Differential Expression of MEK1 and MEK2 during Mouse Development

Alessandro Alessandrini, Barbara K. Brott, and Raymond L. Erikson

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138 [B. K. B., R. L. E.], and Renal Unit, Massachusetts General Hospital East, Charlestown, Massachusetts 02129 [A. A.]

Abstract

MAP/Erk kinase 1 (MEK1) and MEK2 activate the Erk/MAP kinases and have been implicated in cell growth and differentiation. To investigate the role of MEKs during mouse development, we have examined their expression and activity in various murine tissues during embryonic development and in the adult mouse. MEK2 RNA message is expressed at high levels in all embryonic tissues examined, including all neural tissues, and liver. This can be observed by in situ hybridization of tissue sections of 14.5-day-old mouse embryos, as well as by Northern blot analyses. MEK1, on the other hand, is expressed at very low levels in most embryonic murine tissue but can be detected in developing skeletal muscle. It is expressed at higher levels in adult tissue, particularly in brain, where it is expressed at high levels. Western blot analyses of MEK1 and MEK2 in 14.5-day-old embryonic and adult mouse tissues confirm the RNA analysis. Levels of MEK1 kinase activity are particularly high in adult brain tissues as well. These findings suggest that MEK2 may be the primary Erk/MAP kinase activator during development and that MEK1 may play a role in the proliferative or mitogenic response in adult mouse tissues. This study also raises the possibility that MEK1 and MEK2 might not have redundant functions in cells but may possess unique specificity in their interactions with upstream activators or downstream targets.

Introduction

MEK family members are key components in an intracellular kinase signaling pathway called the Erk/MAP kinase pathway, which is implicated in the transition of cells from G0 to G1 (1–3). Members of this kinase cascade are highly conserved between species from yeast to mammals (2, 4). In addition, proteins with sequence similarity to Erk/MAP kinases and MEKs participate in other cellular signaling pathways (e.g., those responding to stresses such as osmotic shock and UV-induced DNA damage; Ref. 4). The MAP/Erk kinase cascade is activated following stimulation of a wide variety of cell types with growth factors, hormones, or mitogens. The binding of these various ligands to the appropriate cell surface receptor results in receptor activation, which in turn leads to GTP binding of Ras complexed to members of the Raf family of serine/threonine kinases. Raf family members then activate the kinases MEK1 and MEK2 by phosphorylating them on serines 218 and 222, for MEK1, or serines 222 and 226, for MEK2. MEKs, as dual specificity kinases, subsequently activate their downstream targets, Erk-1 and Erk-2, by phosphorylating them on threonine and tyrosine. Erks then phosphorylate both cytoplasmic substrates and nuclear transcription factors, which, thus modified, contribute to the early response of the cell after stimulation.

Although there are six known members of the MEK family of kinases at present, only two of them, MEK1 and MEK2, have been shown to play a role in the Erk/MAP kinase pathway (5–10). None of the other known MEKs [MKK3, MKK4 (also called SEK1), MEK5, and MEK6] have been shown to phosphorylate Erk-1 and Erk-2 in vitro (11–16). MEK1 and MEK2 are approximately 90% similar and 80% identical; the differences in these proteins are mainly in the NH2-terminal region outside the kinase domain, and in a proline-rich region between conserved kinase domains IX and X (5, 9, 10). These differences between MEK1 and MEK2 may contribute to differences in interactions and specificity.

The respective contributions of MEK1 versus MEK2 to the Erk/MAP kinase pathway are currently not well defined. In vitro studies suggest that MEK2 may phosphorylate both Erk-1 and Erk-2 to a greater extent than does MEK1 (17). However, relative MEK specificities toward these two known substrates in vivo systems have not yet been rigorously examined. Differential activation of MEK1 and MEK2 by various Raf family members in HELA cells has been described by Wu et al. (18), who find that MEK1 is activated in vitro by A-Raf, as well as Raf-1 and B-Raf. This is in contrast to MEK2, which is only phosphorylated by Raf-1 and B-Raf (19). Jelenik et al. (20) reported that immobilized Ras-Raf-1 and Ras-B-Raf complexes bind MEK1 but not MEK2. However, later studies indicated that both MEK1 and MEK2 interacted with Ras-bound Raf-1 through the proline-rich region in the kinase domain (20). Another point that remains unclear is whether various external stimuli preferentially lead to activation of MEK1 versus MEK2 in cell lines and in tissue or whether their functions are largely redundant. Stimulation of NIH3T3, Rat1, and PC12 cells with TPA, EGF, NGF, FGF, and PDGF failed to show a differential response between MEK1...
and MEK2, suggesting that in some cell types, the functions of these two family members may be redundant. However, Downey et al. (21) have recently reported that MEK2 is more active than MEK1 after stimulation of neutrophils with chemoattractant peptides and is sensitive to the PI-3 kinase inhibitor wortmannin. The roles of MEK1 and MEK2 during development, with respect to organogenesis and tissue differentiation, have not yet been elucidated.

To determine the relative abundance and activities of MEK1 and MEK2 during embryonic development, we examined their levels of mRNA and protein expression in various organs. Enzymatic activities of MEKs in embryonic tissues, compared to activity in adult tissues, were also investigated. We find that MEK1, although present, is expressed to a very low degree in murine embryonic tissues, except in developing muscle tissue of mesodermal origin. MEK1 is expressed at higher levels in adult murine tissues, notably in adult brain, where elevated MEK1 kinase activity is also detected. In contrast, MEK2 is expressed at high levels in most tissues during the embryonic stages examined, as well as in adult tissues. These data suggest a role for MEK2 as the major Erk/MAP kinase activator during embryonic development and imply that MEK1 may have an important function in adult brain, as well as possibly during embryonic skeletal muscle development.

Results

RNA Expression of MEK1 and MEK2 during Embryonic Development. Developmental Northern analysis of total RNA isolated from 9.5–14.5-day-old mouse embryos shows that although both MEK1 and MEK2 are expressed at all stages, MEK2 is expressed at higher levels than MEK1 (Fig. 1). The expression of MEK2 appears to decrease slightly as the age of the embryo increases (Fig. 1).

To investigate MEK expression during development in greater detail, in situ analysis of MEK expression at 14.5 days was performed. Transverse sections of 14.5-day-old mouse embryos were analyzed by in situ hybridization using MEK1 and MEK2 probes made to the 3' untranslated regions of these genes. Results indicate that MEK2 is expressed at high levels in all tissues examined. Fig. 2 shows that MEK2 is present at high levels in the developing spinal cord, dorsal root ganglia, and trigeminal ganglia. It is also expressed throughout the embryonic brain (data not shown). MEK2 RNA levels are also high in the developing eye, with elevated expression in the neural layer of retina (Fig. 3). These findings suggest a role for MEK2 in the developing nervous system. MEK2 expression is also observed in liver (Fig. 4), heart, lung, kidney, and thymus (data not shown). Whereas MEK2 is widely expressed in 14.5-day-old embryos, MEK1 expression appears to be restricted to certain tissues, such as the extrinsic ocular muscle, the long muscles of the neck, and the developing diaphragm (Figs. 3 and 4). Although MEK2 is also expressed in these tissues, there may be a requirement for both MEK1 and MEK2 in developing skeletal muscle.

These data suggest that MEK2 appears to be the predominant MEK family member expressed during embryogenesis.

RNA Expression of MEK1 and MEK2 in Embryonic Tissues Compared to Adult Tissues. To determine the relative levels of MEK1 and MEK2 message in embryonic and adult tissues, total RNA was prepared from liver and brain of adult and 14.5-day-old mouse embryos. After electrophoresis, it was probed with the entire cDNA of either MEK1 or MEK2. Although methylene blue staining indicated that similar levels of RNA were present in each lane, appreciable levels of MEK1 were present only in adult brain, with much lower levels in embryonic brain and in adult and embryonic liver (Fig. 5). In contrast, MEK2 was present in all the tissues examined, with slightly higher levels in embryonic tissue as compared to adult tissue (Fig. 5). This suggests that MEK1 may play a role in signaling in the adult brain but that MEK2 may function not only in adult tissues but in signaling during embryonic organogenesis and differentiation. A heavier message of roughly 3.9 kb was observed in some MEK1 and MEK2 Northern blots; however, this was not reproducibly observed.

Protein Expression of MEK1 and MEK2 in Embryonic and Adult Tissues. Western blot analysis was performed to determine whether levels of MEK RNA message correlated with protein levels in tissue. Tissue lysates from 14.5-day-old embryo and adult mouse brain, heart, and liver were electrophoresed and immunoblotted with antibodies specific for either MEK1 and MEK2. Protein detected by Western blot reflects the Northern blot analysis. Levels of MEK1 protein were detectable but low in all tissues except adult brain (Fig. 6). In contrast, MEK2 was present at relatively high levels in all tissues examined (Fig. 6). Samples were also probed with an antibody against Erk-1 and Erk-2. The anti-Erk Western blot indicated that similar levels of Erks are present in all tissues, except for adult heart, which may have been under-

---

5 B. Brott and R. Erikson, unpublished data.
Fig. 2. MEK2 is expressed in 14.5-day-p.c. murine trigeminal ganglia, spinal cord, and dorsal root ganglia. Tissue sections at 14.5 days p.c. were hybridized with MEK1-specific (A and C) and MEK2-specific (B and D) probes. TG, trigeminal ganglia; SC, spinal cord; DRG, dorsal root ganglia.

Fig. 3. MEK2 is expressed in 14.5-day-p.c. murine eyes. Tissue sections at 14.5 days p.c. were hybridized with MEK1-specific (A and C) and MEK2-specific (B and D) probes. nir, neural layer of the retina; le, lens; eom, extrinsic ocular muscle.

MEK1 and MEK2 Kinase Activity in Embryonic and Adult Tissues. Levels of MEK activity against a kinase-inactive Erk fused to GST (GST-Erk-1 K63M) were assayed. Levels of MEK2 kinase activity were consistent, although not extremely high, in all tissues examined (Fig. 7). MEK1 exhib-
Differential Expression of MEK1 and MEK2

Fig. 4. MEK1, as well as MEK2, is expressed in developing skeletal muscle; MEK2 is expressed in developing liver. A–D, tissue sections at 14.5 days p.c. were hybridized with a MEK1-specific probe. E, tissue section hybridized with MEK2-specific probe. eom, extrinsic ocular muscle; imn, long muscles of the neck; dia, diaphragm.

ited elevated kinase activity primarily in adult brain tissue (Fig. 7). This high level of activity is at least partially accounted for by the higher level of MEK1 protein present in brain tissue. In spite of the low amounts of protein present in embryonic liver, a fair amount of kinase activity was observed (Fig. 7). Although this may indicate an elevated specific activity of MEK1 in embryonic liver, this apparent elevation of kinase activity was not consistently observed.

Discussion
We have analyzed the expression levels of MEK1 and MEK2 in various organs during mouse development. We observed that whereas both MEK1 and MEK2 are expressed in adult tissues, MEK2 is the predominant isoform that is expressed during mouse embryogenesis at the stages examined. This was dramatically illustrated by in situ hybridization of 3' untranslated regions of MEK1 and MEK2 to transverse sections of 14.5-day-old mouse embryos. These data reveal that MEK2 is expressed at elevated levels in every tissue analyzed in the embryo sections (Figs. 2 and 3). This implies that MEK2 and the Erk/MAP kinases may play an important role in organogenesis, promoting cell proliferation and differentiation. In particular, we observe high MEK2 expression in fetal brain, spinal cord, dorsal root ganglia, trigeminal ganglia, and
Fig. 5. MEK1 is expressed in adult brain, whereas MEK2 is expressed at high levels in both embryonic and adult tissue. RNA was isolated from 14.5-day-old embryonic mouse liver or brain or from adult mouse liver or brain. RNAs were electrophoresed and transferred to Hybrid filters and then probed with either the entire MEK1 (Lanes 1–4) or MEK2 (Lanes 5–8) cDNAs. Filters were then washed and exposed to X-ray film. Total RNA was also stained with methylene blue to indicate amounts of RNA in each sample. Lanes 1 and 5, adult liver; Lanes 2 and 6, adult brain; Lanes 3 and 7, embryonic liver; Lanes 4 and 8, embryonic brain.

Fig. 6. MEK1 and MEK2 protein expression correlates with RNA expression. Twenty μg of tissue lysates from 14.5-day-old and adult mice were electrophoresed on an SDS-PAGE gel. Protein was then transferred to membranes and Western blotted with anti-MEK1 serum 724, anti-MEK2 serum 727, or anti-Erk-1/2 antibody (K-23; Santa Cruz). Lane 1, 14.5-day-old heart; Lane 2, adult heart; Lane 3, 14.5-day-old brain; Lane 4, adult brain; Lane 5, 14.5-day-old liver; Lane 6, adult liver.

Fig. 7. MEK1 and MEK2 kinase activity was detected in all tissues examined. MEK1 and MEK2 were immunoprecipitated from tissue lysates from 14.5-day-old or adult mouse with anti-MEK1 serum 725 (A) or anti-MEK2 monoclonal (UBI; B). In vitro kinase assays were performed with 2 μg of GST-Erk1(K63M) as substrate at 30°C for 20 min. Reactions were electrophoresed on a SDS-PAGE gel and transferred to membranes. After exposure of membranes to X-ray film, immunoprecipitates were Western blotted with either anti-MEK1 monoclonal antibody 3D9 or anti-MEK2 antibody 727. Lane 1, 14.5-day-old heart; Lane 2, adult heart; Lane 3, 14.5-day-old brain; Lane 4, adult brain; Lane 5, 14.5-day-old liver; Lane 6, adult liver.

gesting that MEK2 may also function in the development of the immune system.

In contrast, MEK1 could only be detected at low levels in developing skeletal muscle, such as in long neck muscles, extrinsic optic muscles, and the developing diaphragm (Figs. 3 and 4). Inasmuch as MEK2 is also expressed in these tissues, this apparent redundancy in expression may be explained by the recent findings of Wu et al. (18), who showed that whereas Raf-1 and B-Raf activate both MEK1 and MEK2 in vitro, A-Raf activates only MEK1. Also, analysis of the Erk/MAP kinase pathway in neutrophils showed that although both MEK1 and MEK2 were activated after stimulation of neutrophils by chemotactic peptides, only MEK2 activity was sensitive to the PI-3 kinase inhibitor wortmannin (21). This suggests that MEK1 and MEK2 may be involved in different signaling pathways in neutrophils. These studies raise the possibility that in skeletal muscle development, both MEK1 and MEK2 are required to optimally trigger the appropriate pathways leading to full tissue development and differentiation.
Although the data suggest that MEK2 is the predominant Erk1/2 activator during mouse embryogenesis, very high levels of MEK1 expression are detected in adult mouse brain. This high level of expression of MEK1 is observed at the mRNA level, as well as the protein level (Figs. 5 and 6). In addition, assays of MEK1 and MEK2 kinase activity against GST-Erk1(633M) indicate that the elevated MEK1 activity in brain tissue reflects the higher level of MEK1 expressed (Fig. 7). Normalization of the amounts of MEK1 from other tissues in in vitro kinase assays shows, however, that the specific activity of MEK1 in brain, liver, and other tissues is the same and is not greatly activated above basal levels of MEK1 kinase activity observed in MEK1 purified from unstimulated baculovirus-infected Sf9 cells. MEK2 kinase activities were consistently similar in all tissues examined, reflecting the uniform expression levels of MEK2 in both embryonic and adult tissues (Fig. 7). In these analyses, it is important to note that whole tissues were examined, composed of a heterogeneous population of cells, which may mask an increase in kinase activity in either MEK1 or MEK2 within a subset of cells at a given point during development. Isolation of the respective MEK promoters will allow us to further elucidate the variation of MEK1 and MEK2 levels that are observed and to identify transcriptional factors that regulate MEK1 and MEK2 transcription during mouse development.

This is the first study that shows the differential expression of MEK1 and MEK2 in fetal and adult tissues. Our findings suggest that the functions of MEK1 and MEK2 may not be redundant and that MEK2 may play a more dominant role than MEK1 during mouse organogenesis and embryogenesis. MEK1 may be involved in proliferation and signaling in adult tissues. These data, therefore, raise the possibility that MEK1 and MEK2 have alternative activators or substrates, or different affinities for them, to respond differentially to extracellular signals during embryonic development and in the adult organism.

Materials and Methods

In Situ Expression Analysis of MEK1 and MEK2 in 14.5-day-p.c. Mouse Embryos. In situ studies were done on 14.5-day-p.c. mouse embryo cross-sections, which were prepared and hybridized with 35S-UTP-labeled RNA probes according to Wilkinson et al. (23). Adjacent transverse sections, cut at 0.6-μm intervals, were analyzed so as to accurately compare expression for each gene. The 280-bp MEK1 RNA probe, which includes the 3′-most untranslated region, was prepared by linearizing the template with Avall and transcribing using T3 RNA polymerase, as described previously by Echelard et al. (24). The 282-bp MEK2 RNA probe, which includes all of the 3′-untranslated region (158 bp) and 124 bp of the 3′ most coding region, was prepared by linearizing the template with BglII and transcribing using T7 RNA polymerase. Cross-reactivity between MEK1 and MEK2 was minimized by using high-stringency conditions and posthybridization RNase treatment. The same DNA fragments were also used in Northern analysis, in which no cross-reactivity was observed.

RNA Preparation and Northern Analysis. Tissues were isolated from embryonic or adult mice and quick-frozen in liquid nitrogen. Whole embryos ages 9.5-14.5 days p.c. were also isolated and quick-frozen. RNA was prepared using the Ultraspec RNA isolation reagent (Biotex Laboratories, Inc.). Briefly, tissues were Dounce homogenized in Ultraspec reagent, extracted with chloroform, and centrifuged at 12,000 × g for 15 min. The aqueous phase was precipitated with isopropanol and centrifuged again for 10 min. Precipitates were washed with 70% ethanol and resuspended in DEPC-TE (0.1% diethyl pyrocarbonate-10 μM Tris-HCl, pH 8.0, 1 μM EDTA). Twenty μg of each RNA were electrophoresed, transferred to Hybond-N filters (Amersham Corp.), UV cross-linked, and probed with either the 3′ ends of the MEK1 or MEK2 cDNAs, or the entire MEK1 or MEK2 cDNAs, in 1% BSA, 1× TBE, 0.5× NaPO4, pH 7.2, and 7% SDS at 65°C for 18 h. The blot was then washed twice in 0.2× SSC (30 mM NaCl, 3 mM sodium citrate) and 0.1% SDS at 65°C for 20 min. Northern blots were then exposed to X-OMAT AR film (Kodak).

Tissue Lysis and Western Blotting. Tissues were isolated from embryonic or adult mice and quick-frozen in liquid nitrogen. Tissues were lysed by Dounce homogenization in potassium phosphate buffer (10 mM KPO4, pH 7.0, 1× TBE, 5× EGTA, 10 mM magnesium chloride, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 1× TDT, 0.5% NP-40, 0.1% Brij-3, 1× 4-(2-aminoethyl)benzenesulfonfylfluoride (Pefabloc SC, Boehringer Mannheim), 10 μg/ml leupeptin, and 10 μg/ml pepstatin A). Lysates were then centrifuged at 16,000 × g for 10 min. Twenty μg of the supernatants were boiled in 1× sample buffer (50 μM Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, 0.05% bromphenol blue, 1% β-mercaptoethanol) for 5 min and electrophoresed on a 10% SDS polyacrylamide gel. Proteins were transferred to polyvinylidene difluoro immobilon membrane (Millipore). For Western blots, membranes were blocked in 3% BSA in Western buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100) at room temperature for 1 h, incubated with anti-MEK1(724), anti-MEK1(329), or anti-MEK2(272) antisera for 1 h, then washed with Western buffer four times for 10 min each. Membranes were then incubated with protein A-horse-radish peroxidase or goat antimaouse-horse-radish peroxidase (Amersham) at a dilution of 1:2000 at room temperature for 25 min and washed again, four times in Western buffer for 10 min each. Membranes were then subjected to ECL (Amersham) and exposed to X-OMAT AR film (Kodak).

Immunoprecipitations and Kinase Assays. Tissues were lysed as described for Western blots, and immunoprecipitations were performed by the addition of 10 μl of anti-MEK1(724) antiserum or 1 μg of anti-MEK2 monoclonal antibody (UBI) to 400 μg of tissue lysates. Immunoprecipitates were incubated on ice for 1 h, and then protein A-Sepharose beads (Zymed) were added with rocking for 30 min. Immunoprecipitates were then washed 3 times with ST buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0). Immunoprecipitates were then incubated with 25 μM Tris-HCl, pH 7.5, 10 mM MgCl2, 100 μM ATP, and 5 μCi [32P]-ATP with 2 μg of GST-Erk 1K63M) in a total volume of 30 μl for 20 min at 30°C. Samples were then boiled in 1× sample buffer for 5 min and then electrophoresed on a SDS polyacrylamide gel. Proteins were transferred to polyvinylidene difluoro membrane and then exposed to X-OMAT AR film (Kodak).

Materials. Rabbit anti-MEK1 and anti-MEK2 sera were made against the first 32 and 36 amino acids, respectively, of the MEK1 and MEK2 proteins and fused to GST. Anti-MEK1 monoclonal 3D9 was from Zymed; anti-MEK2 monoclonal was from UBI. GST-Erk 1K63M was made as described in Alessandri et al. (25).

Acknowledgments

We are grateful to Mark Bitgood and Drs. Andrew McMahon and George Serbedzija for advice and for providing 14.5-day-p.c. mouse embryo sections.

References