phosphorylation, two significant differences were found in the *in vitro* kinase properties of recombinant MEK2b versus MEK1. MEK2b autophosphorylates to a much lesser extent than MEK1, and MEK2b does not have the major MAPK phosphorylation site found in MEK1. These findings demonstrate that MEK1 and MEK2b may not be similarly regulated in response to growth factors and hormones. The two MEKs may have differences in their ability to sustain activation in that MEK1 may undergo autoactivation more readily than does MEK2b. This observation may in part contribute to acute and prolonged regulation of MAPK activities (Heasley & Johnson, 1992). Similarly, MEK1 may have a form of feedback regulation by MAPK that is not observed with MEK2b. We and others have not yet discovered a regulatory role for MEK1 phosphorylation by MAPK which we have demonstrated occurs *in vivo* in response to EGF stimulation (Gardner et al., 1994b).

Differences in the properties of recombinant MEK1 and MEK2 proteins characterized by Wu et al., Zheng and Guan (1993b), and in the work presented here may be due to the MEK fusion proteins used in each study. Zheng and Guan (1993b) used GST-fusion proteins for all of their studies whereas we used histidine-tagged proteins for bacterial expression and purification. Both groups purified MEKs in the absence of other component proteins in the sequential phosphorylation pathways. In contrast, Wu et al. used COS cell expression for MEKs, which allows possible background phosphorylation events by both upstream and downstream components of the MAPK pathway. The ability to control for MEK alterations in COS cells is difficult if not impossible; in contrast, *E. coli* expressed and recombinant proteins do not encounter this caveat. The findings of Zheng and Guan (1993b) suggest that MEK2 is more active than MEK1, based primarily on autoactivation. We find that both are similarly active toward MAPK when activated by Raf or MEKK. Thus, the autoactivation of GST-MEK2 that we do not detect at any significant level with histidine-tagged MEK2b may be a function of the GST fusion protein versus the histidine-tagged protein. It should be noted, however, that autoactivation is not the primary regulatory mechanism for the control of MEKs. Rather, Raf or MEKK phosphorylation of MEK1 and MEK2b is the primary regulatory mechanism. We have shown for the first time that MEK1 and MEK2b are similarly activated by Raf-1, B-Raf, and MEKK *in vitro*. The role of the two MAPK activators must be overlapping in cellular regulation. MEK1 has been shown to preferentially interact with Ras·GMP-PNP/Raf-1 complexes relative to MEK2 (Jelinek et al., 1994), suggesting that MEK2/2b might preferentially interact with B-Raf or MEKK. If correct, this would suggest specific signal complexes for the different upstream regulators of the MAPK pathway. Such signal complexes would allow differential regulation in response to diverse stimuli. It will be important to understand which MEKs are unique to a specific kinase cascade and which may overlap and play roles in multiple cascades.

## REFERENCES

Blenis, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5889.
Crews, C. M., Alessandrini, A., & Erikson, R. L. (1992) *Science 258*, 478.
Gardner, A. M., Vaillancourt, R. R., & Johnson, G. L. (1993) *J. Biol. Chem. 268*, 17896.
Gardner, A. M., Lange-Carter, C. A., Vaillancourt, R. R., & Johnson, G. L. (1994a) *Methods Enzymol. 28*, 258.
Gardner, A. M., Vaillancourt, R. R., Lange-Carter, C. A., & Johnson, G. L. (1994b) *Mol. Biol. Cell. 5*, 193.
Heasley, L. E., & Johnson, G. L. (1992) *Mol. Biol. Cell. 3*, 545.
Jelinek, T., Catling, A. D., Reuter, C. W. M., Moodie, S. A., Wolfman, A., & Weber, M. J. (1994) *Mol. Cell. Biol. 14*, 8212.
Johnson, G. L., Gardner A. M., Lange-Carter, C., Qian, N.-X., Russell, M., & Winitz, S. (1994) *J. Cell. Biochem. 54*, 415.
Kyriakis, J. M., App, H., Zhang, X.-F., Banerjee, P., Brautigan, D. L., Rapp, U. R., & Avruch, J. (1992) *Nature 358*, 417.
Lange-Carter, C., & Johnson, G. L. (1994) *Science 265*, 1458.
Lange-Carter, C., Pleiman, C., Gardner, A. M., Blumer, K. L., & Johnson, G. L. (1993) *Science 260*, 315.
Seger, R., Seger, D., Reszka, A. A., Munar, E. S. , Eldar-Finkelman, H., Doborowolska, G., Jensen, A. M., Campbell, J. S., Fischer, E. H., & Krebs, E. G. (1994) *J. Biol. Chem. 269*, 25699.
Vaillancourt, R. R., Gardner, A. M., & Johnson, G. L. (1994) *Mol. Cell. Biol. 14*, 6522.
Winitz, S., Russell, M., Qian, N.-X., Dwyer, L., & Johnson, G. L. (1993) *J. Biol. Chem. 268*, 19196.
Wu, J., Harrison, J. K., Dent, P., Lynch, K. R., Weber, M. J., & Sturgill, T. W. (1993) *Mol. Cell. Biol. 13*, 4539.
Zheng, C.-F., & Guan, K.-L. (1993a) *J. Biol. Chem. 268*, 11435.
Zheng, C.-F., & Guan, K.-L. (1993b) *J. Biol. Chem. 268*, 23933.

BI9428991