Partial Purification of a Mitogen-activated Protein Kinase Kinase Activator from Bovine Brain

IDENTIFICATION AS B-RAF OR A B-RAF-ASSOCIATED ACTIVITY*

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A classical biochemical approach was taken to identify mitogen-activated protein kinase kinase (MEK) activators in bovine brain. Fractionation revealed the presence of one major MEK-stimulating activity that was identified as c-Raf-1. Additional results were obtained using bovine adrenal chromaffin cells, and in both cases, immunoblotting and immunoprecipitation experiments demonstrated co-purification of MEK activator with B-Raf. Partially purified MEK activator stimulated phosphorylation of MEK1 on residues tentatively identified as serine 218 and serine 222. Little or no MEK activator was associated with c-Raf-1 in bovine brain or chromaffin cells, although this protein was expressed, suggesting that B-Raf might be the major MEK activator in cells of neural origin.

The MAP kinase pathway is activated by diverse agonists that stimulate cell division, differentiation, and secretion (reviewed in Ref. 1). MAP kinase activation requires phosphorylation on both tyrosine and threonine residues (2, 3), reactions that are catalyzed by a novel family of dual-specificity kinases named MEKs (MAP or Erk kinases) (4–10). MEK activity is in turn regulated by reversible serine/threonine phosphorylation (11–14), implying the existence of MEK kinases that function as MEK activators. A number of candidate MEK-activating kinases have been reported, most notably c-Raf-1. A variety of biochemical (15–20), genetic (9, 15–17, 21–26), and regulatory (27, 28) evidence points to the importance of c-Raf-1 as a MEK activator. For example, partially purified preparations of c-Raf-1 can activate MEK in vitro (15–17, 20, 29); a ras homologue in C. elegans has been shown to function “upstream” of MAP kinase and “downstream” of tyrosine kinases (24), and overexpression of mutant inactive forms of c-Raf-1 have been shown to block MAP kinase activation, presumably by a dominant-negative mechanism (26). In addition, c-Mos (30) and MEK kinase (31) have been shown to be potential MEK activators. The latter is a mammalian homologue of the yeast Ste11 and Bys2 proteins (31), which function as activators of the yeast MEKs, Ste7 and Bys1, respectively.

The regulation of c-Raf-1 activity is clearly complex (for review, see Refs. 32–34). The c-Raf-1 protein becomes phosphorylated during activation by kinases whose identities are uncertain but may include protein kinase C isoforms (34–36). Phosphorylation is apparently necessary for activation of c-Raf-1 (37). c-Raf-1 becomes activated upon activation of p21ras, and c-Raf-1 has been shown to bind directly to p21ras (38–42). However, the Ras-Raf interaction appears insufficient to activate c-Raf-1 kinase activity, and the role of this interaction is likely to recruit c-Raf-1 to its membrane site of activation (43, 44). Disruption of the Ras-Raf interaction correlates with inhibition of the MAP kinase cascade in fibroblasts (45–47) and smooth muscle cells (48).

The studies described above have been carried out predominantly in cultured cells (especially fibroblasts) or in Xenopus oocytes (where Mos was identified as a MEK activator (30)). Moreover, the studies on in vitro activation of MEK suffer from the use of partially purified Raf and/or MEK utilized in high concentrations (where nonspecific reactions might occur). The genetic experiments establish an enzyme sequence, but do not establish proximal partners; and the effectiveness of dominant-negative Raf mutants in blocking MEK and MAP kinase activation may result from the sequestration of p21ras, thereby preventing access of other Ras-dependent effectors. With notable exceptions (49–51), there is little literature reporting classical purification and identification of endogenous MEK activators from cells or tissues and analysis of Raf isoforms or other MEK activators that might be tissue or agonist specific. Here we report partial purification of a MEK activator from bovine brain and the identification of this activity as B-Raf or a B-Raf-associated activity.

B-Raf is a serine/threonine kinase reportedly expressed primarily in brain and in the nervous system (52, 53). B-Raf is 54% homologous to c-Raf-1, with greatest homology in the kinase domain (conserved region 3 or CR3) and CR1 (54). Like c-Raf-1, B-Raf can be rendered oncogenic by mutational events that constitutively elevate Raf kinase activity, indicating a role for these proteins in regulation of cell growth and division (54). Previous reports have indicated an increase in B-Raf phosphorylation and autophosphorylating activity in response to NGF in PC12 phaeochromocytoma and SH-SYSY neuroblastoma cells (55, 56), although activity against a physiologically relevant substrate could not be examined. NGF treatment results in activation of MEK and MAP kinase (11, 57, 58) in PC12 cells, and both this stimulation, and subsequent neurite outgrowth are blocked by dominant negative mutants of Ras (59–61).

MATERIALS AND METHODS

Construction and Expression of Histidine-tagged MEK1—Rat kidney MEK1 (6) was tagged at the N terminus with a polyhistidine sequence,
allowing purification by Ni²⁺-chelate chromatography. Expression was achieved under the control of a cytomegalovirus promoter (62) in CCL39 fibroblasts. Stable transfectants (CCL39hMEKl) were obtained by

**Cell Culture**—CCL39hMEKl cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) each calf and fetal calf serum. Prior to purification of inactive histidine-tagged MEK1, CCL39hMEKl cells were incubated overnight in Dulbecco’s modified Eagle’s medium supplemented with 0.1% (v/v) fetal calf serum. Bovine adrenal chromaffin cells were prepared and maintained as described (63).

**Substrate Purification**—Recombinant wild-type and kinase-defective (K52R) p42κα were purified to apparent homogeneity as described previously (3, 64). Inactive histidine-tagged MEK1 was purified from serum-deprived CCL39hMEKl by sequential Ni²⁺-chelate and strong anion-exchange chromatography. Histidine-tagged MEK1 purified in this manner was homogenous by Coomassie and silver staining (not shown) and had a specific activity of approximately 15 units/mg using the standard assay (see below). This protein could be activated >500-fold by active fractions using the standard assay procedure.

**Assays**—Activation of histidine-tagged MEK1 was measured in a two-step assay using kinase-defective (K52R) p42κα as the final sub-

strate. 10 μl of each fraction (diluted as necessary) or appropriate im-

munoprecipitates were mixed with 30 μl of a reaction mix to give (final concentrations) 25 mM Hepes-NaOH, pH 7.5, 10 mM Mg(CH₂COO)₂, 1 mM MgCl₂, 0.1 mM ATP, 1 μM okadaic acid, 5 μg/ml leupeptin plus 100 ng inactive histidine-tagged MEK1. Reactions were incubated at 30 °C for 20 min, at which time 10 μl of 25 mM Hepes-NaOH, pH 7.5, containing 2 μg of K52R p42κα and [γ³²P]ATP (final specific activity of 1000–2000 cpm/mmol) was added. Incubations were continued for 20 min, and terminated by the addition of 15 μl of 4 × SDS-PAGE sample buffer. Reaction products were resolved by SDS-PAGE and transferred to nitrocellulose, and the radioactivity incorporated into K52R p42κα was quantitated by Cerenkov counting. One unit is that amount of MEK1 activator that raises the K52R p42κα-phosphorylation activity of 100 ng of histidine-tagged MEK1 by 1 pmol/min in the standard assay. At each purification step, the pooled activator was assayed with or without exogenous recombinant MEK1, and these data were normalized to a single column or buffer blank. The activator that raises the K52R p42κα phospho-

rylating activity was absolutely dependent upon the presence of exogenous MEK1 in the assays. Control experiments demonstrated that histidine-tagged MEK1 activated by brain activator was able to stimulate the kinase activity of wild-type recombinant p42κα, i.e. the ob-

served phosphorylation of K52R p42κα was on the regulatory threo-

nine and tyrosine residues.

Protein concentration in column fractions was estimated by Coomassie Blue binding (Bio-Rad). The concentration of purified pro-

tein was estimated after gel electrophoresis by Coomassie Blue stain-

ing in parallel with bovine serum albumin standards.

**Peptides**—Brain, MEK activator activity—The following opera-

tions were performed at 4 °C. Bovine brain (300 g) was diced and 27-29 containing the highest MEK1 activator activity were pooled, dialyzed against buffer A supplemented with 50% (v/v) glycerol but without phenylmethylsulfonyl fluoride, and stored at –20 °C.

**Chromatography of Chromaffin Cell Extracts**—Chromaffin cell fractions were left untreated or stimulated with nicotine (10 μM) and assayed with or without exogenous MEK1, and this demonstrated that the K52R p42κα phosphorylating activity was absolutely dependent upon the presence of exogenous MEK1 in the assays. Control experiments demonstrated that histidine-tagged MEK1 activated by brain activator was able to stimulate the kinase activity of wild-type recombinant p42κα, i.e. the ob-

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Fig. 1. Partial purification of MEK1 activator from bovine brain. Extract was sequentially chromatographed as described under "Materials and Methods." The dotted line indicates protein concentration (right axis). d, assays of pooled activator with (+) or without (-) histidine-tagged MEK1 confirmed that K52R p42\textsuperscript{52}, phosphorylation was absolutely dependent on the presence of exogenous MEK1. Assays were performed with (+) or without (-) activator from S-Sepharose FF peak fractions. K52R indicates kinase-defective p42\textsuperscript{52} phosphate substrate.

Table I

| Partial purification of brain MEK1 activator |
|-----------------------------|
| Vol. | Protein | Activator | Units/mg | Yield | -Fold |
| % |
| Extract | 800 | 8856 | 896 | 0.1 | 100 | 1.0 |
| DEAE-Sephacel | 520 | 782 | 1149 | 1.5 | 128 | 14.7 |
| S-Sepharose FF | 47 | 48.7 | 3601 | 82.4 | 402 | 824 |
| DEAE-Blue F3GA | 32 | 13.3 | 975 | 73.3 | 109 | 733 |
| APIQ | 1.3 | 0.75 | 45.8 | 60.7 | 6.8 | 607 |

* See "Materials and Methods" for unit definition.

Histidine-tagged MEK1 purified to apparent homogeneity from serum-deprived CCL39 MEK1 fibroblasts. MEK1 activation was measured by phosphorylation of homogeneous kinase-defective (K52R) p42\textsuperscript{52}, assays employing homogeneous recombinant wild-type p42\textsuperscript{52} as substrate confirmed that this phosphorylation resulted in stimulation of p42\textsuperscript{52} MBP kinase activity (data not shown).

Bovine Brain Extract Supports Activation of Both MEK1 and MEK2—A MEK1-activating activity could be enriched by successive ion-exchange and dye affinity chromatographies (Fig. 1), with a striking increase in apparent yield over the first two steps (Table I), suggesting the presence of inhibitors in the initial extract. Enhanced phosphorylation of K52R by column fractions was absolutely dependent on the presence of exogenous MEK1 in the assays (Fig. 1d and data not shown), and this activation could be abolished by prior incubation of the column fraction at 95°C for 10 min (data not shown). Recombinant MEK2 could be similarly activated by the same column fractions (data not shown), indicating that the MEK activator shows no marked preference for MEK1 or MEK2 in vitro. The minor MEK1 activating activity in fractions 84–100 after DEAE-Sephacel chromatography was not analyzed further.

Superose 12 gel filtration of MEK stimulating activity from either the S-Sepharose FF peak or the APIQ peak indicates that the activator has an apparent molecular mass of >400 kDa (data not shown).

B-Raf, but Not c-Raf-1 or MEK Kinase, Correlates with MEK-stimulating Activity—Since a number of kinases capable of activating MEK1 have been described in the literature (15–17, 30, 31, 49, 51), we sought to correlate our activity with candidate enzymes by immunoblotting for these known activators. Aliquots of starting material and column fractions were resolved by SDS-PAGE and immunoblotted using antisera specific for c-Raf-1 and MEK kinase. c-Raf-1 could be detected in the hypotonic extract as a partially resolved doublet of approximately 72 and 74 kDa (Fig. 2a, lane 1). Although the majority of c-Raf-1 did not bind to DEAE-Sephacel, a portion was resolved in fractions 64–104 (Fig. 2a) after the peak of MEK activating activity (fractions 60–80, see Fig. 1a). Similarly, MEK kinase (flow-through and fractions 36–64) eluted prior to the majority of MEK activator (Fig. 2b). Thus, weak anion-exchange chromatography partially resolves the brain activator from two previously described MEK1 activators, c-Raf-1 and MEK kinase. Given the tissue-specific distribution of the Raf-family kinases (52, 53), we hypothesized that B-Raf might account for the observed MEK1 activator activity. Indeed, immunoblotting with anti-B-Raf antiserum revealed a 90-kDa product in the hypotonic extract (Fig. 2c, lane 1) and enrichment of this species in fractions 64–72 from the DEAE-Sephacel column, correlating directly with MEK1-stimulating activity. Furthermore, subsequent chromatography on S-Sepharose FF resulted in colution of MEK1 activating activity with a form of B-Raf (Fig. 2d). A small amount of c-Raf-1 was also observed in these fractions (Fig. 2e), although a comparison of lanes 1, 2, and 9 between panels d and e clearly indicates enrichment of B-Raf but not c-Raf-1 with MEK1-activating activity. Note that little or no MEK1-activating activity co-eluted with B-Raf in fractions 28–31 of the S-Sepharose FF column (Fig. 2d).

MEK1 activator was fractionated further by dye affinity (data not shown) and strong anion-exchange chromatography, resulting in partial resolution of the MEK1 activator into two peaks (Fig. 1c). Both peaks contain material reactive with B-
Fig. 2. MEK1 activator resolves from MEK kinase and c-Raf-1 but correlates with B-Raf. Clarified brain extract, DEAE-Sephacel flow-through and DEAE-Sephacel fractions (Fig. 1a) were immunoblotted with antisera specific for c-Raf-1 (a), MEK kinase (b), and B-Raf (c). Immunoprecipitation of MEK1 activator and B-Raf from Pooled Column Fractions—Since the activator was not homogeneous after the chromatographic steps, it was not possible to correlate activity with a stainable protein in the APIQ peaks. Hence, we devised experiments to test the possibility that B-Raf, or a B-Raf-associated activity accounts for the MEK1 activator in these fractions. Pooled material from APIQ fractions 27–29 (see "Materials and Methods") was diluted and immunoprecipitated with either control nonimmune antiserum, anti-A-Raf, anti-B-Raf, or anti-c-Raf-1 antiserum. MEK1 activator was quantitatively removed from the pooled material by B-Raf antiserum (Fig. 2f) with the later eluting minor B-Raf peak containing the greatest MEK1-activating activity. c-Raf-1 was not detectable by immunoblotting of these fractions (data not shown).

These data indicate that B-Raf can be resolved into at least three chromatographically distinct forms, and that MEK1-activating activity correlates with two of these forms.

Immunoprecipitation of MEK1 Activator and B-Raf from Pooled Column Fractions—Since the activator was not homogeneous after the chromatographic steps, it was not possible to correlate activity with a stainable protein in the APIQ peaks. Hence, we devised experiments to test the possibility that B-Raf, or a B-Raf-associated activity accounts for the MEK1 activator in these fractions. Pooled material from APIQ fractions 27–29 (see "Materials and Methods") was diluted and immunoprecipitated with either control nonimmune antiserum, anti-A-Raf, anti-B-Raf, or anti-c-Raf-1 antiserum. MEK1 activator was quantitatively removed from the pooled material by B-Raf antiserum (Fig. 3, lane 8) but not by control antiserum (lane 4), A-Raf antiserum (lane 6), or c-Raf-1 antiserum (lane 10). As expected, MEK1 activator was markedly enriched in the B-Raf immunoprecipitate (lane 16); little activity was found in nonimmune, A-Raf, or c-Raf-1 immunoprecipitates (lanes 12, 14, and 18, respectively). (The relatively high level of activator activity associated with the nonimmune immunoprecipitate was not seen in other experiments utilizing this serum or in experiments employing two other nonimmune sera.) Immunoblotting confirmed that B-Raf protein was quantitatively and specifically depleted from the pooled activator with B-Raf antiserum (data not shown).

MEK1 activator therefore correlates tightly with the presence of B-Raf protein, suggesting that the major MEK1 activator in these bovine brain extracts is B-Raf, or B-Raf-associated.

Fig. 3. B-Raf antiserum immunoprecipitates brain MEK1 activator. Pooled activator or immunoprecipitates thereof were assayed with (+) or without (−) histidine-tagged MEK1 as described under "Materials and Methods." Pool indicates assays containing diluted pool material prior to immunoprecipitation. Blank denotes assay where dilution buffer replaced pool material. Diluted activator was immunoprecipitated with control nonimmune antiserum, A-Raf antiserum, B-Raf antiserum, or c-Raf-1 antiserum to yield supernatants and immunoprecipitates. Supernatants and washed immunoprecipitates were assayed for their ability to activate histidine-tagged MEK1. K52R indicates the position of kinase-defective p42\textsuperscript{mox} substrate. Molecular mass standards (in kDa) are indicated left.
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**Fig. 4.** MEK1 activator co-purifies with B-Raf in chromaffin cell extracts. **a,** bovine adrenal chromaffin cells were left untreated (□) or stimulated with the secretagogue nicotine (■) before fractionation by Mono Q chromatography. Fractions were assayed for their ability to activate histidine-tagged MEK1; values from control assays lacking exogenous MEK1 have been subtracted. The dotted line indicates salt gradient concentration (right axis). Mono Q fractions were immunoblotted for B-Raf (b) or c-Raf-1 (c). Horizontal bars denote MEK1 activator activity. The positions of B-Raf and c-Raf-1 are indicated right, and molecular mass standards (in kDa) are indicated left.

**Fig. 5.** B-raf antiserum immunoprecipitates chromaffin cell MEK1 activator. **a,** Mono Q fractions 14-16 (unstimulated extract, see Fig. 4) were pooled and immunoprecipitated with control nonimmune antiserum, A-Raf antiserum, B-Raf antiserum, or c-Raf-1 antiserum. Washed immunoprecipitates were assayed with (+) or without (−) histidine-tagged MEK1 substrate as described under “Materials and Methods.” K52R indicates the position of kinase-defective p42 Raf substrate. **b,** pool material and supernatants from immunoprecipitates in **a** were immunoblotted for B-Raf and c-Raf-1. The positions of B-Raf and c-Raf-1 are indicated right, and molecular mass standards (in kDa) are indicated left.

B-Raf immunoprecipitation as compared with 29 and 24% depletion following c-Raf-1 and nonimmune immunoprecipitation, respectively (data not shown). Immunoblotting of the same supernatants indicated ~75% depletion of B-Raf and ~100% depletion of c-Raf-1 following immunoprecipitation with cognate antisera (Fig. 5b). Interestingly, the recovery of MEK1 activator in the B-Raf immunoprecipitate was ~2.5-fold greater than expected, reminiscent of the apparent increase in yield of MEK1 activator following chromatography of the brain extract (see Table I).

MEK1 activator in chromaffin cells therefore correlates tightly with the presence of B-Raf protein. These results support the data obtained using bovine brain extracts, and indicate that our failure to measure other MEK1 activator activities in brain is probably not due to the pathophysiological status of the bovine brains used in these studies. However, in experiments to date, we have not demonstrated a regulation of B-Raf activity in response to stimulation under conditions where endogenous MEK1 activity is regulated. Possible reasons for these results are presented under “Discussion.”

**Brain MEK1 Activator Stimulates Phosphorylation of MEK1**—Since MEK1 is known to be regulated by reversible serine/threonine phosphorylation, we undertook experiments to address the role of phosphorylation in the activation of MEK1 by both partially purified brain activator and B-Raf immunopurified from this pooled material. Purified histidine-tagged MEK1 was phosphorylated by appropriate fractions from the APIQ column and resolved by SDS-PAGE. Fig. 6 dem-
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FIG. 6. Brain MEK1 activator stimulates phosphorylation of MEK1. AP1Q fractions were incubated with (+) or without (−) histidine-tagged MEK1 substrate in the presence of labeled ATP. Reaction products were resolved by SDS-PAGE and detected by autoradiography. Maximal MEK1 activator activity was found in fractions 24–28, see staining. The lower arrow indicates the position of MEK1 after reaction with buffer alone, and the upper arrow denotes the retarded form generated after reaction with fractions containing MEK1 activator. Molecular mass standards (in kDa) are indicated left.

FIG. 7. In vitro activation of MEK1 correlates with serine/threonine phosphorylation. Histidine-tagged MEK1 was phosphorylated with pooled MEK1 activator and subjected to phosphoamino acid analysis (panel a) and two-dimensional tryptic phosphopeptide mapping (panel b). a–c are phosphopeptides referred to in the text. Panel c, phosphopeptides a–c were recovered and subjected to phosphoamino acid analysis. o marks the origin, and PS, PT, and PY indicate the positions of ninhydrin-stained phosphoamino acid standards.

FIG. 8. Phosphorylation of 2 serine residues correlates with MEK1 activation in vitro. Phosphopeptide a (1683 cpm) was subjected to repetitive Edman degradation as described under "Materials and Methods," and the radioactivity recovered at each cycle is indicated left.

DISCUSSION

To date, there have been few attempts to define the contribution of various MEK activators in different tissues or in response to various agonists. Most published literature examines the activity of candidate MEK activators without addressing the quantitative role of these enzymes in vivo. We have therefore taken a biochemical approach to identify MEK activators in bovine brain, and our data demonstrate that a MEK1-activating activity co-purifies tightly with a form of B-Raf from this tissue. Immunoprecipitation analysis strongly suggests that this activity is intrinsic to the B-Raf polypeptide, although we cannot exclude the possibility that a co-precipitating activity is responsible in part or fully for the observed activation of...
MEK1. Indeed, B-Raf is resolved into at least three chromatographically distinct forms during partial purification of the brain activator, and these are differentially active against MEK1. Furthermore, gel filtration indicates an apparent molecular mass of >400 kDa for the brain MEK1 activator, rather larger than that expected for monomeric B-Raf (∼90 kDa). Similarly large sizes have been reported for a MEK activator from Xenopus (41, 51) and for c-Raf-1 (72). Conclusive identification of the MEK1 activator can be determined only by purification and sequence analysis of the enzyme responsible or perhaps by expression and purification of B-Raf from a heterologous source.

Since the MAP kinase pathway is highly inducible in response to a number of ligands, we hypothesized that a physiological activator of MEK1 would display transient activity in response to cell stimulation. Hence, we chose to examine bovine adrenal chromaffin cells where the MAP kinase pathway is rapidly induced in response to the secretagogue nicotine (63). Consistent with the identification of B-Raf as the major MEK1 activator in bovine brain, chromaffin cells exhibit a MEK1 activator that co-purifies with B-Raf and that can be immunoprecipitated with an antiserum specific for B-Raf, suggesting that B-Raf is also the major detectable MEK1 activator in this cell type. However, we have been unable to demonstrate a regulation of MEK1 activator/B-Raf activity in response to nicotine under conditions where endogenous MEK1 activity was stimulated. Potential explanations for this result include regulation by relocation within the cell, as has been demonstrated for the c-Raf-1 product (43, 44, 72), facilitating interaction of effector activator that co-purifies with B-Raf and that can be immunoprecipitated with an antiserum specific for B-Raf, suggesting that B-Raf is also the major detectable MEK1 activator in this cell type. However, we have been unable to demonstrate a regulation of MEK1 activator/B-Raf activity in response to nicotine under conditions where endogenous MEK1 activity was stimulated. Potential explanations for this result include regulation by relocation within the cell, as has been demonstrated for the c-Raf-1 product (43, 44, 72), facilitating interaction of effector activator that co-purifies with B-Raf and that can be immunoprecipitated with an antiserum specific for B-Raf, suggesting that B-Raf is also the major detectable MEK1 activator in this cell type.

Our data are suggestive of the latter possibility since the initial purification steps of MEK1 activator from bovine brain gives a 4-fold increase in activity. Furthermore, immunoprecipitation of B-Raf from fractionated chromaffin cell extract yields 2.5-fold more MEK1 activator activity than predicted, indicating the existence of an inhibitor in the active fractions. This enhancement of activity is unlikely to result from binding of the antibody since soluble anti-B-Raf antibody does not alter the activity of partially purified MEK1 activator from bovine brain (data not shown). Cohen and colleagues have described a latent MEK1 activator in PC12 cells that becomes active following prolonged storage, regardless of prior cell stimulation (73). The authors suggest that decay of a co-purifying inhibitor may be responsible for this unusual behavior (73).

The possibility that regulation of B-Raf/MEK1 activator activity results from relocation of the enzyme following cell stimulation awaits clarification, although no evidence in support of this hypothesis was found by Stephens et al. (55). Furthermore, we have been unable to detect translocation of B-Raf polypeptide or MEK1 activator activity to the particulate fraction of bovine adrenal chromaffin cells following stimulation with nicotine (data not shown). However, inhibitors of B-Raf or MEK1 in this fraction might render enzymatic assays inconclusive. Extraction of chromaffin cells with nonionic detergent also fails to reveal a regulation of B-Raf activity following nicotine stimulation, as measured by immune complex phosphorylation of Escherichia coli-produced glutathione S-transferase-MEK1 fusion protein or activation of either fibroblast MEK1 or glutathione S-transferase-MEK1. Our data confirm that activation of MEK1 correlates with phosphorylation of the enzyme on serine and threonine residues. Activation of MEK1 in vitro with partially purified brain MEK activator, and in vivo in NGF-stimulated PC12 cells (20) correlate with the appearance of a serine-phosphorylated peptide. A peptide with similar properties has been described by Kyriakis et al. (27). We have not conclusively identified the sites utilized by the partially purified brain MEK1 activator/B-Raf, although the sites on MEK1 phosphorylated by c-Raf-1 in vitro have been identified genetically (74) and biochemically (20). Alessi et al. (20) isolated a nonconventional tryptic phosphopeptide from c-Raf-1-phosphorylated MEK1 and demonstrated, by amino acid sequencing, phosphorylation of serine residues corresponding to 218 and 222 of rat MEK. Our data are consistent with these results since cleavage at the corresponding position in rat kidney MEK1 would be expected to yield a peptide with phosphorylation sites on serine residues at positions 4 and 8; phosphoamino acid analysis and repetitive Edman degradation indicate that this is the case. Hence, we suggest that B-Raf activates MEK1 through phosphorylation of serine residues 218 and 222.

Serine 218 and 222 are phosphorylated in MEK1 isolated from NGF-stimulated PC12 cells (20). The enzyme(s) responsible for this in vitro phosphorylation and activation is unknown, although previous studies in cells of neural origin (55, 56) and our data indicate that B-Raf is a good candidate. Significantly, expression of an activated c-Raf-1 allele in PC12 cells has little effect on the MAP kinase pathway (60), suggesting that a distinct enzyme is the physiological regulator of MEK in these cells. Relatively little is known regarding the regulation of B-Raf activity. Since NGF-stimulation of MAP kinase in PC12 cells is dependent upon Ras, as determined by studies with dominant negative Ras mutants, one might speculate that B-Raf binds to, and becomes activated upon Ras-GTP. This model could in part explain the differential effects of cyclic AMP elevation in fibroblasts and smooth muscle cells (where MEK activity is inhibited, Refs. 45-48) and PC12 cells (where MEK activity is elevated, Ref. 75) since B-Raf lacks serine 43 (53), a proposed site corresponding position in rat kidney MEK1 would be expected to, and becomes activated upon Ras-GTP. This model could in part explain the differential effects of cyclic AMP elevation in fibroblasts and smooth muscle cells (where MEK activity is inhibited, Refs. 45-48) and PC12 cells (where MEK activity is elevated, Ref. 75) since B-Raf lacks serine 43 (53), a proposed site of protein kinase A-mediated inhibition of c-Raf-1 (46). Note however that the Ras-Raf interaction might be regulated independently of protein kinase A-mediated c-Raf-1 phosphorylation (76).

Although we were able to detect both B-Raf and c-Raf-1 by immunoblotting, MEK1-stimulating activity was associated quantitatively with B-Raf following chromatography of bovine brain homogenate or chromaffin cell extracts. Although we have been unable to quantitate the absolute levels of B-Raf and c-Raf-1, this suggests that c-Raf-1 is not a significant MEK1 activator in brain or chromaffin cells. If this contention is correct, the differential regulation of these Raf isoforms becomes an interesting question. A-Raf and B-Raf are highly homologous to c-Raf-1 in the kinase domain (CR3) and CR1 (53), the conserved region that encompasses a putative zinc finger domain important for association with GTP-Ras (39-41). However, a third (short) region of sequence homology (94%) between A-Raf and c-Raf-1 is only 47% conserved in B-Raf (53), and sequences surrounding CR2 are largely isoform specific. The function of this region is uncertain, but linker insertion mutagenesis of CR2 in c-Raf-1 yields a transforming protein (77). One might speculate that CR2 and surrounding B-Raf-specific sequences could in part explain differential regulation of the Raf isoforms.

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