Cloning and Characterization of Two Distinct Human Extracellular Signal-regulated Kinase Activator Kinases, MEK1 and MEK2*

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Mitogen-induced signal transduction is mediated by a cascade of protein phosphorylation and dephosphorylation. One of the immediate responses of mitogen stimulation is the activation of a family of protein kinases known as mitogen-activated protein kinase or extracellular signal-regulated kinase (ERK). MEK (MAP kinase or ERK kinase) is the immediate upstream activator kinase of ERK. Two cDNAs, MEK1 and MEK2, were cloned and sequenced. MEK1 and MEK2 encode 393 and 400 amino acid residues, respectively. The human MEK1 shares 99% amino acid sequence identity with the murine MEK1 and 80% with human MEK2. Both MEK1 and MEK2 were expressed in Escherichia coli and shown to be able to activate recombinant human ERK in vitro. The purified MEK2 protein stimulated threonine and tyrosine phosphorylation on ERK1 and concomitantly activated ERK1 kinase activity more than 100-fold. The recombinant MEK2 showed lower activity as an ERK activator as compared with MEK purified from tissue. However, the recombinant MEK2 can be activated by serum-stimulated cell extract in vitro. MEKs, in a manner similar to ERKs, are likely to consist of a family of related proteins playing critical roles in signal transduction.

Cellular responses to growth factors involve the stimulation of protein phosphorylation accompanied by the sequential activation of protein kinases. Most of these phosphorylations occur at serine and/or threonine residues and may involve alteration of the activity of serine/threonine protein kinases or phosphatases. Since the growth factor receptors initiating these phosphorylation events are protein tyrosine kinases, they must regulate serine/threonine-specific kinases or phosphatases.

Recent evidence suggested that MAP kinase (mitogen-activated protein kinase) or ERK (extracellular signal-regulated kinase) plays important roles in the growth factor signal transduction (1, 2). ERK, also known as MBP (myelin basic protein) kinase or MAP2 (microtubule-associated protein 2) kinase, is rapidly activated upon growth factor stimulation (1-3). Possible physiological substrates of ERK include transcription factor Jun and the p90 kinase. Thus, ERK is believed to be directly involved in relaying signals from the growth factor receptors to the regulation of gene transcription and translation. Several ERKs have been cloned (4, 5). Rat ERK1 and ERK2 are closely related to each other (90% amino acid sequence identity), while ERK3 is more distantly related (4, 5). Interestingly, ERK1 and ERK2 display significant sequence identity to FUS3 and KSS1, two kinases from yeast Saccharomyces cerevisiae involved in signal transduction of the mating pheromone (4-6). Thus, some aspects of the signal transduction mechanism appear to be conserved from yeast to higher eukaryotes.

One of the most appealing properties of ERK activation is that ERK is activated by both tyrosine and threonine phosphorylation upon growth factor stimulation (5, 7, 8). This phosphorylation event is mediated by a single protein kinase which is known as MAPKK (MAP kinase kinase) or MEK (MAP kinase or ERK kinase). MEK was initially identified as an ERK activator from growth factor-treated cells (9-12). Purification and characterization of the ERK activator indicated that it was a unique protein kinase that phosphorylates ERK on tyrosine and threonine residues. This phosphorylation leads to the concomitant activation of ERK. MEK displays exclusive substrate specificity toward ERK in vitro (11). Partial amino acid sequence of MEK confirmed its protein kinase nature and showed sequence identity to byrl and STE7 gene products of Schizosaccharomyces pombe and S. cerevisiae (10, 12-14). Interestingly, both byrl and STE7 are involved in the yeast signal transduction pathway of mating pheromone. Genetic evidence has indicated that the STE7 gene (MEK homolog) functions immediately upstream of FUS3 and KSS1 (ERK homologs) in S. cerevisiae mating response (6). MEK itself is also activated upon mitogen stimulation presumably via phosphorylation (12). The upstream activator of STE7 is STE11 whose homolog has not been found in high eukaryotes (6). Recent evidence indicated that the protooncogene c-raf may be responsible for MEK activation (15, 16).

To understand the regulation of tyrosine and threonine phosphorylation/dephosphorylation on ERK, we made an effort to isolate the MEK cDNA. A MEK1 cDNA has recently been cloned from a murine pre-B cell cDNA library (17). In this report, we described the cloning and characterization of two distinct human MEK cDNAs. The human MEK1 encodes 393 amino acid residues and shows 99% amino acid sequence identity to the published mouse MEK1 (17). The other MEK, MEK2, encodes 400 amino acid residues with a predicted molecular mass of 44 kDa and shares 80% sequence identity.
with MEK1. To test MEK-dependent ERK activation, both MEK1 and MEK2 were expressed and purified from Escherichia coli. The recombinant MEK proteins could activate ERK1 more than 100-fold. Interestingly, activity of the recombinant MEK2 could be activated by cell extract in vitro.

EXPERIMENTAL PROCEDURES

**Molecular Cloning and Sequencing**—Two degenerate PCR primers were designed based on the conserved amino acid sequence of S. pombe byr1 and the partial amino acid sequence of MEK (12–14). Oligonucleotides CTTGGATCTCCTTATA/CTA/C/TGNTGGNTT (C/T/TA and CTTGGATCC(G/C)/G/C/TTCGNCG(C/G)/A/T)CAT (A/G/T)A are coding for amino acid residues YIVGYF and YMSPER, respectively (the BamHI restriction sites are underlined). These two primers were used in PCR (Perkin-Elmer Cetus) for 35 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min using 1 ng of human brain cDNA as template (Clontech). The PCR products were resolved by agarose gel and subcloned into M13 for sequence analysis. The 0.35-kb PCR product was radiolabeled with a multiprimer DNA labeling kit (Amersham Corp.) and used to screen three X ZAP cDNA libraries of human brain, placenta, and spleen (Stratagene). Standard conditions for hybridization were used. Primary positive clones were purified upon secondary and tertiary screening, and further confirmed by DNA Southern hybridization.

**Bacterial Expression and Purification**—DNA fragments coding for MEK1 and MEK2 were subcloned into plasmid pGEX-2T to express GST fusion protein. BamHI restriction sites were introduced into both 5′- and 3′-ends of MEK1 and MEK2 coding sequence by PCR. The PCR DNA products were subcloned into the BamHI site of pGEX-2T to produce in-frame GST fusion proteins. MEK2 without the N-terminal 32 residues, which is not conserved between MEK1 and MEK2, was also constructed to facilitate expression. The complete coding sequences in expression plasmids were sequenced to eliminate possible PCR mistakes. Expression and purification were performed following published procedure (18). Both GST fusion protein and thrombin-cleaved MEK proteins were isolated and dialyzed against 25 mM Tris, pH 7.5, 0.5% 2-mercaptoethanol. Protein concentration was determined by Bio-Rad protein assay reagent and determined spectrophotometrically in 0.1% SDS-PAGE using bovine serum albumin (Sigma) as standard. Similar procedures were used to express and purify human ERK1.

**Cell Extract**—NIH3T3 cells (2.4 × 10⁶) were grown in Dulbecco’s modified Eagle’s medium with 10% calf serum (Gibco Laboratories) for 3 days. Cells were scraped from 20-cm dishes in the same medium with 0.1% fetal calf serum and then stimulated with 20% fetal calf serum for 5 min. Cells were washed twice with ice-cold phosphate-buffered saline and finally scrapped into 0.5 ml of cell lysis buffer (0.5 ml/plate) (25 mM HEPES pH 7.5, 0.2 mM phenylmethylsulfonyl fluoride, 0.05% 2-mercaptoethanol, 1% Triton X-100). The cell lysis solution was briefly sonicated and directly used in MEK activation assay.

The recombinant GST-MEK2 (5.8 μg) was incubated with 70 μg of cell extract in 100 μl of solution containing 30 mM HEPES pH 7.5, 50 μM ATP, 10 mM magnesium acetate, 0.1 mM phenylmethylsulfonyl fluoride, 0.025% 2-mercaptoethanol, 1 mM NaF, and 1 mM sodium vanadate for 10 min at 30 °C. The reaction mixture was then mixed with 40 μl of glutathione agarose (50% v/v in phosphate-buffered saline containing 0.5% Triton X-100) and incubated at room temperature for 10 min followed by 5 min at 0 °C. The activated GST-MEK2 was purified and eluted with free glutathione. Control experiments for GST-MEK2 without cell extract or cell extract without GST-MEK2 were treated identically.

**Kinase Assay**—ERK kinase activity was determined using myelin basic protein as substrate (11). Reaction was performed in 20 μl of solution containing 18 mM HEPES pH 7.5, 50 μM ATP (5000 cpm), 10 mM magnesium acetate, and 20 pg of myelin basic protein for 20 min at 30 °C. One half of the reaction (20 μl) was transferred onto a 2.5-cm diameter plastic phosphocellulose paper (Whatman). The filters were washed with 180 mM phosphoric acid five times and then rinsed with 95% ethanol. Phosphorylation was quantitated by scintillation counting. The remaining half (20 μl) was analyzed by SDS-PAGE followed by autoradiography.

To activate the inactive ERK, recombinant GST-MEK2 was first incubated with ERK1 in 38 μl of solution similar to the ERK kinase assay for 10 min at 30 °C, and then 2 μl of MBP (10 mg/ml) was added to initiate the MBP kinase reaction for an additional 20 min at 30 °C. The amount of phosphorylation was quantitated as described for the ERK kinase assay above.

**Phosphoamino Acid Analysis**—Phosphorylated proteins were resolved by SDS-PAGE and transferred to Immobilon-P membrane (Amersham Corp.). After autoradiography, 32P-containing bands were excised and hydrolyzed in 6 N HCl for 1.5 h at 105 °C. Phosphoamino acids were separated by one-dimensional electrophoresis on cellulose plates (Whatman) (11).

**RESULTS AND DISCUSSION**

**Cloning and Characterization of MEK cDNA**—To study the functions of MEK in signal transduction and its activation mechanism, experiments were performed to isolate human MEK cDNAs. Degenerate PCR primers based on the conserved yeast STE7 and byr1 sequences and the partial amino acid sequence of MEK (13, 14) were designed and used to amplify human MEK cDNA fragment. When human brain cDNA (reverse transcription product of human brain mRNA) was used as a template, a strong 0.35-kb PCR product was evident. DNA sequencing analysis confirmed that the PCR product contained a sequence similar to STE7, byr1, and the published partial amino acid sequence of MEK (13, 14). This fragment was used to screen human brain, placenta, and spleen cDNA libraries. Sixty positive clones (32 from the spleen library, 18 from the placenta library, and 10 from the brain library) were initially isolated from these libraries, and 14 were further analyzed by restriction analysis followed by Southern hybridization. These clones could be divided into two classes, one with an internal EcoRI site and the other without. DNA sequencing of all 14 clones confirmed that they belonged to two classes of closely related cDNAs. Six clones (three of them contained the entire coding region) were highly related to the murine MEK1 sequence (approximately 99% amino acid sequence identity) and likely represented the human homolog of the murine MEK1 (17). These clones were denoted as MEK1. The longest cDNA of human MEK1 is 2.2 kb in length with an open reading frame of 1179 nucleotides. DNA sequence homology between human and murine MEK1 could not be assessed since the nucleotide sequence of murine MEK1 was not available in the DNA data bank. The deduced MEK1 amino acid sequence and sequence comparison are shown in Fig. 1.

In addition to MEK1 clones, eight other cDNA clones (three of which contained the entire coding region) encoding a polypeptide having 80% amino acid sequence identity to the human and murine MEK1 (Fig. 1) were also identified and designated as MEK2. The longest MEK2 cDNA was 1.6 kb in length with an open reading frame of 1200 nucleotides. The deduced amino acid sequence of MEK2 is 403 residues with a predicted molecular mass of 44 kDa (Fig. 1).

Our data suggest that MEK proteins, like the ERK family, may constitute a family of enzymes that may play a critical role in signal transduction. However, the functional relationship between MEK1, MEK2 and ERK1, ERK2, ERK3 remains to be elucidated. It is possible that different MEKs
the recombinant murine MEK1 could activate ERK cellular signal of Wisconsin GCG. Residues that are not conserved are have cloned and expressed the murine MEK1. They demonstrated that the recombinant murine MEK1 phosphorylated nine. However, no data have been presented to indicate that MEK2 are aligned with the mouse MEK1 (17) using the PILEUP program. The high degree of sequence identity between MEK1 and MEK2 based on their partial amino acid sequences (13, 14). The previously purified MAP kinase activators (MEK) were MEK1 and MEK2 were partially purified and used to activating ERK. To test this hypothesis, human MEK1 and MEK2 were expressed in E. coli using the glutathione S-transferase system. Both MEK1 and MEK2 were partially purified and used to activating ERK. The recombinant MEK1 and MEK2 were able to activate human ERK1 in vitro as much as 100-fold (data not shown). Initial experiments with the full-length MEK2 revealed that the majority of fusion proteins were insoluble and partially degraded in E. coli (TG-1) cells. Amino acid sequence comparison of MEK1 and MEK2 indicates that the N-terminal region is not conserved (Fig. 1). Therefore, a truncated version of MEK2 missing the N-terminal 32 amino acid residues was also constructed and expressed in E. coli as GST fusion to facilitate purification. The purified truncated GST-MEK2 is shown in Fig. 2A along with the human ERK1 that was also expressed and purified from E. coli. An in vitro kinase assay demonstrated that the truncated MEK2, similar to the full-length MEK2, was able to activate ERK1 (Fig. 3). This truncated GST-MEK2 was used for subsequent experiments.

One of the most intriguing events leading to ERK activation is the requirement of both tyrosine and threonine phosphorylation. This phosphorylation occurs on tyrosine 185 and threonine 183 residues (19). Because MEK2 and ERK1 have similar molecular weights, they are not easily separated on SDS-PAGE. Therefore, we used GST-MEK2 fusion protein (67 kDa) and ERK1 (44 kDa) to study phosphorylation. The recombinant ERK1 autophosphorylated itself predominantly on tyrosine residues and to a less extent on serine residues (Fig. 2, B and C) consistent with a previous observation (20–22). However, this autophosphorylation failed to activate the MBP kinase activity of ERK1. When recombinant MEK2 was incubated with ERK1 in vitro, the phosphorylation of ERK1 was elevated (Fig. 2B). Phosphoamino acid analysis showed that ERK1 was also phosphorylated on threonine in the presence of GST-MEK2. Furthermore the tyrosine phosphorylation of ERK1 was significantly enhanced (Fig. 2C). GST-MEK2 autophosphorylated mainly on serine and on a
the MBP kinase activity of ERK1 was measured. The ERK1 preincubated with various amounts of GST-MEK2, and then phosphorylation on threonine and tyrosine could not be excluded (open circle). As a control, GST-MEK2 was assayed for MBP kinase activity in the absence of ERK1 (closed circle). Data were obtained from triplicate experiments. The MBP kinase activity of ERK1 is indicated by the y axis, and the amount of GST-MEK2 protein is indicated by the x axis.

less extent on threonine (Fig. 2C). Thrombin cleavage of the autophosphorylated GST-MEK2 showed that the phosphorylation was exclusively associated with the MEK2 moiety, not the GST in the fusion protein (data not shown). It is likely that the recombinant MEK2 phosphorylated ERK1 on threonine and tyrosine residues and hence activated ERK1 although the possibility that MEK2 stimulated ERK autophosphorylation on threonine and tyrosine could not be excluded from this experiment. It is noteworthy that ERK1 activation did not correlate with ERK1 phosphorylation. This result suggested that in the presence of MEK2 only was a fraction of the ERK1 phosphorylation responsible for activation.

The recombinant human ERK1 purified from E. coli had a MBP kinase activity of 0.056 nmol/min/mg. MEK2 can greatly enhance the MBP kinase activity of ERK1 more than 100-fold (Fig. 3). A dose response of ERK1 activation by GST-MEK2 was determined (Fig. 3). ERK1 (0.216 μg) was preincubated with various amounts of GST-MEK2, and then the MBP kinase activity of ERK1 was measured. The ERK1 activation was linearly correlated with the increasing amount of GST-MEK2 protein (Fig. 3, open circles). To confirm that the increased MBP kinase activity was due to ERK1 activation rather than the endogenous MBP kinase activity of recombinant GST-MEK2, assay without ERK1 was also performed under identical conditions. The data showed that GST-MEK2 alone had no significant contribution to the increased MBP kinase activity (Fig. 3, closed circles).

In Vitro Activation of MEK—MEK is activated during mitogen-induced signal transduction. Some evidence indicated that the activation of MEK was also mediated by serine/threonine phosphorylation (12, 15, 16). However, the mechanism of MEK activation is not completely understood. We have noticed that a relatively large amount of MEK2 was needed to activate ERK1 (Fig. 3). This observation suggested that the recombinant MEK2 had rather low specific activity as an ERK activator. Therefore, in vitro experiments were carried out to test whether the recombinant MEK2 could be activated. GST-MEK2 was first incubated with NIH3T3 cell extract. The treated GST-MEK2 was then purified using glutathione-agarose affinity resin and used in ERK1 activation assay (Fig. 4). The cell extract-treated GST-MEK2 and the control GST-MEK2 were analyzed by SDS-PAGE and shown to be identical to each other (data not shown). Fig. 4 showed that the recombinant GST-MEK2 was activated by NIH3T3 cell extract in vitro. Approximately 10-fold activation of MEK2 was observed. Because the amount of recombinant MEK2 used in the activation was likely exceeding the physiological MEK concentration, we thought that the observed 10-fold activation was probably an underestimated number. Furthermore, the activated MEK2 had increased autophosphorylation (data not shown).

Data from several laboratories have documented that MEK was the immediate upstream activator kinase of ERK and phosphorylated ERK on tyrosine as well as threonine (5–7). Three distinct ERKs have been cloned from rat, and evidence suggested that additional ERK may exist (4). The two human MEKs reported here indicated that MEK also consists of a family of proteins. With recombinant MEKs and ERKs, it will be possible to test the functional relationship between individual MEK and ERK proteins. Furthermore, it has been indicated that the protooncogene c-raf kinase can activate MEK (15, 16). The availability of recombinant MEK proteins and the ability to activate MEK in vitro should allow the dissection of MEK activation by mitogenic growth factors.

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REFERENCES

FIG. 3. Activation of ERK1 by MEK2. Activation of MBP kinase of ERK1 depends upon the amount of GST-MEK2. The same quantity (0.216 μg) of ERK1 was incubated with different amounts of GST-MEK2 for 10 min, and then MBP kinase activity was determined (open circle). As a control, GST-MEK2 was assayed for MBP kinase activity in the absence of ERK1 (closed circle). Data were obtained from triplicate experiments. The MBP kinase activity of ERK1 is indicated by the y axis, and the amount of GST-MEK2 protein is indicated by the x axis.

FIG. 4. In vitro activation of MEK2. The recombinant GST-MEK2 was treated with NIH3T3 cell extract and then purified by glutathione-agarose affinity chromatography. Activation of MEK2 was measured by its ability to activate ERK1. Data were obtained from duplicate experiments. Lane E, ERK1 alone; lane C, cell extract control (see "Experimental Procedures" for detail); lane E + C, ERK1 plus control; lane M, control GST-MEK2; lane E + M, ERK1 plus control GST-MEK2; lane Ma, activated GST-MEK2; lane E + Ma, ERK1 plus activated GST-MEK2. The same amount of protein of ERK1 was used in lanes E, E + C, E + M, and E + Ma. The quantity of control GST-MEK2 protein in lanes M and E + M was identical to that of the activated GST-MEK2 in lanes Ma and E + Ma.