Molecular Cloning of Mouse ERK5/BMK1 Splice Variants and Characterization of ERK5 Functional Domains*

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The mitogen-activated protein kinases (MAPKs) play important roles in regulation of cell growth and survival. Human MAPK 5 (ERK5) or Big MAP kinase 1 (BMK1) is a recently cloned member of the MAPK family. To identify ERK5-related kinases, we searched the GenBank® expressed sequence tag (EST) data base for mouse cDNAs with homology to human ERK5. A full-length mouse cDNA that was highly homologous to the human ERK5 was identified. Further analysis of ERK5 polymerase chain reaction products generated from mouse embryo cDNA yielded three mouse ERK5 cDNAs (mERK5a, mERK5b, and mERK5c). Sequence analysis showed that these cDNAs are alternative splice products of the mouse ERK5 gene. Interestingly, expressed mERK5b and mERK5c act as dominant negative inhibitors based on inhibition of mERK5a kinase activity and mERK5a-mediated MEF2C transactivation. However, the physiological significance of mERK5b and mERK5c is not fully understood. Further investigation using these mouse ERK5 splice variants and other constructed mutants identified functional roles of several regions of mERK5, which appear to be important for protein-protein interaction and intracellular localization. Specifically, we found that the long C-terminal tail, which contains a putative nuclear localization signal, is not required for activation and kinase activity but is responsible for the activation of nuclear transcription factor MEF2C due to nuclear targeting. In addition, the N-terminal domain spanning amino acids (aa) 1–77 is important for cytoplasmic targeting; the domain from aa 78 to 139 is required for association with the upstream kinase MEK5; and the domain from aa 140–406 is necessary for oligomerization. Taken together, these observations indicate that ERK5 is regulated by distinct mechanisms determined by its unique structure and presumably the presence of multiple splice variants.

Mitogen-activated protein kinases (MAPKs)1 are serine/threonine protein kinases that play important roles in signal transduction pathways activated by extracellular stimuli. MAPKs regulate many cellular processes, including cell proliferation, cell differentiation, cell death, and stress responses (1, 2). MAPKs constitute a superfamily of highly related serine/threonine kinases. At least seven family members of the MAPK family have been identified in mammals: ERK1/2 (extracellular signal-regulated kinase 1 and 2) (3, 4), JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase) (5–7), p38 (a mammalian equivalent of the yeast high-osmolarity glycerol kinase) (6), Big MAP kinase 1 (BMK1, also known as ERK5) (8, 9), ERK6 (mitogen-activated protein kinase 6) (10), and ERK7 (extracellular signal-regulated kinase 7) (11). MAPKs are activated by phosphorylation on threonine and tyrosine residues in a Thr-X-Tyr (TXY) motif involving upstream dual-specificity protein kinases (MAPKs) and phosphatases. The TXY activation motif is used to classify the MAPK superfamily into three main groups. The TXY (Thr-Glu-Tyr) group consists of extracellular signal-regulated kinases ERK1/2, ERK5, and ERK7. The TPY (Thr-Pro-Tyr) family consists of JNK/SAPK (5). The TGY (Thr-Gly-Tyr) family includes p38 and ERK6. Each MAPK pathway generally consists of three kinase modules composed of a MAPK, a MAPK kinase (MAPKK), and a MAPKKK kinase (MAPKKKK) (12). These kinase modules are differentially activated by a variety of cellular stimuli and contribute to distinct cellular function (13). The ERK1/2 module includes Raf isoforms, MEK1/2 and ERK1/2, which are highly responsive to mitogenic signals such as growth factors and cytokines. In contrast, JNK/SAPK and p38 are stress-sensitive pathways activated by MEK 4/7 and MEK3/6, respectively. ERK5 is specifically activated by MEK5 (14). Human ERK5 was recently cloned by two groups (8, 9). Human ERK5 contains 816 amino acid residues with a primary structure distinct from other MAPK members. ERK5 has a unique long C-tail and a distinct loop-12 domain. ERK5 is activated by reactive oxygen species (15), hyperosmolarity (16), and fluid shear stress (16). Most recently, it has been shown that ERK5 is required for EGF-induced cell proliferation and progression through the cell cycle (17). Although ERK5 has a

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; MEK, MAPK/ERK kinase; NLS, nuclear localization signal; aa, amino acid(s); JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; BMK1, Big MAP kinase 1 (also known as ERK5); EGF, epidermal growth factor; EST, expressed sequence tag; PCR, polymerase chain reaction; RT, reverse transcription; GFP, green fluorescence protein; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagles’ medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); MBP, myelin basic protein; DN, dominant negative; FAK, focal adhesion-associated protein tyrosine kinase.

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mERK5a in the PcDNA3.1/His vector as the downstream of the codon aa 539 were generated. PCR reaction was performed using the entire mERK5a in the PcDNA3.1/His vector as the template. Several PCR fragments were found in the first-strand cDNAs of a mouse embryo (purchased from CLONTECH) and were subcloned into the TA vector (Invitrogen) for sequencing. Combining analysis of mouse ERK5 RT-PCR clones with mouse EST clones, three artificial restriction sites were constructed by removing aa 407–806 with artificial restriction sites EcoRI. mERK5a(-NLS), lacking aa 505–806, was cloned into the mammalian expression vector (pmEGFP-N1, from CLONTECH) as a negative control. The pEGFP-N1 vector was digested with BamHI and EcoRI and ligated with the mERK5a(-NLS) fragment. The recombinant vector was transfected into CHO-K1 cells along with the GAL4-responsive reporter plasmid pG5E1bLUC, which contains five GAL4 sites cloned upstream of a minimal promoter driving a luciferase gene (14). For transfection, CHO-K1 cells (0.2 × 10^6 cells per well) were seeded into 24-well plates the day before transfection. Cells were transfected with 0.5 μg of DNA in total per well using LipofectAMINE Plus (Life Technologies, Inc.). The PCR reaction was performed as follows: initial denaturation at 94 °C for 3 min and 30 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 1.5 min). Products were separated by 15% SDS-PAGE, transferred to a nitrocellulose, and visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech).

Immunoprecipitation and Immune Complex Kinase Assay—Immunoprecipitation assays were performed by ectopically expressing tagged ERK5 proteins from CHO-K1 cells as previously described (19), except 2 μg of MBP was used per reaction in kinase buffer. Proteins were separated by 15% SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography. The presence of epitope-tagged proteins in immunoprecipitates was verified by Western analysis with antibody against the tag.

RNA Isolation and RT-PCR—Total RNA was prepared from multiple adult mouse tissues and mouse embryos (15 days) using an RNasey Mini Kit (Qiagen) according to the manufacturer’s instructions. Two degenerate oligonucleotide primers were designed corresponding to splicing junction sequences of three cDNAs: the sense primer 5'-ACGATGAGATCTGACGACC-3' and antisense primer 5'-GGTCAACAGGATGACGTA-3'. The first-strand cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies, Inc.) with antisense primer. The amplification was carried out in a 100-μl mixture containing 2 μl of the first-strand cDNA product, 10 μl each of the sense and antisense primer, and 5 μl of Taq DNA polymerase (Life Technologies, Inc.). The PCR reaction was performed as follows: initial denaturation at 94 °C for 3 min and 30 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, extension at 72 °C for 1.5 min). Products were separated by 15% SDS-PAGE, transferred to a nitrocellulose, and visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech).
FIG. 1. Schematic representations of mouse ERK5 splice variants and genomic structure at the splicing junction. A, schematic diagram of mouse ERK5 cDNAs. I-1, I-2, and I-3 refer to the insertions of DNA sequence. The asterisk indicates the stop codon. B, schematic diagram of partial genomic DNA flanking the splicing region of mouse ERK5 gene. The splicing donor gt sites (D1, D2, and D3) and splicing acceptor ag sites (A1 and A2) are underlined. Exon and intron regions are represented by capital and lowercase letters, respectively.

To explore the mechanism for producing these three different mouse ERK5 cDNAs, PCR and sequencing of the mouse genomic DNA flanking the splicing region of the ERK5 gene was performed. The genomic structure of the splicing junction of mouse ERK5 gene is shown in Fig. 1B. Sequence analysis of genomic DNA showed an additional 429-bp (I-3) insert with consensus “gt/ag” just after I-2. It is likely that the variations in N-terminal sequences are produced by alternatively choosing different splicing donors and acceptors within a single intron that is composed of I-1, I-2, and I-3. mERK5a is likely generated by use of the splicing donor D1 and the acceptor A2, whereas mERK5b is generated by use of D2 and A1 and mERK5c is generated by use of D3 and A2. The I-1 intron introduces a stop codon and causes mERK5b and mERK5c to have shorter N termini than mERK5a.

To confirm the presence of different species of ERK5 cDNAs in the mouse, mouse embryo first-strand cDNA (from CLONTECH) was used for PCR with primers from conserved regions of human and mouse ERK5. Sequence analysis showed that at least three different ERK5 cDNAs were present, which we will designate as mERK5a, mERK5b, and mERK5c (Fig. 1A). mERK5a is the mouse cDNA most homologous to human ERK5. mERK5b and mERK5c cDNAs contain one or two inserts compared with mERK5a, respectively. Although mERK5b has a 50-bp insert (I-1) (Fig. 1A), mERK5c, which corresponds to the mouse EST clone mentioned above, contains both the 50-bp (I-1) insert found in mERK5b and a 91-bp (I-2) insert (Fig. 1A). Both inserts start with consensus splicing donor “gt,” and the second insert (I-2) ends with an acceptor sequence “ag,” suggesting that they are likely to be intron sequences that were not spliced out.

To explore the mechanism for producing these three different mouse ERK5 cDNAs, PCR and sequencing of the mouse genomic DNA flanking the splicing region of the ERK5 gene was performed. The genomic structure of the splicing junction of mouse ERK5 gene is shown in Fig. 1B. Sequence analysis of genomic DNA showed an additional 429-bp (I-3) insert with consensus “gt/ag” just after I-2. It is likely that the variations in N-terminal sequences are produced by alternatively choosing different splicing donors and acceptors within a single intron that is composed of I-1, I-2, and I-3. mERK5a is likely generated by use of the splicing donor D1 and the acceptor A2, whereas mERK5b is generated by use of D2 and A1 and mERK5c is generated by use of D3 and A2. The I-1 intron introduces a stop codon and causes mERK5b and mERK5c to have shorter N termini than mERK5a.

Protein Sequence Analysis—mERK5a contains a putative open reading frame (ORF) from nucleotide 27 to nucleotide 2447 that encodes a protein of 806 amino acids with a predicted molecular mass of 88 kDa, whereas mERK5b (putative ORF:
nucleotides 284–2497) contains 737 amino acid with a predicted molecular mass of 80 kDa and mERK5c (putative ORF: nucleotide 864–2867) consists of 667 amino acid residues with a predicted molecular mass of 73 kDa. The predicted N-terminal amino acid sequences for the three isoforms of ERK5 are consistent with their relative mRNA levels, mERK5a weights corresponding to mERK5a, mERK5b, and mERK5c, respectively. Relative protein levels of three splice variants are consistent with their relative mRNA levels, mERK5a > mERK5c > mERK5b.

Kinase Activities and Effects of Different Mouse ERK5 Isoforms on Transactivation of the Transcription Factor MEF2C—Because mERK5b and mERK5c lack the GXXGXXG domain required for ATP binding, it is very likely that mERK5b and mERK5c have no kinase activity. To further explore the function of mERK5b and mERK5c, phosphorylation of myelin basic protein (MBP) was evaluated by an in vitro kinase assay. Immunoprecipitated mERK5a, isolated from the cells coexpressing constitutively active MEK5(D), rapidly phosphorylated MBP (Fig. 4). In contrast, mERK5b and mERK5c failed to phosphorylate MBP, which is similar to results using human dominant negative ERK5 (DN-hERK5) (14). To investigate the possibility that mERK5b and mERK5c act as dominant negative isoforms, mERK5b or mERK5c were cotransfected with mERK5a and kinase assays were performed. As shown in the last two lanes of Fig. 4, mERK5b inhibited mERK5a kinase activity. mERK5c behaved in a similar manner (data not shown).

Because the transactivation of the transcription factor MEF2C is stimulated by human ERK5-induced phosphorylation (14), we measured MEF2C activity as a means to determine the kinase activities of different ERK5 isoforms. Utilizing fusion proteins containing the transactivation domain of MEF2C fused to the DNA binding domain of the yeast tran-

Presence of Mouse ERK5 Splice Variant Expression in Mouse Embryo and Adult Mouse Tissues—To confirm the existence of the three forms of mouse ERK5 mRNAs, we performed RT-PCR with a different source of mouse embryo mRNA. Three bands corresponding to PCR products from the three recombinant cDNAs were detected (Fig. 3A). To determine the presence of three mouse ERK5 mRNAs in adult mouse tissues and examine tissue-specific expression, RT-PCR was performed for multiple adult mouse tissues. PCR products encoding the three mouse ERK5 mRNAs were detected in all mouse adult tissues examined (Fig. 3B).

To confirm the presence of the endogenous protein products corresponding to the three splice variants, we used a polyclonal antibody against ERK5, which was made using amino acids EGHGMNPADESLQREIQMDSPML of the human ERK5 as antigen. This human ERK5 peptide is 100% similar to the corresponding amino acids of the three mouse isoforms. Preliminary data showed that it recognized mouse and human ERK5 equally well. Immunoblotting of mouse embryonic proteins revealed three distinct bands (Fig. 3C) with the molecular weights corresponding to mERK5a, mERK5b, and mERK5c, respectively. Relative protein levels of three splice variants are consistent with their relative mRNA levels, mERK5a > mERK5c > mERK5b.

Presence of Mouse ERK5 Splice Variant Expression in Mouse
Splice Variants and Functional Domains of ERK5/BMK1

mERK5b and mERK5c were examined for their ability to inhibit mERK5a-induced MEF2C expression. As expected, MEF2C-dependent reporter gene expression was enhanced dramatically when mERK5a and MEK5(D) were cotransfected into CHO-K1 cells (Fig. 7A). To test whether mERK5a(-tail) was catalytically active, an Xpress-tagged ERK5 truncated at Gln-406, termed mERK5a(-tail), was generated. When mERK5a(-tail) and ERK2 amino acid sequences are aligned, the length of mERK5a(-tail) is comparable to that of ERK2. To test whether mERK5a(-tail) was catalytically active, an in vitro kinase assay using MBP as a substrate was performed. Using MEK5(D) to activate ERK5, mERK5a(-tail) was able to phosphorylate MBP in vitro similar to mERK5a (Fig. 7A, upper panel). These results suggest that the kinase activity of mERK5a(-tail) is comparable to mERK5a.

Another indication of kinase activity is autophosphorylation. The anti-Xpress antibody recognized both the full-length mERK5a and the truncated mERK5a(-tail) in cell lysates from transfected CHO-K1 cells, suggesting that mERK5a(-tail) protein was stably expressed (Fig. 7A, lower panel). Upon activation by coexpression of MEK5(D), mERK5a(-tail) exhibited an electrophoretically shifted band similar to the full-length mERK5a (Fig. 7A, lower panel), suggesting that mERK5a(-tail) was phosphorylated by MEK5. The shifted band of the full-length ERK5 is consistent with the phosphorylation of ERK5 and is generally thought to correspond to the activated form (14, 15). Together, these data indicate that the C-terminal domain was not needed for kinase activity in vitro (9).

Finally the effects of mERK5a(-tail) on the activity of transcription factor MEF2C fusion protein were assessed by meas-
uring the luciferase activity from CHO-K1 cells cotransfected with a construct containing five copies of the GAL4-binding site upstream of a luciferase reporter gene. As expected, MEF2C-dependent reporter gene expression was enhanced dramatically when the full-length mERK5a and MEK5(D) were cotransfected into CHO-K1 cells (Fig. 7B). However, the mERK5a(-tail), which can act as an active kinase, did not stimulate MEF2C activity (Fig. 7B).

The C-terminal Tail Containing the NLS Plays a Role in Nuclear Translocation of ERK5—Like other MAPKs, ERK5 is localized in cytoplasm in the unstimulated state and translocates into the nucleus upon activation (14). A possible role for the C-terminal domain is to facilitate the translocation of activated ERK5 to the nucleus, because this domain is required for the transactivation of the transcription factor MEF2C and it has a possible nuclear localization sequence. To explore this possibility, immunocytochemistry was performed to test whether the C-terminal domain influences the nuclear translocation of mERK5a. In the absence of MEK5(D), cells expressing mERK5a or mERK5a(-tail) had predominantly cytoplasmic staining (Fig. 8, A and C). In the presence of MEK5(D), a significant amount of mERK5a localized in the nucleus (Fig. 8B). In contrast, mERK5a(-tail) was unable to translocate to the nucleus (Fig. 8D). In CHO cells, recombinant GFP was distributed in both the cytoplasm and the nucleus (Fig. 8E). However, GFP-tail, a fusion protein containing the GFP and the C-tail of mERK5a (aa 407–806), was exclusively localized in the nucleus (Fig. 8F), suggesting that the C-tail of mERK5a is able to drive GFP to the nucleus.

Searching the mouse ERK5 sequence against a currently available profile data base, a putative bipartite nuclear localization signal (NLS) (aa 505–539) was found in the C-tail. To determine whether the putative NLS is biologically important, GFP-NLS, a fusion protein containing GFP and the NLS domain of mERK5a (aa 505–539), as well as mERK5a(-NLS), an NLS-lacking mutant of mERK5a (deleting aa 505–539), were constructed. As expected, the NLS domain was able to drive GFP to the nucleus (data not shown). In contrast, mERK5a(-NLS), lacking the NLS domain, was localized in the cytoplasm under unstimulated conditions (Fig. 8G) but failed to move to the nucleus efficiently upon activation by MEK5(D) (Fig. 8H), indicating that the NLS domain is biologically active (Fig. 8, compare B and H). Taken together, these findings suggest that
translocation into nucleus via the NLS in the C-terminal tail is essential for biological activity of ERK5 in vivo.

The N-terminal Domain of ERK5 Is Responsible for Its Oligomerization and Association with MEK5—It has been reported that ERK1/2 oligomerizes upon phosphorylation. To determine whether ERK5 oligomerizes, we coexpressed Xpress-tagged mouse ERK5 (Xp-mERK5a) and FLAG-tagged mouse ERK5a (FLAG-mERK5a) in CHO-K1 cells and performed coimmunoprecipitation assays. Immunoprecipitation of Xp-mERK5a with the Xpress antibody brought down FLAG-mERK5a (Fig. 9A) and vice versa (data not shown), suggesting that ERK5a forms oligomers in the cells. Oligomerization of ERK5 was observed in cell lysates from both activated and control cells, because coimmunoprecipitation was found in cells with or without expression of MEK5D, different from reported data for ERK1/2 (20). The observation, that the wild type FLAG-mERK5a and truncated Xp-mERK5a(-tail) could also be coimmunoprecipitated (Fig. 9B) but tail-GFP could not be coimmunoprecipitated (data not shown), suggests that the N-terminal domain but not the C-terminal domain of ERK5 is involved in the oligomerization. Furthermore, the N-terminal truncated isoforms, mERK5b and mERK5c, were able to be coimmunoprecipitated with wild type mERK5a (Fig. 9B), suggesting that the region aa 140–406 but not the region aa 1–139 of the N terminus of ERK5 is important for the oligomerization.

The ability of the N-terminal or C-terminal truncated ERK5 mutants to bind to MEK5 was also examined by coimmunoprecipitation. The observation that mERK5a(-tail) and mERK5b but not mERK5c were able to bind to MEK5 (Fig. 10) indicates that the region aa 78–139 in the N-terminal domain is important for the association of ERK5 with MEK5.

**DISCUSSION**

In this report we have identified three differentially spliced mouse ERK5 cDNAs (mERK5a, mERK5b, and mERK5c), which appear to play unique functional roles in regulating ERK5 and MEF2C activity. mERK5b and mERK5c function as dominant negative kinases blocking mERK5a activity and ERK5-mediated MEF2C activation. In addition, we have investigated the functional roles of several regions of mouse ERK5, which appear to be important for protein-protein interactions and intracellular localization. Specifically, we found that the N-terminal domain aa 1–77 is important for the upstream kinase MEK5; and domain aa 140–406 is necessary for oligomerization. The C-terminal tail, which contains a putative NLS, was found to be required for nuclear translocation of mERK5a upon activation (Fig. 11).

We identified three mouse ERK5 cDNAs by homology analysis, mERK5a (a mouse homologue of human ERK5), and two truncated mouse ERK5 isoforms termed mERK5b and mERK5c. Analysis of mouse genomic DNA sequences adjacent to the splicing junctions suggests that the three mERK5 cDNAs are generated by alternative splicing using different splicing donors and acceptors from a single gene encoding mouse ERK5. Several different ERK5 transcripts have been shown in human tissue (8), but these were generated by alternative splicing occurring at the 5′-noncoding region. The sequence of mERK5a is the same as that of the mouse ERK5 reported previously by Kamakura et al. (21) except for six scattered amino acid mismatches within the entire open reading frame. Possible explanations are a sequencing error or a DNA polymorphism causing the sequence variation.

The most abundant cDNA was mERK5a, which shares 91% identity with human ERK5. Compared with mERK5a, mERK5b and mERK5c lacked 69 and 139 amino acids at their N terminus, respectively. It appears likely that mERK5b and mERK5c perform functions different from mERK5a, because these proteins are unable to bind ATP and therefore are not active kinases. Our observations, that mERK5b and mERK5c lack kinase activity, inhibit mERK5a kinase activity, and inhibit mERK5a-mediated MEF2C transactivation, suggest that mERK5b and mERK5c may act as endogenous dominant negative kinases if mERK5b and mERK5c are expressed endogenously to a significant extent under some conditions. Immunoblotting results indicate that mERK5a is expressed to a greater extent than mERK5c and much greater than mERK5b. It is possible that the proteins encoded by mERK5b and mERK5c mRNAs could be expressed to different extents under some conditions or selectively expressed in some cell types. It is also possible that the mRNAs of mERK5b and mERK5c are not efficiently translated to the protein products in vivo as predicted, because the ribosome may initiate and terminate the translation early by the stop codon in the first insert. Thus, the expression of alternatively spliced mERK5b and mERK5c mRNAs may provide a mechanism to regulate ERK5 protein expression by preventing the translation of ERK5 in some cells at specific developmental stages or pathological conditions. Future studies are necessary to clarify the biological signifi-
ncence of mERK5b and mERK5c mRNAs in the regulation of mERK5a function.

Several other kinases exhibit independent expression of non-cata
dalytic domains resulting from alternative splicing, which function as endogenous dominant negative inhibitors. For ex-

ple, the primary transcript of a calmodulin-dependent protein
kinase is alternatively spliced to generate mRNAs encoding
either the full-length kinase or the calmodulin binding
domain alone (22). Focal adhesion-associated protein tyrosine
kinase (FAK) has an independent, C-terminal, noncatalytic
domain (FRNK, FAK-related nonkinase) (23). Finally, the C
terminus of the smooth muscle myosin light chain kinase is
also expressed as an independent protein, telokin (24). Thus,

the truncated, catalytically inactive forms of the mouse ERK5,
mERK5b, and mERK5c may function as endogenous dominant
negative inhibitors if they are expressed endogenously to a
significant extent under some conditions.

Searching the mouse ERK5 sequence against a currently
available profile data base to identify known functional re-
gions, a proline-rich region and a NLS were found in the C-
terminal domain. Proline-rich regions exist widely in both pro-
karyotes and eukaryotes. Studies have shown that proline-rich
regions may act as Src-homology 3-binding motifs. These re-
gions can directly interact with other proteins containing Src-
homology 3 domains to regulate cellular localization and/or
modulate enzymatic properties (1, 2). A proline-rich sequence
unique to MEK1 and MEK2 is required for Raf binding and
MEK function (25). The function of proline-rich regions re-
mains largely unclear. Interestingly, a significant difference
between mouse ERK5a and human ERK5 sequences is present
in the proline-rich region. Thus, the cloned mouse ERK5a,
which differs significantly only in the proline-rich region com-
pared with the human ERK5, may be a useful gene to deter-
nine the function of proline-rich regions. Differences between
human and mouse with regard to intracellular signal transduc-
tion by highly related proteins have been reported (26, 27).

For example, the BAS-like Fas-associated phosphatase-1 inter-
acts with the human Fas receptor, but not with the mouse Fas
receptor. The C terminus of the Fas receptor, which is required
for this interaction, is not conserved between mouse and hu-
man. Elucidation of the role of the mouse ERK5 proline-rich
region may also reveal differences in signaling mechanisms
between human and mouse.

The putative bipartite NLS in ERK5 located in the C-termi-
nal tail is very likely to be important for nuclear translocation
of ERK5. A bipartite NLS was described initially in the nucleo-
some assembly factor nucleosomin, which consists of two
basic amino acids, a spacer region of any 10–12 amino acids,
and a basic cluster in which at least three out of the next five
amino acids must be basic (28). Although the bipartite motif is
a considerably more reliable indicator of nuclear localization,
because less than 5% of non-nuclear proteins have a sequence
that fits this motif (29), it is important to demonstrate that the
putative NLS is necessary for nuclear targeting of the parent
protein and sufficient to direct a non-nuclear protein to the
nucleus. Our observations, that the NLS of mERK5 is required
for the nuclear targeting of mERK5 upon activation and that
this NLS itself is sufficient to drive GFP to the nucleus, indi-
cate that the mERK5 NLS is biologically functional.

The mechanisms for the nuclear translocation and cytoplas-
mic anchoring of ERK1/2 must be different from ERK5. It has
been suggested that ERK1/2 could cross the nuclear envelope
by passive diffusion if it does not have either a NLS or a nuclear
export signal. The cytoplasm retention of ERK1/2 in unstimu-
lated cells likely involves specific association with MEK1/2, and
nuclear translocation of ERK1/2 upon stimulation is accompa-
 nied by dissociation from MEK1/2 (30). A nuclear export signal
in the N terminus of MEK1/2 has also been identified (31).
These data indicate that MEK1/2 is a cytoplasmic anchoring
protein for ERK1/2. The molecular size of ERK5 is beyond the
limit for passive diffusion through the nuclear envelope pore.
The nuclear targeting of ERK5 seems to be mediated by the
NLS. The observation that mERK5b or mERK5c was present in
the nuclear portion suggest that the N-terminal domain aa
1–77 is responsible for the cytoplasmic localization. The role for
the N-terminal domain aa 1–77 in cytoplasmic localization of
mERK5 is not clear. It is possible that the N-terminal domain
aa 1–77 associates with other cytoplasmic components other
than MEK5.

ERK5, unlike ERK1/2, exists as oligomer in unstimulated
cells. ERK1/2 oligomerizes in a phosphorylation-dependent
manner (32). In addition, ERK1/2 dimers are composed of ei-
ther two phosphorylated molecules or one phosphorylated
and one unphosphorylated molecule. In contrast, ERK5 expres-
sed from transfected plasmids was able to form oligomers in both
stimulated and unstimulated conditions, suggesting that the
oligomerization of ERK5 is not dependent on its phosphyra-
lation status. We found that the region aa 140–406 in ERK5 is
important for oligomerization, homologous to the dimer inter-
face of ERK1/2, which is localized to amino acids 170–359 (32).

Among the MAPKs, ERK1/2, ERK5, and ERK7 share the
same signature TEY activation motif. However, these three
MAPKs are completely different in terms of their structure,
activation, and mechanisms of regulation, mediated in part by
the fact that ERK5 and ERK7 have unique C-terminal tails
compared with ERK1/2. Activation by TEY phosphorylation
of ERK2 leads to nuclear import even though ERK1/2 does not
have any classic nuclear localization signal. It has been sug-
gested that interaction of ERK1/2 with upstream kinases may
control nucleocytoplasmic transport (30). ERK7 is a constitu-
tively activated and permanently nuclear-localized enzyme.
Its activation and kinase activity seem to be dependent on nuclear
targeting, which requires the C-terminal tail (11). In the case of
ERK5, nuclear translocation occurs upon activation. However,
its activation and kinetic activity are not dependent on its
C-terminal tail.

In summary, we have identified three differentially spliced
mouse ERK5 cDNAs whose unique structures result in differ-
ent roles in the regulation of ERK5 and MEF2C activity. Using
these mouse ERK5 splicing variants and other constructed
mutants, we have also located regions of ERK5 that are respon-
sible for cytoplasmic targeting, nuclear translocation, oligo-
merization, and MEK5 binding. Further studies are required to
characterize in detail the precise role of these three isoforms in
the specific function of ERK5.

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Fig. 11. Schematic diagram showing the organization of func-
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