Selective Activation of MEK1 but Not MEK2 by A-Raf from Epidermal Growth Factor-stimulated Hela Cells*

(Received for publication, August 28, 1995, and in revised form, October 31, 1995)

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Activation of the mitogen-activated protein kinase cascade is a critical event in mitogenic growth factor transduction. Mitogen-activated protein kinase is directly activated by a dual specific kinase, MEK, which itself is activated by serine phosphorylation. The c-Raf kinase has been implicated in mediating the signal transduction from mitogenic growth factor receptors to MEK activation. Recently, the B-Raf kinase was shown to be capable of phosphorylating and activating MEK as a result of growth factor stimulation. In this report, we used the yeast two-hybrid screening to isolate MEK interacting proteins. All three members of the Raf family kinases were identified as positive clones when the mutant MEK1S218/222A, in which the two phosphorylation serine residues were substituted by alanines, was used as a bait, whereas no positive clones were isolated when the wild type MEK1 was used as a bait in a similar screening. These results suggest that elimination of the phosphorylation sites of a target protein (MEK1 in our study) may stabilize the interaction between the kinase (Raf) and its substrate (MEK1), possibly due the formation of a nonproductive complex. These observations seem to suggest a general strategy using mutants to identify the upstream kinase of a phosphorylating or the downstream targets of a kinase. Although c-Raf and B-Raf have been implicated in growth factor-induced MEK activation, little is known about A-Raf. We observed that stimulation of Hela cells with epidermal growth factor resulted in a rapid and transient activation of A-Raf, which is then capable of phosphorylating and activating MEK1. Interestingly, A-Raf does not activate MEK2, although c-Raf can activate both MEK1 and MEK2. Our data demonstrated that A-Raf is, indeed, a MEK1 activator and may play a role in growth factor signaling.

Binding of growth factors to their respective receptors results in the activation of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase. Activation of the receptor tyrosine kinase triggers an increase of the intrinsic tyrosine kinase.
Activation of MEK1 by A-Raf

Materials and Methods

Cell Culture—Hela cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Cells were cultured to 80–90% confluence and then stimulated with 100 nM PMA (Sigma), 100 ng/ml EGF (Sigma), 10 ng/ml tumor necrosis factor (Calbiochem), or 0.5 μM ionomycin (Calbiochem). For serum stimulation, Hela cells were cultured to 50% confluence and starved in minimal essential medium with 0.1% fetal bovine serum for 24 h. The serum-starved cells were stimulated with 10% fetal bovine serum in minimal essential medium.

Immunoprecipitation—Cells were washed twice with ice-cold phosphate-buffered saline before harvest. The washed cells were scraped into NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40, 0.5 mM Na3VO4, 50 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM chymostatin, 1 μM trypsin inhibitor, 5 μM aprotinin), sonicated for 10 s, and centrifuged at 15,000 × g for 10 min at 4 °C. The cell lysates were incubated with 1 μg of anti-Raf antibody for 2 h, and then protein A agarose (Pierce) was added for an additional hour with shaking. The immunoprecipitates were washed three times with NETN buffer and once with 20 mM HEPES, pH 7.5, 0.05% 2-mercaptoethanol, 0.2 mM EDTA. The anti-A-Raf, B-Raf, and c-Raf antibodies (Santa Cruz Biotechnology) were raised against the synthetic peptides corresponding to the C-terminal 20 amino acid residues of each protein. For peptide competition, 2 μg of a synthetic antigenic peptide was incubated with 1 μg of affinity purified antibody for 10 min before immunoprecipitation. Western blotting was performed with anti-Raf antibodies (1:1000 dilution).

Kinase Assay—Recombinant human extracellular signal-regulated kinase 1 was expressed as GST fusion (42). MEK1, MEK2, and corresponding mutants were also expressed as GST fusions and purified as described previously (43). Immunoprecipitated Raf was used to activate 0.08 μg of GST-MEK1 in 20 μl of kinase buffer (18 mM HEPES, pH 7.4, 10 mM magnesium acetate, 50 μM ATP). The reactions were incubated at 30 °C for 30 min with gentle shaking. The samples were briefly spun in a microfuge, and 10 μl of the activated GST-MEK1 was used in a kinase assay (43). When GST-MEK2 was used, 0.02 μg of recombinant GST-MEK2 was used for in vitro activation by immunoprecipitated Raf.

To assay for the phosphorylation of MEK by A-Raf, the kinase-deficient GST-MEK1 was used. The MEK1 mutant has the catalytic essential lysine residue 97 substituted by an arginine residue and thus cannot activate 1 μg of GST-MEK1 was phosphorylated by the immunoprecipitated Raf (30) and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Yeast Two-hybrid Screening—The coding sequences of wild type MEK1 and MEK1S218/222A were subcloned into the BamHI site of pAS-CYH vector (44) to produce pAS-MEK1 and pAS-MEK1S218/222A. These vectors express the fusion of the yeast DNA-binding domain with the entire MEK protein. The pAS-MEK1 and pAS-MEK1S218/222A were introduced into the yeast strain Y190 (MATa leu2-3, 112 ura3-52 trp1-901 his3-A200 ade2-101 gal4 gal80 lys2::Gal-HIS3 Gal-laZ cyh) by the lithium acetate method (45). The transformants were selected on yeast synthetic complete medium lacking the amino acid and tryptophan (SC-Trp). A two-hybrid cDNA library prepared from Hela cells (Clonetech) was then introduced into the yeast Y190 cells containing the bait vector to screen for MEK1 interactive proteins. Transformants were selected on yeast dropout medium lacking histidine, leucine, and tryptophan and containing 50 mM 3-amino-1,2,4 triazole (SC-His, Leu, Trp+ 3-AT) for 5–7 days. His+ colonies, which denote yeast cells that contain both the bait and interacting targets, were purified and tested for lacZ expression by β-galactosidase staining. In order to isolate the library plasmid, the positive yeast colonies were streaked on SC-Leu plates containing cycloheximide (2.5 μg/ml), which selects for cells containing only the library plasmids and not the bait plasmid (pAS-MEK1S218/222A). Total chromosomal DNA was prepared from positive colonies and electroporated into DH5α Escherichia coli cells to recover the library plasmid. Positive clones were further analyzed by DNA sequencing.

Results

Substitution of the Phosphorylation Sites in MEK1 Facilitates Its Interaction with A-Raf in the Yeast Two-hybrid System—Several MEK activators have previously been identified, including c-Raf, B-Raf, c-mos, and MEK kinase 1 (27–29, 33, 34, 38–40). Weak interaction between c-Raf and MEK1 has been observed (46). To identify MEK1 interacting proteins, we performed a screening using MEK1 as a bait by the yeast two-hybrid system. When the wild type MEK1 was initially used as a bait in the screening for interacting proteins, no positive clone was isolated among several libraries screened (Table I). Activation of MEK1 requires the phosphorylation of serine residues 218 and 222. Mutation of these two serine residues produced a dominant negative mutant of MEK1 that can block the MAP kinase signal transduction pathway in cultured cells (47) as well as in the Caenorhabditis elegans vulval development (48). This dominant negative effect of MEK1S218/222A could be due to the possibility that the amino acid mutant MEK1 blocks the function of upstream activators by forming a stable complex with the activators. The MEK1S218/222A mutant was subcloned into the yeast pAS-CYH vector and used as a bait to screen the Hela cell cDNA library by the yeast two-hybrid system. Among 1.2 × 107 colonies screened, 29 positive clones were isolated and purified. DNA sequence analysis demonstrated that every one of the positive clones encoded a member of the Raf family kinases (Fig. 1). The majority of the positive clones are A-Raf (16 clones) or c-Raf (11 clones), and only two B-Raf clones were isolated. The number of different Raf clones isolated is consistent with the relative levels of Raf mRNA in Hela cells. The shortest clone of A-Raf isolated started at amino acid residue 310. Therefore, it consisted of a sequence that was little more than the kinase domain (Fig. 1). Similarly, the shortest c-Raf clone started at amino acid residue 323 and contained only the C-terminal kinase domain (Fig. 1). Although many clones with various N-terminal truncation were isolated, no positive clone contained sequence less than the kinase domain, suggesting the entire kinase domain structure is necessary and sufficient for the interaction with MEK1S218/222A. This observation is consistent with the notion that the kinase domain of Raf has to
bind its substrates, including MEK1. Interestingly, Ras and MEK interact with Raf at different sites.

We were interested in the observation that no positive clone was isolated when the wild type MEK1 was used in the identical screening by the yeast two-hybrid system (Table I). To test if the wild type MEK1 can interact with Raf in the yeast two-hybrid system, plasmid pAS-MEK1 was cotransformed with A-Raf, B-Raf, and c-Raf clones in the yeast strain Y190. All transformants were selected on synthetic complete medium lacking leucine and tryptophan (Fig. 2A), which selected for the presence of both MEK1- and Raf-containing plasmids. The transformants were also tested on a selective medium lacking histidine and supplemented with 50 mM 3-AT, which selects for positive interaction between the bait (MEK) and the target (Raf) (Fig. 2C). As shown in Fig. 2, positive interactions between MEK1S218/222A and all three Raf were evident by the growth of transformants on SC-His, Leu, Trp, 50 mM 3-AT medium. By contrast, no positive interaction between MEK1 and A-Raf or B-Raf was observed. The interaction between wild type MEK1 and c-Raf was much weaker than that of MEK1S218/222A and c-Raf. This result further supports the notion that the interaction between MEK1 and its activator, Raf, can be stabilized by the elimination of the phosphorylation sites in MEK1. A similar screening was performed with the MEK5 kinase (49). Extracellular signal-regulated kinase 5 clones were isolated with the inactive MEK5' mutant but not with the wild type MEK5 (Table I).

Phosphorylation and Activation of MEK1 by A-Raf—Both c-Raf and B-Raf have been demonstrated to phosphorylate and activate MEK1. However, little is known about the role of A-Raf in MEK activation. We immunoprecipitated A-Raf with a specific antibody that recognizes the C-terminal 20 amino acid residues of A-Raf that are distinct from c-Raf and B-Raf. When the immunoprecipitated A-Raf was tested in a coupled in vitro kinase assay, A-Raf activated the recombinant GST-MEK1 (Fig. 3A). In the in vitro kinase assay, the immunoprecipitated A-Raf was used to phosphorylate and activate the purified GST-MEK1. The activated GST-MEK1 was then used to activate the purified recombinant extracellular signal-regulated kinase 1, whose activity was measured by the [γ-32P]-ATP incorporation into MBP, an extracellular signal-regulated kinase 1 substrate. A-Raf activity in Hela cells was acutely increased by stimulation of the cells with serum. Maximal activation of A-Raf was observed 5 min after serum stimulation. To test if A-Raf phosphorylates MEK1, the kinase-deficient MEK1" mutant (to reduce background by eliminating autophosphorylation) was subjected to in vitro phosphorylation by immunoprecipitated A-Raf. Fig. 3B shows that A-Raf could phosphorylate GST-MEK1" and that the phosphorylation activity was stimulated by serum. It is worth noting that the effect of serum on A-Raf phosphorylation of MEK1" (Fig. 3B) was much greater than the corresponding increase of MEK1 activity (Fig. 3A). This was likely due to the phosphorylation of MEK1" by the residual amount of MAP kinase co-immunoprecipitated with Raf. MAP kinase phosphorylates MEK1 on threonine residues 292 and 386 but does not activate MEK1.

To confirm the specificity of the antibody used in the immunoprecipitation experiments, the antigen peptide (corresponding to the C-terminal 20 amino acid residues of A-Raf) was used for competition in immunoprecipitation. Preincubation of the anti-A-Raf antibody with competing peptide completely eliminated A-Raf in the immunoprecipitates (Fig. 4B), whereas competition with a corresponding c-Raf peptide had no effect on the level of A-Raf precipitated (data not shown). The peptide also
stimuli including tumor necrosis factor
mine if growth factor can activate A-Raf. Several extracellular
phosphorylate but not activate MEK.

because a nonspecific kinase in the immunoprecipitate may
measuring MEK activation is a more specific assay than meas-
activity in the immunoprecipitate. Our data also indicate that
GST-MEK1* in
phosphorylation of MEK1* (Fig. 4
A-Raf was competed by the antigen peptide. The immunoprecipitated A-
Raf was subjected to Western blotting with anti-A-Raf antibody and
detected by alkaline phosphatase-conjugated second antibody. Immu-
noprecipitated A-Raf from unstimulated Hela cells (lane 1), serum-
stimulated cells (lane 2), competition with 2 μg of antigen peptide (lane 3)
in immunoprecipitation. The arrow indicates A-Raf.

extent with PMA (Fig. 5, A and B). EGF activated A-Raf by as
much as 16-fold, which was much greater than the effect of
serum (Fig. 3A). EGF and PMA both activated A-Raf activity in
a rapid and transient manner (Fig. 5, A and B). However,
stimulation of cells by EGF resulted in an increase of A-Raf
activity stronger than that by PMA. Furthermore, maximal
activation by EGF was observed at 2 min, whereas maximal
activation by PMA occurred at 5 min after stimulation. The
time course of A-Raf activation was similar to that of c-Raf
(data not shown). These results indicate that A-Raf may play
an important role in growth factor mediated MEK activation in
Hela cells. We have also found that EGF stimulated A-Raf
activity in human epidermoid carcinoma A431 cells (data not
shown).

Activation of c-Raf by EGF has been observed in numerous
cell types. We compared the relative contributions of A-Raf and
c-Raf to MEK activation in Hela cells. B-Raf was not tested
because it is expressed at a very low level in Hela cells. The
MEK-activating activity of A-Raf and c-Raf was measured in
EGF-stimulated Hela cells. Our data demonstrated that c-Raf
had a significantly higher MEK activating activity than A-Raf
in EGF-stimulated Hela cells (Fig. 5C). Nevertheless, A-Raf
constitutes a significant fraction of the MEK-activating activ-
ity, approximately 40% of that of c-Raf.

c-Raf activates MEK1 by phosphorylating at serine residues
218 and 222 (30–32). We wanted to test if these two serine
residues are also required for A-Raf-dependent MEK1 activa-
tion. Purified GST-MEK1S218A and GST-MEK1S222A mu-
tants were treated with immunoprecipitated A-Raf, and their
ability to be activated by A-Raf was compared with that of the
wild type MEK1. Elimination of either serine 218 or 222 com-
pletely abolished A-Raf-dependent activation (Fig. 5D). It is
worth noting that mutation of either serine residue decreases
the basal activity of MEK1, consistent with previous observa-
tions (30). Our data suggest that serine residues 218 and 222
are the phosphorylation residues targeted by A-Raf. The bio-
chemical mechanisms of MEK1 activation by A-Raf is thus
similar to that by c-Raf.

A-Raf Preferentially Activates MEK1 over MEK2—MEK1
and MEK2 are two closely related MAP kinase activators. The
functional redundancy between MEK1 and MEK2 is not clear.
We tested the activation of MEK2 by A-Raf in vitro. Purified
recombinant GST-MEK2 was incubated with immunoprecipi-
tated A-Raf. The activity of treated GST-MEK2 was deter-
mined by the extracellular signal-regulated kinase activation
assay. We were surprised to find that A-Raf did not activate
MEK2 (Fig. 6, columns 3 and 4). In a parallel experiment, the

competed with the MEK activating activity in A-Raf immuno-
precipitate. Similarly, phosphorylation of GST-MEK1* by A-
Raf was competed by the antigen peptide, suggesting that
A-Raf or an associated kinase was responsible for the phosho-
phorylation of MEK1 (Fig. 4A). The low level phosphorylation
of GST-MEK1* in lane 3 of Fig. 4A was likely due to nonspecific
activity in the immunoprecipitate. Our data also indicate that
measuring MEK activation is a more specific assay than meas-
uring phosphorylation of MEK in determining the Raf activity
because a nonspecific kinase in the immunoprecipitate may
phosphorylate but not activate MEK.

Activation of A-Raf by PMA and EGF —We wished to deter-
mine if growth factor can activate A-Raf. Several extracellular
stimuli including tumor necrosis factor α, PMA, ionomycin,
EGF, and UV treatment were tested in stimulation of A-Raf
activity. Treatment of Hela cells with tumor necrosis factor α,
ionomycin, or UV irradiation did not cause significant activa-
tion of A-Raf (data not shown). In contrast, A-Raf activity was
strongly activated in cells treated with EGF and to a lesser
similarly immunoprecipitated A-Raf-activated MEK1 effectively (Fig. 6, columns 1 and 2). Immunoprecipitated c-Raf, however, activated MEK2 as effectively as MEK1 (Fig. 6, lanes 5–8). Similar observation that immunoprecipitated B-Raf from EGF-stimulated Swiss3T3 cells could activate both MEK1 and MEK2 was also obtained (data not shown). These data suggest that unlike c-Raf, A-Raf is specific to MEK1 in vitro. The immunoprecipitated A-Raf has also been tested in the activation of MEK3 and MEK5 to determine if A-Raf may activate other members of the MEK family kinases. Neither MEK3 nor MEK5 was activated by A-Raf (data not shown).

**DISCUSSION**

Protein phosphorylation plays critical roles in the regulation of many cellular activities, including growth, differentiation, and metabolism. Protein kinases are key enzymes in signal transduction, typified by the MAP kinase pathway, which involves a cascade of kinases. Identification of upstream regulators and downstream effectors of protein kinases is a challenge for signal transduction research. The yeast two-hybrid system have been widely and successfully used to identify protein-protein interaction (11, 12, 44, 50). Because many of the components in the signal transduction pathway physically interact with each other, the two-hybrid system may be the ideal approach to the identification of kinase-interacting proteins. However, it has often been difficult to identify interaction between a kinase and its substrates because of the transient nature of the interaction. We reasoned that a kinase may be able to form a stable complex with its substrate if the target

![Graph](image)

**Fig. 5. Activation of A-Raf by EGF and PMA.** A, time course of A-Raf activation by EGF. Hela cells were stimulated with 100 ng/ml EGF for various time periods (indicated by x axis, min). A-Raf was immunoprecipitated and assayed for MEK1 activation. The A-Raf activity in immunoprecipitate of unstimulated cells was set at the value of one. B, time course of A-Raf activation by PMA. C, determination of the relative contributions of A-Raf and c-Raf to MEK1 activation in EGF-stimulated Hela cells. Level of MBP phosphorylation plotted on the y axis reflects the relative MEK-activating activity of immunoprecipitated Raf. Hela cells were stimulated with EGF for 2 min and immunoprecipitated by either anti-A-Raf or anti-c-Raf antibodies. The immunoprecipitates were assayed for GST-MEK1 activation. D, activation of MEK1 by A-Raf requires the presence of serine residue 218 and 222. The arrow indicates the phosphorylated MBP by the coupled kinase assay. All reactions contained extracellular signal-regulated kinase 1. Lanes 1, 2, and 3 represent the basal activity of GST-MEK1, GST-MEK1S218A, and GST-MEK1S222A, respectively. A-Raf could not activate either GST-MEK1S218A or GST-MEK1S222A (comparing lanes 2 and 6 and lanes 3 and 7), in contrast to the wild type (lanes 1 and 5).
phosphorylation residues of the substrates are eliminated. Based on this rationale, we have successfully isolated all members of the Raf family kinases by using the MEK1S218/222A mutant.

The mutant MEK1S218/222A functions as a dominant negative in cultured cells (47). Similarly, the corresponding mutations in the C. elegans mek-2 also resulted in a dominant negative mutant that blocked the Ras/Raf-dependent vulva induction (48). MEK1S218/222A could display the dominant negative effect because the mutant MEK1S218/222A may stably bind to its upstream activator, such as Raf, and sequester the Raf from activating the endogenous MEK1. This idea is consistent with our observation that MEK1S218/222A interacted strongly with Raf in the yeast two-hybrid system, whereas the wild type MEK1 failed to isolate Raf by the same screening (Fig. 2 and Table I).

Using a mutant as the bait in the yeast two-hybrid system may have a general application in identifying upstream kinases or downstream substrates. One such example is that the extracellular signal-regulated kinase 5 was isolated by the yeast two-hybrid system using mutants that lack the target phosphorylation residues as a bait. Similarly, it may be feasible to identify downstream substrates of a kinase using the kinase-deficient mutant as a bait in the yeast two-hybrid screening. The general application of these ideas remains to be tested with many of the kinases or phosphoproteins available.

MEK1 and MEK2 are the only two identified MAP kinase activators. MAP kinase activity can be stimulated by a wide variety of stimuli, which may be mediated by different MEK activators. Identification of c-Raf as a MEK activator provided an essential link between the growth factor receptor tyrosine kinases and the MAP kinase cascade (27–29). Raf is a family of protein kinases consisting of c-Raf, B-Raf, and A-Raf. Recently, B-Raf has been indicated to play an important role in MEK activation. B-Raf activity is rapidly stimulated by growth factors in several cell types (38–40). Early transformation experiments of NIH3T3 cells by A-Raf supported a role of this kinase in cell growth regulation. Furthermore, interaction of A-Raf with the activated/oncogenic Ras strongly suggests that A-Raf may function in Ras signaling. Data from this study unambiguously demonstrated the function of A-Raf as a MEK1 activator, indicating a role of A-Raf in mitogenic growth factor and protein kinase C-induced MAP kinase activation.

Activation of MEK1 by A-Raf apparently required the phosphorylation of serine residues 218 and 222, which are also the common phosphorylation sites of different MEK activators including B-Raf, c-Raf, c-mos, and MEK kinase 1. Interestingly, A-Raf preferentially activated MEK1 but not MEK2 (Fig. 6). In contrast, the c-Raf kinase can effectively activate both MEK1 and MEK2 kinases. Although extracellular signal-regulated kinase 1 and extracellular signal-regulated kinase 2 are the only two identified substrates of MEK1 and MEK2, it is possible that MEK1 may have other physiological substrates not shared by MEK2. Currently, it is unclear if MEK1 and MEK2 have the identical physiological functions. Genetic studies in C. elegans and Drosophila demonstrated that a single MEK gene fulfills the critical role in receptor tyrosine kinase signal transduction (48, 52, 53), suggesting that the mammalian MEK1 and MEK2 may have overlapping as well as distinct functions. Activation of A-Raf may lead to specific activation of MEK1 but not MEK2, possibly eliciting cellular responses different from those elicited by the activation of c-Raf.
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