Growth Factor Induced MEK Activation Is Primarily Mediated by an Activator Different from c-raf†

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Received January 27, 1994; Revised Manuscript Received March 10, 1994®

ABSTRACT: Activation of the mitogen-activated protein kinases (MAPKs) is a common event of many signal transduction pathways. MAPKs are phosphorylated and activated by an immediate upstream activating kinase, MEK. The proto-oncogene c-raf, encoding a serine/threonine kinase, has been reported to be a direct activator of MEK. In this paper, it is shown that growth factors activate MEK by stimulating c-raf and a raf-independent MEK activator. Treatment of Swiss3T3 cells with epidermal growth factor (EGF) rapidly increased the activity of MEK activator. Maximal activation was detected by 2.5 min and declined to the prestimulated level within 10 min. This stimulation of the MEK activator was temporally followed by increased activities of MEK and MAPK. The activation of MEK was accompanied by phosphorylation of this protein. To determine the relationship of this MEK activator and the c-raf kinase, cell lysates were immunoprecipitated with anti-raf antibody and assayed for MEK activation. Only a fraction (<20%) of the MEK activating activity was detected in anti-raf immunoprecipitates from EGF-stimulated Swiss3T3 cells. Similar experiments with nerve growth factor stimulated pheochromocytoma 12 (PC-12) cells revealed that the raf kinase contributed less than 5% of the total MEK activating activity while the overwhelming majority of MEK activating activity remained in the postimmunoprecipitation supernatant in which the raf protein had been quantitatively depleted. These data demonstrate that Swiss3T3 and PC-12 cells contain at least two different growth factor sensitive MEK activators, one residing in anti-raf immunoprecipitates and a second activity that is separate from raf. This raf-independent MEK activator appears to play a major role in the activation of MEK.

Protein phosphorylation plays an important role in cellular growth and differentiation. Mitogenic growth factors stimulate many phosphorylation events through a cascade of protein kinases (Cobb et al., 1991). Among the best characterized are the mitogen-activated protein kinases (MAPKs)[1] or the extracellular signal-regulated kinases (ERKs). These enzymes are activated by numerous mitogenic stimuli via protein kinase C dependent and independent pathways (Cobb et al., 1991). Although the cascade of events leading to activation of these kinases has been extensively studied, their precise role in cellular regulation has yet to be determined. Studies of substrate specificity revealed that microtubule-associated proteins (Gotoh et al., 1991), other protein kinases (Sturgill et al., 1988), transcription factors (Pulverer et al., 1991; Seth et al., 1991), and growth factor receptors (Northwood et al., 1991) are good substrates for MAPK.

MAPK is modulated by phosphorylation and dephosphorylation on tyrosine and threonine residues (Haycock et al., 1992; Payne et al., 1991). These phosphorylations are catalyzed by a single protein kinase known as MAPKK or MEK which is highly specific for MAPK (Crews et al., 1992; Seger et al., 1992a). Both biochemical purification and molecular cloning indicated that MEK constitutes a multiple enzyme family with sequence homology to the byrl and *STE7* gene products of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Crews et al., 1992; Zheng & Guan, 1993a). Recombinant MEKs expressed in *Escherichia coli* can activate MAPK *in vitro* (Zheng & Guan, 1993b). Moreover, overexpression of MEK in cultured cells increased MAPK activity (Seger et al., 1992b). Although the specific pathway by which growth factors and phorbol esters regulate MEK is not fully understood, phosphorylation of serine and possibly threonine residues may be responsible for MEK activation (Ahn et al., 1993; Gomez & Cohen, 1991), most likely due to the activity of an upstream kinase or kinases.

Some evidence has indicated a role of the raf proto-oncogene product (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992), a serine/threonine kinase activated by both receptor and nonreceptor tyrosine kinases via activation of p21ras. Both MAPK and MEK are constitutively activated in v-raf transformed cells. Furthermore, immunoprecipitated c-raf activates MEK *in vitro* by serine and threonine phosphorylation.

To evaluate whether growth factor dependent MEK activation was directly due to the activity of raf kinase, we studied the effect of growth factors in two separate cell lines known to exhibit these phosphorylation responses. Results in this paper indicate that the majority of the growth factor induced MEK stimulating activity was not associated with the raf kinase, suggesting the involvement of another protein kinase.

† This work is supported in part by grants from the American Cancer Society (BE-171), the University of Michigan Geriatrics Center (NIH AG-08808), and the Gastrointestinal Peptide Research Center (to K.L.G.).

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® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

[1] Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK or ERK kinase; EGF, epidermal growth factor; NGF, nerve growth factor; PC-12, pheochromocytoma 12; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified eagle medium; MEKK, MEK kinase.

0006-2960/94/0433-5595$04.50/0

MATERIALS AND METHODS

Expression and Purification of Recombinant MEK1, MAPK, and MEKK. $p44^{mapk}$ and MEK1 were expressed as GST fusion and purified as described (Zheng & Guan, 1993b). The kinase domain of mouse MEKK (amino acid residues 362–672) was amplified by polymerase chain reaction and expressed as GST fusion (Guan & Dixon, 1991). The recombinant GST-MEKK was purified and used to prepare antibodies in rabbits.

Cell Culture. Swiss3T3 cells were cultured in DMEM with 10% calf serum (Gibco BRL). Cells were cultured to confluence and starved in DMEM with 0.1% serum for 24 h. The starved cells were stimulated with EGF (100 ng/mL, Sigma) for various times and harvested in cell lysis buffer (25 mM HEPES, pH 7.5, 0.2 mM PMSF, 0.05% 2-mercaptoethanol, 1% Triton X-100, 2 mM sodium vanadate, 50 mM NaF). To inhibit translation, cells were preincubated with cycloheximide (15 μg/mL) for 30 min prior to stimulation by EGF. PC-12 cells were cultured in DMEM with 10% fetal calf serum.

Kinase Assays. Recombinant GST-MAPK (2 μg) was incubated in a 200-μL reaction containing 160 μL of cell extract (2.15 mg/mL total protein) and 40 μL of 5× MBP kinase buffer (90 mM HEPES, pH 7.5, 50 mM magnesium acetate, 250 μM ATP) at 30 °C for 20 min. The activated GST-MAPK was purified from cell extracts by glutathione agarose affinity chromatography as described (Zheng & Guan, 1993b) and eluted with 20 μL elution buffer (10 mM Tris, pH 8.0, 10 mM glutathione). The activated GST-MAPK (5 μL) was used in MBP kinase assay as described (Zheng & Guan, 1993b). Controls without cell extract were performed under identical conditions.

Assay of MEK activation by cell lysates or immunoprecipitates was performed under the same conditions described above for MAPK activation except that 0.72 μg of GST-MEK1 was used. The activated GST-MEK1 was purified from cell extracts by glutathione affnity chromatography and eluted in 20 μL of elution buffer. Three microliters of activated GST-MEK1 was used in MAPK activation as described (Zheng & Guan, 1993b). Phosphorylation of GST-MEK1 by cell lysates was performed under the same conditions described above, except that [^{32}P]-γ-ATP was included in the reaction.

Immunoprecipitation. Cells were scraped into the cell lysis buffer, sonicated for 10 s on ice, and centrifuged at 100000*g* for 20 min at 4 °C. The cell lysate (0.3 mL of Swiss 3T3, 2.15 mg/mL total protein, or PC-12, 13.6 mg/mL) was incubated with 10 μL of anti-raf serum, which was raised against the C-terminal peptide of c-raf, for 1 h on ice with gentle shaking, and 40 μL of protein A agarose (Pierce) was then added and incubated for 30 min on ice with shaking. After 2 min of centrifugation at 4000 rpm in a microfuge, the supernatant was subjected to two more rounds of immunoprecipitation with 10 μL of antiserum. The pellets from immunoprecipitation were washed three times with 1 mL of cell lysis buffer and assayed for MEK activation or Western blotting. The supernatants obtained after the third immunoprecipitation were assayed directly for MEK activation.

Western blotting with anti-raf serum was as described (Ohmichi et al., 1992). The anti-serum used for Western blotting was raised against the recombinant c-raf protein in rabbits. The anti-MEKK antibody was affinity purified following standard procedure (Sambrook et al., 1989). The purified antibody was used in immunoblotting.

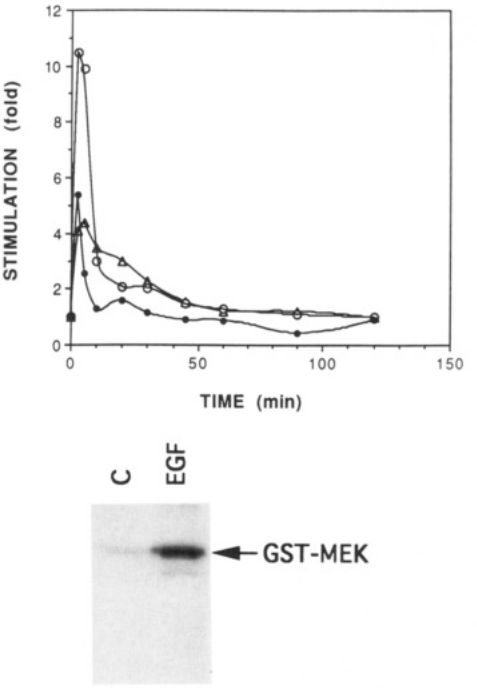

FIGURE 1: Transient activation of MEK activator. (A, top) Serum-starved Swiss3T3 cells were stimulated with 100 ng/mL EGF for various times. Activities of MAPK (open triangles), MEK (open circles), and MEK activator (solid circles) were determined. Stimulation (fold) is expressed relatively to the unstimulated level, which is considered as a value of 1. (B, bottom) Phosphorylation of GST-MEK1 by cell lysates. Cell lysates prepared from serum-starved (C) or EGF-stimulated (EGF) Swiss3T3 cells were used to phosphorylate GST-MEK1. The phosphorylated GST-MEK1 was purified from cell extracts by glutathione agarose affinity chromatography and subjected to SDS–PAGE followed by autoradiography.

RESULTS AND DISCUSSION

Activity of the MEK Activator Is Transiently Increased in Response to Growth Factors. Stimulation of various cell types with mitogens acutely increases the activity of MAPK and MEK due to phosphorylation. To study this activation mechanism in more detail, we compared the activities of MAPK, MEK, and MEK activator in Swiss3T3 cells exposed to EGF (Figure 1A). All of these activities were transiently increased in response to EGF. MAPK activity increased approximately 4-fold 5–10 min after EGF stimulation and declined thereafter. The activity of MEK was stimulated over 10-fold by EGF. The MEK activator activity was also rapidly and transiently activated by EGF, reaching a maximum 5–6-fold stimulation within 2.5 min after EGF stimulation and declining to basal level by 10 min. The activation patterns of these activities stimulated by EGF were not inhibited by pretreatment with cycloheximide (data not shown), suggesting that the MEK activator was regulated by a posttranslational mechanism. Similar results were obtained with PC-12 cells treated with NGF (data not shown).

It has been shown that MEK is phosphorylated on serine and threonine residues in response to growth factors (Ahn et al., 1993; Gomez & Cohen, 1991). To determine whether the MEK activator described above stimulates MEK phosphorylation, the phosphorylation of GST-MEK was tested in lysates of EGF-treated Swiss3T3 cells. Figure 1B shows that GST-MEK phosphorylation was dramatically stimulated by EGF. Cleavage of the fusion protein with thrombin indicated that all of the phosphorylation was associated with MEK1, but not GST in the fusion protein. The time course of this phosphorylation was similar to that of MEK activation (data

not shown), indicating that the growth factor dependent activation was due to stimulation of MEK phosphorylation. These data suggest that the MEK activator itself is a protein kinase, although it remains possible that this activator associates with MEK to induce autophosphorylation that activates the enzyme.

The Predominant Growth Factor Dependent MEK Activator Is Not the raf Proto-oncogene Product. Previous papers have suggested a role for proto-oncogene c-raf in directly phosphorylating and activating MEK (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992). However, these experiments relied on assay of MEK activity with mutated recombinant raf *in vitro* or by overexpression of the oncogenic active raf. Moreover, the relative contribution of raf to MEK activation stimulation has not been addressed. To explore in more detail the role of c-raf kinase in MEK activation in response to growth factors, we evaluated the MEK activating activity of c-raf immunoprecipitated from growth factor treated cells. Swiss3T3 cells were treated with EGF for 2.5 min. Cell lysates were sequentially immunoprecipitated with anti-raf or preimmune serum, and the immune complexes were subjected to *in vitro* activation assay using recombinant GST-MEK1 fusion protein as a substrate. MEK activating activity was observed in the first anti-raf immunoprecipitation but not in the subsequent immunoprecipitations (Figure 2A), suggesting that c-raf has been quantitatively precipitated by the anti-raf serum. However, the majority of the MEK activating activity was not precipitated by the anti-raf serum (Figure 2A). Preimmune or nonspecific serum did not precipitate the raf activity (Figure 2). Identical experiments were performed in NGF-treated PC-12 cells. Although a trace of MEK activator was detected in the anti-raf immunocomplex, the great majority of activity was found in the supernatant after three sequential immunoprecipitations (Figure 2B). MEK activating activity in both anti-raf immunoprecipitate and raf-depleted supernatant was stimulated by NGF.

Phosphorylation of GST-MEK1 was examined to demonstrate that the NGF-dependent MEK activating activity in the raf-depleted supernatant was due to protein phosphorylation. As described above for activation of MEK, phosphorylation of GST-MEK1 in the raf-depleted supernatant was stimulated by NGF (Figure 3).

To confirm that c-raf protein was present in the immunoprecipitates and was depleted in the supernatants after three sequential immunoprecipitations, the immunoprecipitates and supernatants were subjected to SDS–PAGE, followed by Western blotting with c-raf antibody (Figure 4A). The anti-raf serum used for Western blotting was prepared independently from that used in immunoprecipitation, thus excluding the possibility that the activity remaining in the supernatant represented a different population of raf not recognized by the precipitating anti-serum. Western blotting showed that virtually all raf protein (the 72-kDa band slightly above the 68-kDa marker) was precipitated in the first and second immunoprecipitations (Figure 4A, lanes 1–4). Little raf protein was found in the third immunoprecipitation and the supernatant (Figure 4A, lanes 5–8), yet the majority of MEK activating activity remained in the supernatant (Figure 2B). The lower molecular weight bands in the supernatants (Figure 4A, lanes 7 and 8) were due to proteins in the anti-raf serum recognized by the goat anti-rabbit second antibody. It is worth noting that treatment of PC-12 cells with NGF resulted in a mobility shift of the raf protein (Figure 4A, lanes 1 and 2), indicative of the phosphorylation and activation of this kinase, as previously demonstrated (Ohmichi et al. 1992).

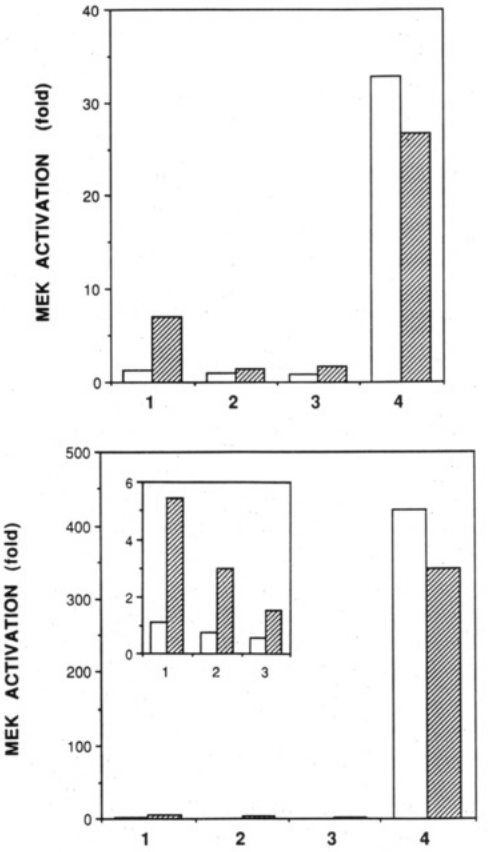

FIGURE 2: (A, top) MEK activating activity of anti-raf immunoprecipitates and supernatant from EGF-treated Swiss3T3 cells. The c-raf protein was immunoprecipitated from EGF-stimulated Swiss3T3 cell lysates and used in MEK activation assays. Open bars denote results of control serum. Hatched bars denote results of anti-raf serum. Columns 1, 2, and 3 represent MEK activating activity in the first, second, and third immunoprecipitations, respectively. Column 4 represents the MEK activating activity remaining in the supernatant after three immunoprecipitations. Results are representative of three independent experiments. Although the total MEK activating activity differs from one experiment to another, the ratio of anti-raf immunoprecipitable and raf-independent activity remains constant. The reason for the observed variation of total MEK activating activity lies in the transient nature of MEK activation (see Figure 1 for the activation kinetics). Therefore, cells stimulated with growth factors for 2 or 3 min (1 min is within experimental error of this procedure) may show a significant difference. (B, bottom) MEK activating activity of anti-raf immunoprecipitates and supernatant from NGF-treated PC-12 cells. Open bars denote results of control serum. Hatched bars denote results of anti-raf serum. Columns 1, 2, and 3 represent MEK activating activity in the first, second, and third immunoprecipitations, respectively. Column 4 represents the MEK activating activity remaining in the supernatant after three immunoprecipitations. To see the activity of raf, data of columns 1, 2, and 3 are also shown on a lower scale as an insert.

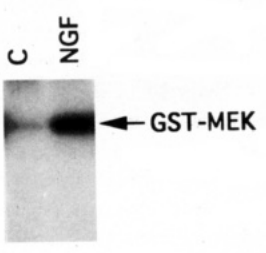

FIGURE 3: Phosphorylation of GST-MEK1 by raf-depleted serum-starved (lane C) or NGF-stimulated (lane NGF) cell lysates, in which raf was depleted by immunoprecipitation.

Other MEK activators besides raf have been described (Lange-Carter et al., 1993; Posada et al., 1993). MEKK (for MEK kinase) was isolated as a mammalian homolog of the yeast STE11 gene. MEKK has been expressed in cultured

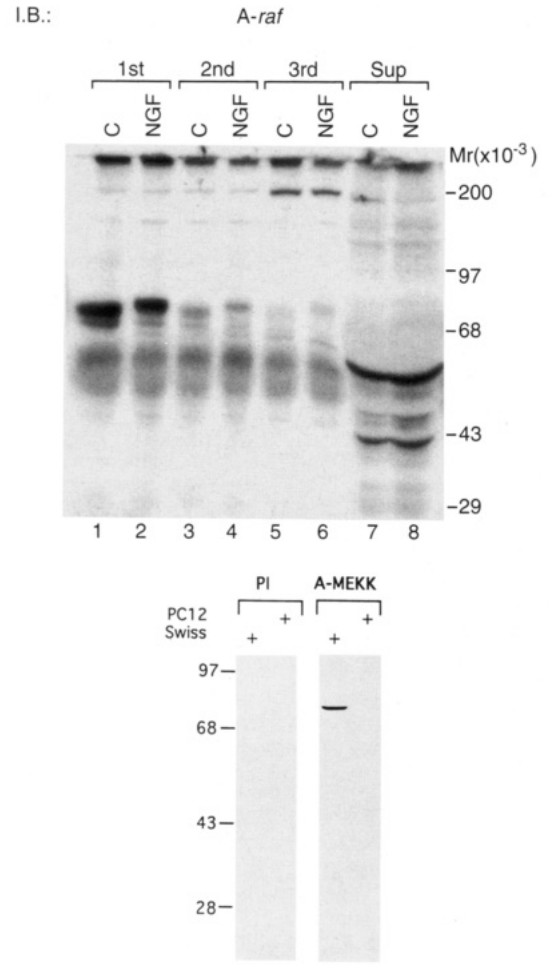

FIGURE 4: (A, top) Depletion of c-raf by immunoprecipitation. Western blotting was performed with anti-raf serum. Samples from serum-starved (C) or NGF-stimulated (NGF) are indicated. Pellets from the first (lanes 1 and 2), second (lanes 3 and 4), and third (lanes 5 and 6) immunoprecipitation were resolved on a SDS gel for Western blotting. Supernatant after three immunoprecipitations is denoted Sup (lanes 7 and 8). The c-raf protein (72 kDa, slightly above the 68-kDa marker) was most evident in the first immunoprecipitation (lanes 1 and 2). (B, bottom) Immunoblotting of MEKK in Swiss3T3 and PC-12 cells. Western blotting was performed with affinity-purified anti-MEKK antibody (A-MEKK) or preimmune (PI) as indicated. Cell lysates (40 μg of protein) from Swiss3T3 (denoted Swiss) or PC-12 cells are indicated in the figure. The MEKK has a predicted molecular weight of 78 000, which is consistent with the immunoreacting band detected by the anti-MEKK antibody.

cells and shown to phosphorylate and activate MEK (Lange-Carter et al., 1993). To determine the relationship between the MEK activator described above and MEKK, immunoblotting of Swiss3T3 and PC-12 cell lysates was performed. The affinity-purified anti-MEKK antibody detected a MW 78 000 protein which is consistent with the predicted molecular weight of MEKK in Swiss3T3 cells. Interestingly, this MW 78 000 protein was barely detectable in PC-12 cells under the identical conditions (Figure 4B). The immunoreacting band was not detected in the preimmune serum (Figure 4B), supporting the conclusion that the MW 78 000 protein is MEKK. Furthermore, detection of the MW 78 000 protein was completely eliminated if the antibody was preincubated with purified MEKK protein (data not shown). These observations show that the MEK activating activity in PC-12 cannot be contributed by MEKK and suggest the existence of a novel NGF-stimulated MEK activator in PC-12 cells.

We have shown that MEK activating activity was acutely stimulated by growth factors. The proto-oncogene c-raf constitutes approximately 20 and 5% of the total MEK activating activity in EGF-treated Swiss3T3 and NGF-treated PC-12 cells, respectively. The raf-independent activity was stimulated by growth factors in these cell lines. Wood et al. have demonstrated that expression of an activated raf in PC-12 cells does not result in significant activation of MAPK (Wood et al., 1992). Our data may be consistent with this observation, suggesting that the major MEK activator is different from c-raf, although this putative MEK activator could be activated downstream from raf. The precise identity of this growth factor dependent MEK activator is unknown; however, it is likely to be a kinase since activation was correlated with phosphorylation. The recombinant MEKK could activate MEK and was initially suggested to mediate the trimeric G-protein but not the tyrosine kinase induced MEK activation (Lange-Carter et al., 1993). However, recent evidence indicates that raf is activated in the trimeric G-protein mediated MAPK activation (Winitz et al., 1993). Our data indicate that the NGF-sensitive MEK activator identified in this paper and the *STE11* homolog MEKK are likely to be different proteins because little MEKK was found in PC-12 cells.

The proto-oncogene c-mos, a Ser/Thr kinase, has been demonstrated to phosphorylate and activate MEK (Posada et al., 1993). However, the c-mos kinase cannot be the MEK activator in Swiss3T3 and PC-12 cells because the c-mos mRNA is found only in germ cells and early embryonic cells (Propst & Vande Woude, 1985; Keshet et al., 1988). Preliminary experiments to purify the MEK activator in PC-12 cells showed that it could be indeed chromatographically separated from raf (Pang and Saltiel, unpublished data). Future experiments to further characterize the MEK activator will shed light on the mechanism of MEK activation in response to mitogenic growth factors.

ACKNOWLEDGMENT

We thank Drs. R. Jove (University of Michigan) and S. Decker (Parke-Davis Pharmaceutical Research) for anti-raf serum, S. L. Pelech for ERK1 plasmid, J. E. Dixon for advice, and X. Wu for technical assistance.

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