Volume 320, number 3, 246–250 FEBS 12317 April 1993

Isolation of two members of the rat MAP kinase kinase gene family

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Received 23 February 1993

Mitogen-activated protein (MAP) kinase kinase (MAPKK) is a recently characterized activator of MAP kinase (MAPK), and is considered to be regulated by a protooncogene product c-Raf-1. It is, however, unclear whether the signals originating from c-Raf-1 utilize this phosphorylation cascade to lead to oncogenesis. To clarify this point, we isolated rat MAPKK cDNAs, and identified two distinct cDNAs encoding MAPKK and a highly related kinase, both with molecular weights of ~45 kDa (MEK1 and MEK2). Genomic Southern blot analyses suggested that MAPKK may form a large gene family.

Signal transduction; MAP kinase kinase; Molecular cloning

1. INTRODUCTION

Stimulations by many growth factors rapidly activate a protein serine/threonine kinase named mitogen-activated protein (MAP) kinase (MAPK) or extracellular-signal response kinase (ERK) (reviewed in [1]). This kinase, in turn, activates S6 kinase II (RSK) [2], MAP kinase-activated protein kinase-2 [3], and transcription factors such as c-Jun [4] . Recent studies have identified and characterized an upstream activator of MAP kinase, MAP kinase kinase (MAPKK) [5–10], which has the activity of phosphorylating both serine/threonine and tyrosine residues [11]. To be active, MAPKK itself requires phosphorylation on its serine/threonine residues [5,10,12], indicating the presence of a further upstream phosphorylation system regulating MAPKK.

Another serine/threonine protein kinase, c-Raf-1, encoded by the cellular homologue of oncogene v-*raf*, is also activated by stimulations with many mitogens [13,14]. Although the substrates for c-Raf-1 have long remained unidentified [13,14], very recent data suggest that MAPKK is a substrate candidate [10,15–18]. This finding is substantiated by a genetic observation that Dsor1 (for downstream of Raf) is a MAPKK homologue and is located downstream of raf-1 in *Drosophila melanogaster* [19].

Using rat NRK cells and its mutants, we previously demonstrated that mitogenesis can be dissociated from oncogenesis as measured by the anchorage-independent cell growth, and that c-Raf-1 is a downstream component of the pathway for oncogenic signals induced by many oncogenes [20,21]. To identify the signalling cascade leading from c-Raf-1 to oncogenesis, more specifically to clarify the involvement of MAPKK in the c-Raf-1 oncogenesis pathway, we tried to isolate rat MAPKK cDNAs based on the peptide sequences obtained from *Xenopus* MAPKK [5]. In this communication we describe two distinct cDNAs encoding rat MAPKK and a highly related protein.

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Abbreviations: MAP: Mitogen activated protein, PCR: polymerase chain reaction.

The nucleotide sequences of MEK1 and MEK2 have been submitted to the DNA Data Bank of Japan (DDBJ) and will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the following accession numbers: ratMEK1 — D14591; ratMEK2 — D14592.

2. EXPERIMENTAL

2.1. *PCR-amplification of rat MAP kinase kinase cDNA fragment*

Based on the published peptide sequence of *Xenopus* MAP kinase kinase, IMHRDVKPSNILVNSRGEXKLXDFGVSGQ (X=unassigned) [5], two oligonucleotides were synthesized with the Applied Biosystems 394 DNA/RNA synthesizer. Oligo 1. 5′-ATGCA(T/C)(C/A)G(T/C/A/G)GA(T/C)GT(T/C/A/G)AA(A/G)CC-3′ (sense) for MHRDVKP. Oligo 2: 5′-TG(T/C/A/G)CC(T/C/A/G)GA(T/C/A/G)AC(T/C/A/G)CC(A/G)AA(A/G)TC-3′ (antisense) for DFGVSGQ. Polymerase chain reaction (PCR) was carried out with plasmid DNA isolated from the rat NRK cDNA library (see below) as a template and with Vent DNA polymerase (New England Biolab) according to the manufacturer's suggestion. After 40 cycles of denaturation at 94°C for 1 min followed by primer annealing at 55°C for 1 min and primer extension at 72°C for 2 min, the resulting ~85 bp amplified product was separated by agarose gel electrophoresis, and isolated. The product

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was then reamplified under the same conditions as in the first round of PCR, but this time using the sense primer with an *Eco*RI site and the antisense primer with a *Xba*I site. Subsequently, the reamplified product was purified, subcloned into the *Eco*RI–*Xba*I sites of pGEM3Zf(+) (Promega), and sequenced to confirm the identity.

2.2. *Screening of rat NRK cDNA library for MAPKK cDNAs*

A NRK cDNA library composed of 5×10^6 independent clones was prepared with the pcD2 vector [22]. 1×10^6 colonies from the library were screened with a mixture of two oligonucleotides. Oligo 3: 5′-TCCAACATTCTGGTGAACTCT-3′ (sense) for SNILVNS. Oligo 4: 5′-GCACAGCTTAATCTCTCCACG-3′ (antisense) for RGEIKLC. These oligonucleotides were derived from the sequence of the PCR product. They were labelled at the 5′ ends with [γ-^{32}P]ATP (Amersham) and T4-kinase (Takara, Japan). The procedures for library plating and filter preparation (Millipore) have been described [23]. Hybridization was done in 6× SSPE, 0.5% SDS, 10× Denhardt solution, 100 μ/ml denatured herring sperm DNA, and the radiolabelled oligonucleotides. Filters were washed twice in 6× SSC, 0.1% SDS at 42°C for 10 min, and then exposed for 2 days By repeating these steps, single colonies were finally isolated. Their DNA sequences were determined using the dideoxy chain termination method with Sequenase (USB), and primer walking with synthesized oligonucleotides.

2.3. *Northern blot analysis*

3 μg of poly(A)$^+$ RNA isolated from NRK cells was electrophoresed in a formamide/formaldehyde gel, and transferred to a nylon membrane (Biodyne, PALL). Hybridization was carried out at 42°C in the solution as described [23] with MEK1 and MEK2 as probes, separately. The probes used for MEK1 and MEK2 were the 0.45 kb *Bst*XI–*Sph*I and the 0.5 kb *Kpn*I–*Not*I fragments, respectively, as shown in Fig. 1. They were labelled with [α-^{32}P]dCTP (Amersham) by the random priming method. Filters were washed at room temperature in 2× SSC, 0.1% SDS for 15 min, twice, then at 65°C in 2× SSC, 0.1% SDS for 15 min, twice, and finally at 65°C in 0.2× SSC, 0.1% SDS for 15 min, twice, followed by exposure to a XAR5 film (Kodak) at −70°C for 12 h

2.4. *Southern blot analysis*

3 μg of genomic DNA (purchased from Promega) was digested with several restriction enzymes, run in 0.5% agarose gels and transferred to nitrocellulose filters (Schleicher & Schuell) using standard methods [23]. Hybridization was carried out in a solution containing 1 M NaCl, 10× Denhardt solution, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.1% SDS, 100 μ/ml denatured herring sperm DNA and a radiolabelled probe. Probes used for this experiment were the 0.7 kb *Pst*I–*Pst*I fragment for MEK1 and the 0.75 kb *Stu*I–*Asp*718 fragment for MEK2, as shown in Fig. 1. They were labelled with [α-^{32}P]dCTP (Amersham) by the random priming method (Amersham). Filters were hybridized with MEK1 probe, and then with MEK2 after washing off the first probe. Filters were washed in 0.5× SSC, 0.1% SDS at 50°C and exposed to a XAR-5 film for 2 days, then washed further in 0.1× SSC, 0.1% SDS at 65°C and exposed for 7 days.

3. RESULTS

3.1. *Isolation of a cDNA fragment for rat MAP kinase kinase*

Since only the peptide sequences for *Xenopus* MAPKK were available [5] when we started this work, the PCR technique was employed to isolate a cDNA fragment for rat MAPKK. PCR amplifications of a rat NRK cDNA library with degenerate oligonucleotides (oligo's 1 and 2, see Experimental) synthesized on a *Xenopus* peptide sequence yielded a band of ~85 bp, which was exactly the size expected from the *Xenopus* peptide sequence. This amplified fragment was subcloned, and the sequence was determined. Of seven clones isolated, two clones contained an open reading frame encoding the amino acid sequence identical to the *Xenopus* peptide sequence (data not shown). The remaining five clones had some sequence homologies to protein kinases. This was because the amplified region was among the well-conserved motifs in many protein kinases [24].

3.2. *Identification of two types of MAP kinase kinase cDNAs*

The internal nucleotide sequence of the PCR-amplified rat MAPKK cDNA fragments was then used to prepare oligonucleotides (oligo's 3 and 4, see Experimental) for the isolation of full-length cDNA clones. After screening ~10^6 clones of the NRK cDNA library with these oligonucleotides, finally seven positive clones were obtained. By mapping and sequencing, they were classified into two groups (Fig. 1). The first group, com-

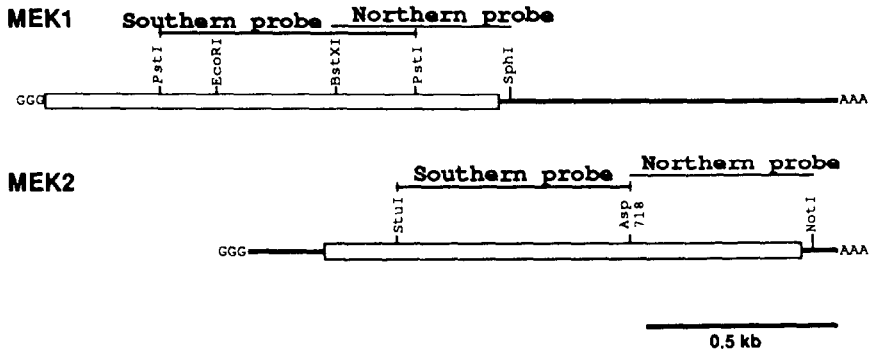

Fig. 1. Schematic representation of the structures of rat MEK1 and MEK2 cDNAs. Open boxes and bold lines indicate open reading frames and untranslated regions, respectively. Major restriction enzyme sites used in this article are shown. DNA fragments used as probes for Southern and Northern analyses are shown by bars.

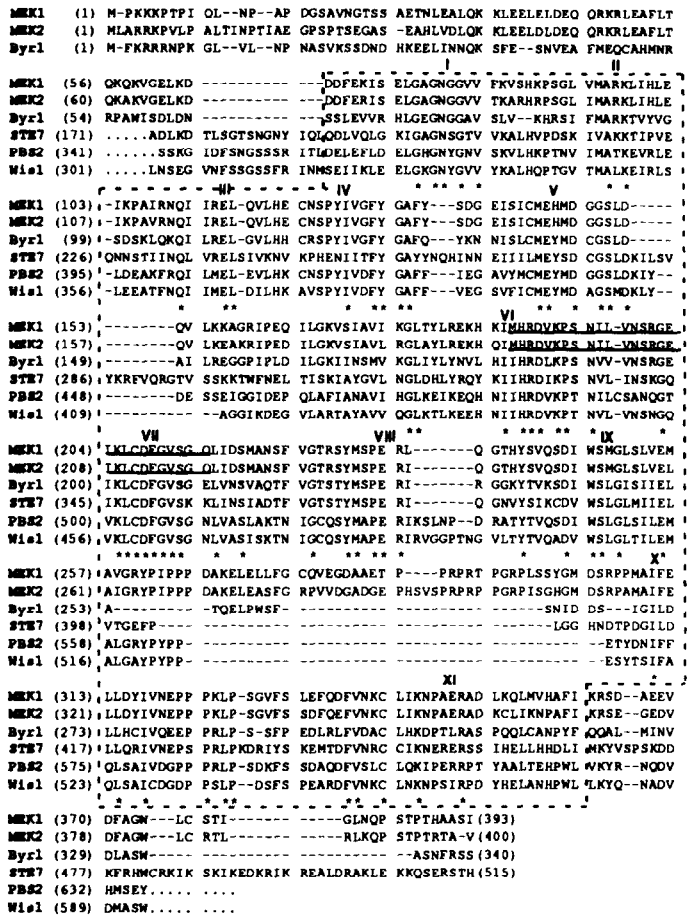

Fig. 2. Amino acid sequence comparison between rat MEK1, MEK2, Byr1, STE7, PBS1 and Wis1. They were aligned using the GENETYX MaxMatching 1.0.1 program with some modification. For MEK1 and MEK2 as well as Byr1, the entire amino acid sequences are shown. For STE7, PBS2 and Wis1, only parts of their amino acid sequences are shown. On the left side, residue numbers are indicated. Lines and dots indicate gaps and continued residues, respectively. Identical residues between six sequences are marked with asterisks. The area surrounded by dotted lines is the catalytic domain, and their subdomains marked with roman numbers as proposed by Hanks et al [24]. Underlined residues in MEK1 and MEK2 are the region used for PCR amplification (see the text).

posed of two clones, could encode a 393 amino acid protein identical to the recently published mouse MAPKK (MEK1) except for one residue (residue 278, His in mouse was Gln in rat) [25] (Fig. 2). This rat MEK1 protein had an estimated molecular mass of 43,437 Da. The second group, composed of five clones, had 76% nucleic acid identity to MEK1 cDNA, and could encode a 400 amino acid protein with an estimated molecular mass of 44,253Da, which was 80% identitical in amino acid content to MEK1. This protein was, therefore, named MEK2. The sizes of both proteins were similar to those of the purified MAPKKs [5–9].

3.3. *Structural features of two members of the MAP kinase kinase gene family*

The central region of MEK1 and MEK2 were very similar to each other (86%), and had remarkable similarity to other protein kinases, suggesting that this region contained the catalytic domain (Fig. 2) [24]. Among the many kinases, MEK1 and MEK2 were particularly similar to yeast kinases involved in signal transductions: Byr1 [26] (38% identical to both in the entire coding region; 45% identical to both in the kinase domain) and Wis1 [27] (25% and 38%, respectively) of *Schizosaccharomyces pombe*, and STE7 [28] (27% and 38%, respectively) and PBS2 [29] (24% and 42%, respectively) of *Saccharomyces cerevisiae*. Remarkable differences between MEK1 and 2, however, were found in two regions: one was the N-terminal ~35 amino acid sequence, and the other the region between subdomains IX and X of the catalytic region [24]. In these two regions, they were also diverged from yeast MAPKK homologues (Fig. 2). The functional significance of the sequence differences remains to be clarified.

Northern blot analyses of rat NRK cells demonstrated discrete bands for MEK1 and MEK2 (Fig. 3). MEK1 probe identified a single ~2.6 kb band, which was

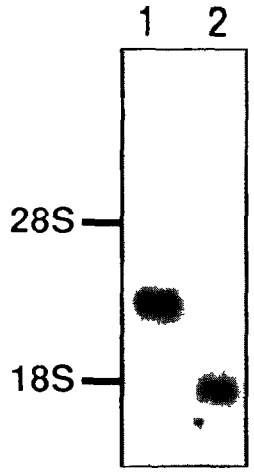

Fig. 3. Northern blot analysis of MEK1 and MEK2. 3 μg of rat NRK poly(A)$^+$ RNA was electrophoresed, transferred to nylon filters and hybridized with MEK1 and MEK2 probes (shown in Fig. 1), separately. After washing under stringent conditions, filters were exposed to an X-ray film. The positions of ribosomal RNA markers are shown to the left of the blots.

similar in size to mouse MEK1 [25]. MEK2 probe detected a single ~1.8 kb band. The abundance of both messages was comparable.

3.4. *Evidence for the presence of more MEK1 related genes*

Since MAPK forms a family [1], we examined whether the MEK family had other members. Rat, human and mouse genomic DNAs were analyzed by Southern blot hybridization with MEK1 and MEK2 cDNA fragments (see Fig. 1), as shown in Fig. 4. Hybridization was carried out under stringent conditions (see Experimental). When rat DNA was analyzed after appropriate restriction enzyme digestions, each probe recognized the band(s) corresponding to its own gene after high stringency washing. Under low stringency washing, each recognized the other. Moreover, they recognized additional bands, some of which were hybridized only with either probe. Similar results were obtained with human and mouse DNAs. These data suggest the presence of more members of the MAPKK gene family, although some of bands may represent pseudogenes.

4. DISCUSSION

In this report, we describe rat MAP kinase kinase MEK1 and a highly related kinase named MEK2. Comparisons of nucleotide and deduced amino acid sequences between the two MEKs show that they are distinct entities, but not those generated from one gene such as by alternative splicing [30]. Southern and Northern analyses clearly demonstrate that MEK1 and MEK2 constitute a gene family, possibly with additional members, in similarity to other intracellular signal transducers, such as protein kinase C and phospholipase C. Two reports have already suggested the presence of at least two related molecules with MAPKK activity, 45 and 46 kDa proteins [6,9]. Molecular cloning has revealed that the 45 kDa protein is encoded by MEK1 [25,30,31]. At present it is unclear whether the protein encoded by MEK2 described here corresponds to the 46 kDa protein. MEK1 contains all the peptide sequences so far described for MAPKKs of *Xenopus* [5,31], mouse [6], rabbit [7,8] and human [9,30]. While some of the peptide sequences, including those used for PCR amplification, were shared with MEK2, no MEK2-specific peptide sequences have been reported (see Fig. 2). This indicates that the purified MAPKK is MEK1, not MEK2.

Significant structural differences between MEK1 and MEK2 reside in the N-terminal region and the region between protein kinase subdomains IX and X. This may

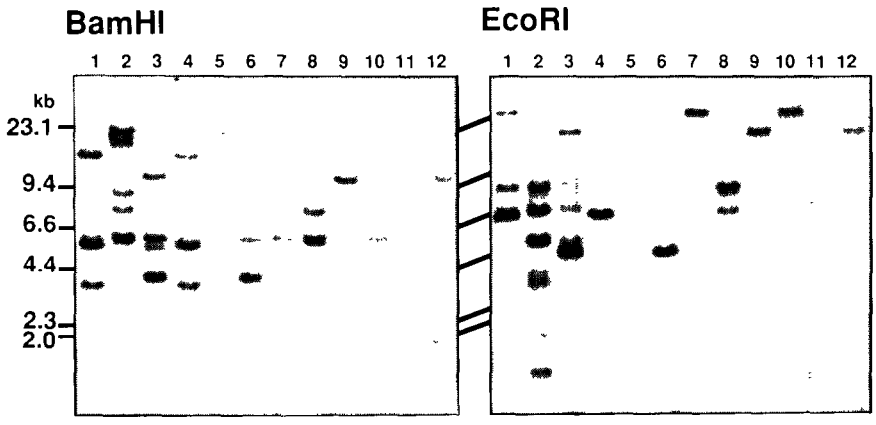

Fig. 4. Genomic Southern blot analyses. After digestion with *Bam*HI (left) or *Eco*RI (right), genomic DNAs from rat (lanes 1, 4, 7, 10), human (lanes 2, 5, 8, 11) and mouse (lanes 3, 6, 9, 12) were separated by agarose gel electrophoresis and transferred to nitrocellulose filters. They were hybridized with MEK1 probe (see Fig. 1: lanes 1–6), washed at low stringency (lanes 1–3), and finally washed at high stringency (lanes 4–6). After washing off the MEK1 probe, they were hybridized with MEK2 probe (see Fig. 1: lanes 7–12), washed at low stringency (lanes 7–9) and finally washed at high stringency (lanes 10–12). The positions of λ*Hin*dIII size marker (kb) are shown to the left of the blots.

suggest that MEK1 and MEK2 might differ in their biological functions. In fact, the yeast homologues Byr1, Wis1, STE7 and PBS2, which differ in function, share the least homology in these regions with one another. Since phosphorylation cascades involving MAPKK have been well studied in yeasts [10,31–33], it may be interesting to know whether MEK1 and MEK2 could substitute for the yeast MAPKK homologues. These studies may provide some clue to finding the functional differences between the two MEKs, as will biochemical analyses.

Several groups have reported that c-Raf-1 is an upstream regulator of MAPKK (MEK1) in growth signalling cascades [10,15–18]. Although this is an intriguing finding, several questions remain unsolved. One major question to be addressed is whether this signalling cascade from c-Raf-1 through MAPKK (MEK1) to MAPK is utilised for oncogenesis. Some reports describe that the expression of v-*raf* or activated c-Raf-1 do not always activate MAPK [34–36]. Our recent observation also suggests that the activation of MAP kinase is not dierectly involved in oncogenic transformation of NRK cells (Kizaka-Kondo and H.O., unpublished observation). If c-Raf-1 could activate different MAPKKs, the interesting possibility arises that the oncogenic signal of c-Raf-1 could be mediated by some other MAPKK family member, such as MEK2 or a putative member yet to be identified. It is, therefore, particulary interesting to determine whether MEK2 is activated by c-Raf-1 and what the biological effector of MEK2 is.

Acknowledgements We would like to thank to Dr. Y Nishida (Aichi Cancer Center Research Institute, Nagoya, Japan) for making results available prior to publication.

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NOTE ADDED IN PROOF

Sturgill's group has published their rat MAP kinase kinase cDNA sequence in Proc. Natl. Acad. Sci. USA, Vol 90, pp. 173–177, 1993. Their deduced amino acid sequence is identical to our rat MEK1. Their 1.7 kb band observed in Northern blot analysis may correspond to that of our rat MEK2.