ERK8, a New Member of the Mitogen-activated Protein Kinase Family*

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The ERKs are a subfamily of the MAPKs that have been implicated in cell growth and differentiation. By using the rat ERK7 cDNA to screen a human multiple tissue cDNA library, we identified a new member of the ERK family, ERK8, that shares 69% amino acid sequence identity with ERK7. Northern analysis demonstrates that ERK8 is present in a number of tissues with maximal expression in a kidney and lung. Fluorescence in situ hybridization localized the ERK8 gene to chromosome 8 band q24.3. Expression of ERK8 in COS cells and bacteria indicates that, in contrast to constitutively active ERK7, ERK8 has minimal basal kinase activity and a unique substrate profile. ERK8, which contains two SH3-binding motifs in its C-terminal region, associates with the c-Src SH3 domain in vitro and co-immunoprecipitates with c-Src in vivo. Co-transfection with either v-Src or a constitutively active c-Src increases ERK8 activation indicating that ERK8 can be activated downstream of c-Src. ERK8 is also activated following serum stimulation, and the extent of this activation is reduced by pretreatment with the specific Src family inhibitor PP2. The ERK8 activation by serum or Src was not affected by the MEK inhibitor U0126 indicating that activation of ERK8 does not require MEK1, MEK2, or MEK5. Although most closely related to ERK7, the relatively low sequence identity, minimal basal activity, and different substrate profile identify ERK8 as a distinct member of the MAPK family that is activated by an Src-dependent signaling pathway.

The mitogen-activated protein kinases (MAPKs)1 are a superfamily of serine/threonine protein kinases that are evolutionarily conserved and whose members have been implicated in key cellular processes including cell proliferation, differentiation, apoptosis, and stress responses (1–4). Regulation of the activity of MAPKs involves phosphorylation of the threonine and tyrosine residues in a Thr-X-Tyr (TXY) motif within the kinase domain. This canonical MAPK TXY motif has also been used to classify MAPKs into three major groups. Members of one group, the extracellular signal regulated kinases (ERKs), can be identified by their TEY (Thr-Glu-Tyr) activation motif and include ERK1, ERK2, ERK5, and ERK7. Recently, p97, an ERK5-related kinase identified through affinity purification that is selectively recognized by anti-pT(PEpY) antibodies, was shown to be involved in a signaling pathway leading to differentiation (5). ERK1 and ERK2 were the first members of the ERK family to be identified and are the most widely studied. The 44-kDa ERK1 and the 42-kDa ERK2 have been implicated in cellular pathways that mediate growth factor regulation of proliferation and/or differentiation in most cell types (2, 3).

The recently identified ERK5 and ERK7 are significantly larger than the originally identified ERK1 and ERK2 due to an extended C-terminal domain. ERK5, also known as big mitogen-activated kinase 1 (6, 7), is 110 kDa, and ERK7 is 61 kDa. Recent information indicates that the C-terminal regions of these ERKs have important regulatory functions. The C-terminal region of ERK5 appears to regulate negatively its kinase activity (6) and contains a putative bipartite nuclear localization signal required for nuclear translocation of ERK5 in vivo following activation (8). The C-terminal region of ERK5 also contains a myocyte enhancer-binding factor 2-interacting region and a potent transcriptional activation domain (9). ERK7 is activated by autophosphorylation, which is regulated through interactions with the C-terminal domain (10). In addition, the C-terminal region is required for the ability of ERK7 to localize to the nucleus and inhibit growth (11). The recent identification and characterization of these larger ERKs suggest that there may be additional members of the ERK family with extended C-terminal regions.

To explore this possibility, we screened a multiple human tissue library using ERK7 cDNA as a probe, and we identified a protein kinase with the characteristic TEY activation motif. The relatively low sequence identity shared between this new ERK, termed ERK8, and the previously identified rat ERK7 suggests that it is a novel MAPK. In contrast to ERK7, ERK8 does not display significant constitutive kinase activity, does not phosphorylate c-Fos, and is activated in response to stimulation by Src or serum. These divergent biochemical characteristics indicate that ERK8 may play a different role in cellular responses to extracellular signals. Further characterization of ERK8 should provide insights into the roles of this kinase in normal and pathological cell function.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AY065978.

1 The abbreviations used are: MAPKs, mitogen-activated protein kinases; ERKs, extracellular signal-regulated kinases; BSA, bovine serum albumin; MBP, myelin basic protein; HA, hemagglutinin; FBS, fetal bovine serum; PBS, phosphate-buffered saline; T/LB, Triton-based lysis buffer; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; pAb, polyclonal antibody; EST, expressed sequence tags; SH, Src homology.

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teristics confirm that ERK8, although most closely related to ERK7, represents a new member of the ERK family.

EXPERIMENTAL PROCEDURES

Materials

Bovine serum albumin (BSA), myelin basic protein (MBP), triethylamine, glycine, glycerol, peroxidase-conjugated goat anti-rabbit IgG, peroxidase-conjugated goat anti-rat IgG, and peroxidase-conjugated goat anti-mouse IgG were purchased from Sigma. Fibronectin and collagen were purchased from BD PharMingen. Dulbecco’s modified Eagle’s medium, modified Eagle’s medium, FBS, trypsin, penicillin, streptomycin, and the eukaryotic TA cloning kit were purchased from Invitrogen. Protein A-Sepharose was purchased from RepliGen Corp. (Needham, MA). Protein G-Sepharose, CNBr-activated Sepharose, the unique site elimination mutagenesis kit, and the GST purification system was purchased from Amersham Biosciences. The Human Universal cDNA Library was purchased from Stratagene (La Jolla, CA). The Marathon-ReadyTM Human Testis cDNA, Advantage-HF 2, and Advantage-GC 2 PCR kits were purchased from CLONTECH (Palo Alto, CA). High affinity rat monoclonal antibody (3F10) against the hemagglutinin (HA) epitope was purchased from Roche Diagnostics. Mouse monoclonal antibody (HA.11) against the HA epitope was purchased from Covance Research Products, Inc. (Richmond, CA). Affinity-purified peroxidase-conjugated goat anti-rabbit IgG was purchased from Jackson ImmunoResearch. Phospho-p44/42 MAPK polyclonal antibody and GST-ATF2 were purchased from Cell Signaling Technology (Beverly, MA). Enhanced chemiluminescence reagents and [γ-32P]ATP (6000 Ci/mmol) were purchased from PerkinElmer Life Sciences. PCR and sequencing primers were synthesized by the University of Chicago Oligonucleotide Facility or purchased from Integrated DNA Technolo-

FIG. 1. Primary structure of ERK8. The nucleotide and deduced protein sequence of ERK8 cDNA. The threonine and tyrosine residues within the activation motif are marked with asterisks. The two putative SH3-binding motifs, PXXP, within the C-terminus are underlined.

| Table I | Sequences at the exon-intron junctions of the partially processed ERK8 clone |
|---|---|
| Exhibit | Nucleotide Position in ERK8 (bp) | Intron Length (bp) | Sequence at Exon-Intron Junction |
| 1-4 | 185 | RIV | T1CAG/CACAG |
| 7 | 747 | 至SNR | CCG/GACAGAC |
| 8 | 415 | 至SNR | CCG/GACAGAC |
| 9-14 | 780 | 至SNR | CCG/GACAGAC |
gies (Coralville, IA). The University of Florida DNA Sequencing Core Lab (Gainesville, FL) and the University of Chicago Cancer Research Center DNA Sequencing Facility performed the sequencing.

Methods

Library Screening—The entire rat ERK7 cDNA (GenBank™ accession number AF078798) was used to screen the Stratagene Human Universal cDNA Library array according to the manufacturer’s instructions. An identified 3208-bp clone was found to contain four introns and a partial coding region. 5'-Rapid amplification of cDNA ends and PCR were used to obtain the full-length ERK8 cDNA from human testis first strand cDNAs (purchased from CLONTECH). The sequence of the full-length clone was verified by sequence comparison to the putative coding region of the original 3208-bp clone.

Tissue Northern Analysis—ERK8 cDNA was radiolabeled with 32P using a nick translation method and was used to probe a poly(A)+ RNA human multiple tissue Northern blot (purchased from CLONTECH) using ExpressHyb hybridization solution according to the manufacturer’s instructions (CLONTECH). The blot was washed extensively with 0.1x SSC (15 mM NaCl, 1.5 mM sodium citrate) -0.1% SDS at 65 °C, and exposed to x-ray film at -80 °C.

Table II

Sequences at the exon-intron junctions of ERK8

The nucleotide position corresponds to the cDNA sequence beginning at the start codon and indicates the position of the first nucleotide of the exon. The exon and intron base pair lengths are listed. The exon coding sequences are in capital letters. The deduced amino acid single-letter code is indicated above the first nucleotide of the corresponding codon. The GT-AG consensus splice donor-acceptor sites are underlined.

| Exon | Exon Length (bp) | Nucleotide Position in cDNA | Intron Length (bp) | Sequence at Exon-Intron Junction |
|------|------------------|-----------------------------|-------------------|----------------------------------|
| 1    | 96               | 1267                        |                   | M TV ... G Q G                     |
| 2    | 69               | 254                         |                   | G D A Q G A G G A G A G A G A       |
| 3    | 106             | 135                         |                   | F L L Q A G G A G A G A G A G A     |
| 4    | 96               | 472                         |                   | E F G ... F M G                     |
| 5    | 287             | 87                          |                   | T D L ... D Q R G                   |
| 6    | 414             | 185                         |                   | P S N ... S R R                     |
| 7    | 582             | 577                         |                   | E F T L F G A G A G A G A G A G A    |
| 8    | 78              | 159                         |                   | R Q ... V Q R G                     |
| 9    | 918             | 125                         |                   | P H C ... V Y Q G                   |
| 10   | 1024            | 137                         |                   | M E L ... E E G                     |
| 11   | 1255            | 78                          |                   | F L L E G A G A G A G A G A G A G    |
| 12   | 1530            | 194                         |                   | V F P H V A G A G A G A G A G A G A   |

Plasmid Construction and Preparation—A hemagglutinin antigen tag, YPYDVPDY, was inserted immediately following the start methionine of ERK8 using PCR and was verified by sequencing. The HA-ERK8 was subcloned into the mammalian expression vector pCRII.1 (Invitrogen). Unique site elimination mutagenesis (12) was performed to generate ERK8 mutants (K42R, T175A, Y177F, and T175A/Y177F). The mutated sequences were verified by sequencing. The ERK7 constructs were prepared as described previously (11). The c-Src, kinase-deficient c-Src (K295R), constitutively active c-Src (Y527F), and v-Src plasmids were a gift from D. Foster (13–15). The pCMV5c-Src plasmid was a gift from J. Brugge. Plasmid DNAs were prepared by CsCl-ethidium bromide gradient centrifugation or by purification through columns according to the manufacturer’s instructions (Qiagen, Chatsworth, CA).

Cell Culture—COS cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). Quiescent cells were obtained by incubating cells in serum-free medium supplemented with 0.5% BSA for at least 18 h. 16HBE14o—human bronchial epithelial cells were a gift from D. Gruenert (16). The 16HBE14o—cells were cultured on tissue culture plasticware coated with fibronectin, collagen, and BSA. The cells were grown in modified Eagle’s medium supplemented with 10% FBS, l-glutamine (4 mM), and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). Both cell types were maintained at 37 °C in a 95% air, 5% CO2 atmosphere.

FIG. 2. Sequence alignment of ERK8 with ERK7. Computer-generated alignment (Vector NTI, InforMax, Bethesda, MD) of ERK8 and ERK7. Roman numerals indicate the 11 conserved protein kinase domains. The GenBank™ accession number for ERK7 is AF078798.
ERK8 was performed as described previously (17). Proteins were transferred to a nitrocellulose membrane. Membrane resolved on an 8 or 10% acrylamide separating gel by SDS-PAGE. The presence of HA-tagged or TEY-phosphorylated proteins in the scintillation counter or by the STORM system (Molecular Dynamics). Quantitation of substrate phosphorylation was determined either by gel. The gels were either stained with Gelcode Blue (Pierce) and dried using the 1896 base pair ERK8 cDNA sequence (GenBank™ accession number AY065978).

**TABLE III**

| GenBank™ accession no. | Tissue source | EST length | ERK8 position | Score (bits) | E value |
|------------------------|--------------|------------|---------------|-------------|---------|
| AL37138                | Fetal brain  | 858        | 812–1515      | 1330        | 0.0     |
| AL37137                | Fetal brain  | 827        | 913–1538      | 1017        | 0.0     |
| BE464560               | Wilms' tumor | 513        | 1363–1875     | 1017        | 0.0     |
| AI049667               | Wilms' tumor | 407        | 1485–1873     | 785         | 0.0     |
| AI476756               | Fetal lung, testis, B-cell | 378        | 1506–1875     | 733         | 0.0     |
| AJ403115               | Larynx carcinoma | 378        | 1063–1440      | 718         | 0.0     |
| AI921666               | Pancreatic adenocarcinoma | 429        | 1490–1875     | 680         | 0.0     |
| BI916334               | Fetal brain  | 577        | 12–521        | 609         | e-171   |
| AI050380               | Uterus adenocarcinoma | 341        | 1557–1875     | 601         | e-169   |
| BI016617               | Lung tumor   | 379        | 1523–1826     | 539         | e-150   |
| AA284718               | Ovarian tumor | 255        | 1620–1872     | 255         | e-128   |

**FIG. 4.** ERK8 is localized to chromosome band 8q24.3. *In situ* hybridization of a biotin-labeled ERK8 probe to human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes. The chromosome 8 homologues are identified with *arrows*; specific labeling was observed at 8q24.3. The inset shows partial karyotypes of two chromosome 8 homologues illustrating specific labeling at 8q24.3 (*arrowheads*). Images were obtained using a Zeiss Axioshot microscope coupled to a cooled charge coupled device camera. Separate images of 4,6-diamidino-2-phenylindole dihydrochloride-stained chromosomes and the hybridization signal were captured and merged using image analysis software (IP Lab Spectrum).
RESULTS

Identification of the ERK8 Sequence—The ERK8 cDNA is 1896 bp long and contains a 544-codon open reading frame (1632 bp) (Fig. 1). Initially, we identified ERK8 by hybridization of rat ERK7 cDNA with a 3208-bp clone from the Stratagene Human Universal cDNA Library. Sequence analysis of this clone indicated that it contained three introns and 537 codons (of 544 codons) suggesting that it represented immature mRNA (Table I). The full-length ERK8 cDNA was obtained through a combination of 5′-rapid amplification of cDNA ends and PCR using human testis first strand cDNA as a template. A BLAST search (19) of the Celera Consensus Human Genome data base did not identify any matches. However, a Blast search (19) of the National Center for Biotechnology Information public human genome data base did not identify any matches. Hence, the human ERK8 gene consists of 14 exons (Table II).

The overall amino acid identity of the human ERK8 and rat ERK7 sequences is 69% (Fig. 2). Comparison of the kinase domains reveals a sequence identity of about 82%, whereas the amino acid sequence identity of the C-terminal regions is only 53%. By contrast, sequence identity between other ERK orthologues is significantly higher. Rat and human ERK1 and ERK2 share 97 and 99% overall sequence identity, respectively. Even mouse and human ERK5 share 92% sequence identity (20).

Therefore, sequence comparison of ERK8 and ERK7 suggests that they are related, but distinct, members of the ERK family.

FIG. 5. ERK8 is expressed in mammalian cells and is a functional kinase. A, whole cell lysates from a human bronchial epithelial cell line, 16HBE14o, and from COS cells transfected with ERK8 were prepared and analyzed by immunoblotting with the affinity-purified anti-ERK8 polyclonal antibody with (+) and without (−) ERK8 blocking peptide at a final concentration of 10 μM. B, whole cell lysates from 16HBE14o− cells were analyzed by immunoblotting with the peroxidase-conjugated, affinity-purified anti-ERK8 antibody with (+) and without (−) ERK8 blocking peptide at a final concentration of 10 μM. C, COS cells were transfected with pcDNA3, HA-ERK8, HA-ERK7, and their kinase-inactive mutants. The kinase-inactive ERK8 mutant, K42R, and the kinase-inactive ERK7 mutant, K43R, were made by replacing the highly conserved lysine in the ATP-binding region with arginine. Lysates from transfected cells were prepared and analyzed by immunoblotting with either the anti-HA 3F10 monoclonal antibody (top) or the anti-phospho-ERK antibody (middle and bottom). A longer exposure of the anti-phospho-ERK Western analysis is depicted at the bottom. D, COS cells were transiently transfected either with pcDNA3, HA-ERK8, K42R, or the HA-ERK8 T20Y mutants. Lysates from transfected cells were prepared and analyzed by immunoblotting with either the anti-HA 3F10 monoclonal antibody (top) or the anti-phospho-ERK antibody (bottom). E, COS cells were transiently transfected either with pcDNA3, HA-ERK8, K42R, HA-ERK7, or K43R and lysed with 1% TLB. Enzyme activity of proteins immunoprecipitated using the anti-HA monoclonal antibody (HA.11) was measured by an in vitro kinase assay using 2 μg of GST-c-Fos as a substrate (top). The immunoprecipitated proteins were analyzed by Western blotting with the anti-HA 3F10 monoclonal antibody (bottom). F, COS cells were transiently transfected either with pcDNA3, HA-ERK8, or K42R and lysed with 1% TLB. The ability of proteins immunoprecipitated using the anti-HA monoclonal antibody (HA.11) to autophosphorylate was measured by an in vitro kinase assay. The data shown are representative of three experiments.
Tissue Distribution of Human ERK8—The tissue distribution of human ERK8 was determined by Northern analysis of poly(A)$^+$ RNA from multiple human tissues (Fig. 3). Three transcript sizes of 5.3, 3.5, and 2.0 kb were identified that could represent immature mRNA. ERK8 mRNA appears to be expressed in multiple tissue types, and, of those evaluated, it is most highly expressed in lung and kidney. A Blast search (19) of the National Center for Biotechnology Information public human expressed sequence tags (ESTs) database revealed several ESTs with high sequence identity with ERK8 (Table III). These ESTs were generated from a variety of tissue sources and like the Northern analysis suggest that ERK8 is expressed in multiple tissues.

Mapping of ERK8 to Chromosome Band 8q24.3—To localize the ERK8 gene, we performed fluorescence in situ hybridization of a biotin-labeled ERK8 probe to normal human metaphase chromosomes. Hybridization of the ERK8 cDNA probe resulted in specific labeling only of chromosome 8 (Fig. 4). Specific labeling of 8q24.2–24.3 was observed on four (6 cells), three (13 cells), or two (6 cells) chromatids of the chromosome 8 homologues in 25 cells examined. Of 75 signals observed, 5 (7%) signals were located at 8q24.2, and 70 (93%) signals were located at 8q24.3. No background signals were observed at other chromosomal sites. We observed specific signal at 8q24.3 in an additional hybridization experiment using this probe. These results suggest that the ERK8 gene is localized to chromosome 8, band q24.3.

Human ERK8 Expression—To verify that ERK8 is expressed in cells, an antibody against a 14-amino acid peptide from the C-terminal region of ERK8 was generated. Western analysis of a human bronchial epithelial cell line, 16HBE14o (16), using this antibody revealed the presence of a band of 60-kDa (Fig. 5, A and B). Recognition of this band by Western analysis was prevented when the anti-ERK8 antibody was incubated with the ERK8 peptide (Fig. 5, A and B) indicating that endogenous ERK8 is expressed in this human lung cell line. Comparison of the signal strength of endogenous ERK8 to that of exogenously expressed ERK8 (Fig. 5A) suggests that endogenous ERK8 is expressed at a very low level in this cell type. Western analysis of primary human bronchial epithelial cells also revealed an extremely low level of endogenous ERK8 (data not shown).

To study ERK8 in intact cells, an HA-tagged human ERK8 construct and the HA-ERK8 K42R kinase-deficient mutant were expressed in COS cells. For comparison, the wild-type and kinase-deficient HA-ERK7 were also expressed. Both HA-ERK8 and HA-ERK8 K42R migrated on SDS-PAGE as a single band. Figure 6 illustrates the bacterial expression and purification of GST-ERK8 and its variants. The middle panel of Figure 7 demonstrates the association of ERK8 with c-Src. Figure 7 also shows the results of an immunoprecipitation experiment (A) and Western blot analysis (B).
band of ~60-kDa when analyzed by immunoblotting with the 3F10 anti-HA monoclonal antibody (Fig. 5C, top). In contrast, wild-type ERK7 is typically detected as a doublet suggesting that it is multiply phosphorylated (Fig. 5C, top). Western analysis using the anti-phospho-ERK polyclonal antibody (pAb) revealed that only ERK7 is TEY-phosphorylated to a significant degree (Fig. 5C, middle). The wild-type ERK8 could be identified by Western analysis with the anti-phospho-ERK pAb only with significantly longer exposure times (Fig. 5C, bottom) or increased protein amounts, and neither kinase-deficient mutant cross-reacted even after prolonged exposure. These findings are consistent with our data published previously (10, 11).
indicating that ERK7 has significant constitutive activity. Therefore, the difference in recognition by the anti-phospho-ERK pAb suggests that the basal activity level of ERK8 is significantly lower than that of ERK7.

To determine the specificity of the anti-phospho-ERK pAb for the dually phosphorylated TEY ERK8, the TEY activation domain was mutated either by changing the threonine residue to alanine (T175A), changing the tyrosine residue to phenylalanine (Y177F), or mutating both residues to T175A/Y177F. COS cells were transfected either with pcDNA3, HA-ERK8, or the mutant forms of ERK8. Western analysis of lysate from transfected cells revealed expression of all the mutants (Fig. 5D, top). Under identical conditions, GST-c-Fos, a known substrate for ERK8, was phosphorylated by immunoprecipitated HA-ERK7 but not HA-ERK8. However, HA-ERK8 was able to autophosphorylate under these conditions (Fig. 5F) indicating that it is a functional kinase.

Because GST-c-Fos-(210–313) is not a substrate for ERK8, identification of an in vitro substrate was attempted using bacterially expressed GST-HA-ERK8 and GST-HA-ERK8 K42R. Despite the use of protease inhibitors, both GST-HA-ERK8 and GST-HA-ERK8 K42R were highly degraded when purified from bacteria (Fig. 6A, top). Bacterially expressed GST-HA-ERK7 and its kinase-deficient mutant were similarly degraded (Fig. 6A, top). Despite being degraded, both GST-HA-ERK8 and GST-HA-ERK7 were recognized by the anti-phospho-ERK pAb (Fig. 6A, bottom). Similar to results obtained with the mammalian expression system, GST-HA-ERK8 was recognized with significantly lower affinity. The ability of GST-HA-ERK8, but not the GST-HA-ERK8 K42R mutant, to autophosphorylate (Fig. 6B, top) and phosphorylate MBP (Fig. 6B, bottom) indicates that it is a functional kinase when expressed in bacteria. However, bacterially expressed GST-HA-ERK8 did not phosphorylate GST-c-Fos (Fig. 6B, top) or c-Fos cleaved from the GST tag by thrombin (data not shown). Additional substrates tested that were not efficiently phosphorylated by bacterially expressed GST-HA-ERK8 included GST-c-Jun-(1–93), histone H1, Ets-1, GST-c-Myc, GST-c-Max, GST-Erk1-(307–428), and GST-paxillin (data not shown). Although MBP could be phosphorylated by bacterially expressed GST-HA-ERK8, it was a poor substrate for immunoprecipitated HA-ERK8 from mammalian cellular lysates due to a high level of background phosphorylation (data not shown). The fact that bacterially expressed ERK8 could phosphorylate MBP but not c-Fos confirms that ERK8 is a functional kinase with an in vitro kinase assay. The HA-tagged proteins were immunoprecipitated from COS cells transfected either with the empty vector, HA-ERK8, HA-ERK8 K42R, HA-ERK7, or HA-ERK7 K43R. In vitro kinase assays were performed using GST-c-Fos, a known substrate for ERK7 (Fig. 5E, top). Under identical conditions, GST-c-Fos was phosphorylated by immunoprecipitated HA-ERK7 but not HA-ERK8. However, HA-ERK8 was able to autophosphorylate under these conditions (Fig. 5F) indicating that it is a functional kinase.
FIG. 10. ERK8 kinase domain phylogenetic tree. A computer-generated phylogenetic tree of the kinase domains of ERK8 and various serine/threonine kinases was constructed using the Neighbor Joining method (34) with Kimura’s correction (InforMax, Inc. Bethesda, MD). The multiple sequence alignment used was generated using the ClustalW algorithm (35). The amino acid identities of the kinase domains between ERK8 and the other kinases are indicated at the right as percentages. The GenBank accession numbers are human CDK2 (CAA43807), human ERK1 (X60188), human ERK2 (M84489), human ERK5 (U25278), human ERK8 (AY065978), rat ERK7 (AF078798), human STK9 (XM_010185), human KKIALRE (X66358), and human MOK (AB022694).

ERK8, a New MAPK

**ERK8 Associates with c-Src**—Sequence analysis of ERK8 revealed the presence of two potential SH3 domain binding motifs, PXXP, within its C-terminal region (Fig. 1). To determine whether ERK8 can interact with proteins containing SH3 domains, an in vitro binding assay was performed using whole cell lysate from HA-ERK8-transfected COS cells and a GST-c-Src SH3 domain fusion protein purified from bacteria. HA-ERK8 specifically bound to GST-c-Src SH3 but not GST (Fig. 7A). To determine whether ERK8 can also associate with c-Src in vivo, COS cells were transiently co-transfected with HA-ERK8 and either c-Src or the control vector. After immunoprecipitation with an anti-c-Src antibody, the immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting with the anti-HA monoclonal antibody. As shown in Fig. 7B, HA-ERK8 co-precipitated with c-Src. HA-ERK8 was seen in immunoprecipitates from both control and c-Src-transfected cells, suggesting that ERK8 associates with the endogenous c-Src as well as the exogenous c-Src. However, expression of exogenous c-Src dramatically increased the level of HA-ERK8 co-immunoprecipitated (Fig. 7B, compare lanes 3 and 4). Surprisingly, when HA-ERK8 was immunoprecipitated first from cells co-transfected with HA-ERK8 and c-Src, c-Src could not be detected (data not shown). It is possible that the anti-HA antibody bound to protein G beads sterically inhibits HA-ERK8 from interacting with c-Src. Two different anti-c-Src antibodies gave identical results, and Western analysis of whole cell lysates containing HA-ERK8 indicated that neither of the anti-c-Src antibodies cross-reacted with ERK8 (data not shown). These results indicate that ERK8 can associate with c-Src in cells.

**ERK8 Can Be Activated by a Src-dependent Signaling Pathway**—To determine whether ERK8 can be activated by a pathway involving Src, COS cells were transiently co-transfected with HA-ERK8 and either v-Src, constitutively active c-Src (c-Src Y527F), kinase-deficient c-Src (c-Src K295R), or the control vector. Western analysis using the anti-HA antibody indicated that the level of HA-ERK8 protein expression was comparable between transfection groups (Fig. 8A, top). However, Western analysis with the anti-phospho-ERK pAb revealed a marked increase in TEY-phosphorylated ERK8 when co-transfected with the constitutively active c-Src or v-Src (Fig. 8A, bottom). Exogenous ERK8 activation by Src was also seen in NIH3T3 and 16HBE14o— cells (data not shown). In addition, an increase in the TEY phosphorylation of the ERK8 kinase-deficient mutant was observed when the mutant was co-expressed with v-Src (Fig. 8B, bottom), consistent with phosphorylation by an upstream kinase. An in vitro kinase assay based on ERK8 autophosphorylation was used to verify increased kinase activity following co-transfection with v-Src. Presumably the majority of activated ERK8 is phosphorylated in vivo prior to the in vitro kinase assay; therefore, the amount of autophosphorylation measured in this assay understimates the level of ERK8 activation. As shown in Fig. 8, C and D, a small but consistent increase in ERK8 autophosphorylation was seen when ERK8 was co-transfected with v-Src. Taken together, these results indicate that ERK8 binds to and is activated by c-Src in cells.

Other than ERK7, members of the ERK family require an upstream MEK for activation. We used a potent MEK inhibitor to determine whether MEK1, MEK2, or MEK5 are involved in the activation of ERK8 by Src. Cells co-transfected with HA-ERK8 and either the empty vector, c-Src Y527F, or v-Src were pretreated with 10 μM U0126 or Me6SO for 15 min. Cell lysates were analyzed by Western blotting with either the anti-HA antibody (Fig. 8E, top) or the anti-phospho-ERK antibody (Fig. 8E, middle and bottom). Both c-Src Y527F and v-Src increased ERK8 activation as determined by increased TEY phosphorylation (Fig. 8E, middle). However, whereas U0126 inhibited TEY phosphorylation of ERK1 and ERK2 (Fig. 8E, bottom), no difference in TEY phosphorylation was seen between cells pretreated with or without U0126 (Fig. 8E, middle). These results indicate that ERK8 activation by c-Src or v-Src is independent of MEK1, MEK2, and MEK5.

**ERK8 Can Be Activated by Serum**—To identify physiologic activators of ERK8, COS cells transfected with HA-ERK8 were treated with 20% FBS following serum starvation. When normalized for ERK8 protein amount, Western analysis using the anti-phospho-ERK antibody revealed a 2.5-fold increase in ERK8 TEY phosphorylation (Fig. 9A). This increase, observed between 30 and 60 min following serum stimulation, was sustained for at least 2 h. Additional measurements done at 1, 10, and 15 min showed no change in TEY phosphorylation from base line (data not shown). To determine whether Src family members play a role in serum activation of ERK8, cells were pretreated with the Src inhibitor PP2 (21). At a concentration of 10 μM PP2, a small but consistent reduction in ERK8 TEY phosphorylation following stimulation with 20% FBS was seen (Fig. 9B). At a concentration of 50 μM PP2, a 50% reduction in ERK8 TEY phosphorylation was observed (Fig. 9B, middle). PP2 inhibited the serum-induced increase, because pretreatment with PP2 alone did not reduce the basal level of ERK8 TEY phosphorylation (data not shown). At these concentrations of PP2, similar reductions in ERK1 and ERK2 TEY phos-
phorylation were seen (Fig. 9B, bottom). Western analysis also detected a similar reduction in the Tyr-416-phosphorylated form of exogenously expressed c-Src in these cells (data not shown). This activation loop tyrosine is phosphorylated in active forms of c-Src (22). To determine whether MEK1, MEK2, or MEK5 play a role in the serum activation of ERK8, cells transfected with HA-ERK8 were pretreated with 10 μM U0126. No difference in TEY phosphorylation was seen between cells pretreated with or without U0126 (Fig. 9C, top and middle). On the other hand, increased TEY phosphorylation of ERK1 and ERK2 was inhibited completely (Fig. 9C, bottom). These results suggest that activation of ERK8 following serum stimulation requires Src family kinases but occurs independently of MEK1, MEK2, and MEK5.

**DISCUSSION**

ERK8, identified through a library screen using the ERK7 cDNA as a probe, is a newly identified member of the ERK family. Analysis of the evolutionary tree (Fig. 10) suggests that ERK7 and ERK8 form a discrete branch within the ERK family of MAPKs. Although ERK8 and ERK7 share an overall amino acid sequence identity of 69%, this sequence identity is significantly lower than that shared between the orthologues of ERK1, ERK2, or ERK5 which are highly conserved across mammalian species. For example, rat and human ERK1 and ERK2 share 97 and 99% overall sequence identity, respectively. Even the much larger ERK5 with its extended C-terminal region shares an overall sequence identity of 92% between mouse ERK5 and human ERK5 (20). The sequence identity shared between the kinase domains of ERK7 and ERK8 is only 82%, and the isolated C-terminal regions share only 53% amino acid identity. The primary sequences, taken in the context of the other ERK family members, indicate that ERK7 and ERK8 are related but distinct molecules.

The biochemical properties of ERK8 verify this distinction. ERK7 is unique because it displays a high level of constitutive activity even in serum-starved cells (10, 11). ERK8, on the other hand, does not have the same degree of constitutive activity. Because we have not yet identified a suitable in vitro substrate, a direct comparison of their kinase activities could not be made. However, Western analysis of lysates from mammalian cells using an antibody that preferentially recognizes the dually phosphorylated TEY motif suggests that, under similar conditions, ERK8 is not as highly TEY-phosphorylated as ERK7. By contrast, both serum and Src increase TEY phosphorylation of ERK8 but not ERK7. These results indicate that, unlike ERK7, a significant fraction of ERK8 is not TEY-phosphorylated in the basal state. Although lack of an effective ERK8 substrate limits our ability to characterize ERK8 biochemically, it also supports the conclusion that ERK8 and ERK7 are distinct members of the ERK family. GST-c-Fos is an in vitro substrate for ERK7 (11). However, ERK8 does not phosphorylate GST-c-Fos in vitro even following activation by v-Src (data not shown). Not surprisingly, orthologues of ERK1, ERK2, and ERK5, as well as other members of the MAPK family share the same activation and substrate profiles across several species. The relatively low sequence identity and different substrate profile indicate that ERK8 is a distinct member of the ERK family.

Accessible clues to the cellular function of ERK8 may come from the finding that v-Src and c-Src can activate ERK8. The Src family of nonreceptor tyrosine kinases, like the MAPKs, has been implicated in a wide range of cellular pathways involving signals initiated from G-protein-linked receptors, growth factor receptor protein tyrosine kinases, integrins, cytokine receptors, and even ion channels (23). There are at least nine members of the Src family (Btk, Hck, Fgr, Fyn, Lck, Lyn, Src, Yes, and Yrk). The common structural features include an SH3 domain and an SH2 domain that are N-terminal to the kinase domain. The crystal structure of c-Src indicates that these domains interact to regulate the kinase activity of Src (24, 25). In addition to regulating its activity, the SH3 and SH2 domains are also involved in interactions with its substrates. For example, phosphatidylinositol 3-kinase can bind to c-Src through an interaction within its p85 subunit and the SH3 domain of c-Src (26), as does paxillin (27). Similarly, Sam68 and actin filament-associated protein p110 interact with the SH3 and SH2 domains of c-Src (28, 29), and cortactin and focal adhesion kinase interact with the SH2 domain (30, 31). SH3 domains are known to bind to proteins that contain a proline-rich motif, PXXP (32). ERK8 contains two of these motifs within its C-terminal region that could facilitate its interaction with SH3 domain-containing proteins such as c-Src. The ability of the specific Src family inhibitor PP2 to partially inhibit ERK8 activation following serum stimulation supports the notion that activation of ERK8 by c-Src is physiologically relevant. Taken together with the observation that Src both associates with and activates ERK5 in cells, these results suggest that ERK8 is a downstream effector of one or more Src family kinases.

It seems unlikely that c-Src, a tyrosine kinase, activates ERK8 directly because ERK activation requires phosphorylation of the threonine residue in addition to the tyrosine residue within the TEY activation motif. For ERK1, ERK2, and ERK5, this dual phosphorylation is performed by MEK1, MEK2, or MEK5. The inability of the specific MEK inhibitor U0126 to block ERK8 activation by Src or serum indicates that these MEKs are not the upstream activators of ERK8 (33). However, it is possible that Src activates ERK8 through a classical signaling cascade involving an unidentified MEK. The ability of ERK8 to interact directly with c-Src suggests that they are components of a larger signaling complex that may serve as a selective activation mechanism, a potential feedback regulator, or a means for cellular localization of ERK8.

Because c-Src has been implicated in several intracellular signaling pathways including those originating from receptor protein tyrosine kinases, integrins, G-protein-coupled receptors, and cytokine receptors, additional studies will be required to determine the specific pathway(s) that involve ERK8. Identification of natural substrates will certainly provide insight into its cellular function. Nonetheless, the identification of ERK8 expands the ERK family of MAPKs and reinforces the notion that additional members with regulatory C-terminal regions may exist.

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