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ISOLATION OF LERK-5: A LIGAND OF THE EPH-RELATED RECEPTOR TYROSINE KINASES

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Abstract—Hek and elk are members of the eph-related family of receptor tyrosine kinases. Recently we isolated four cDNAs encoding membrane-bound ligands to hek and elk [Beckmann *et al.* (1994) *EMBO J.* **13,** 3757–3762; Kozlosky *et al.* (1995) *Oncogene* **10,** 299–306]. Because of the promiscuous nature of their binding, we have termed these proteins ligands of the eph-related kinases or LERKs. A search of GenBank revealed an expressed sequence tag (EST) with homology to the LERKs. Using this EST as a probe, we have isolated human and murine cDNAs that encode a protein which we call LERK-5. The human and murine cDNAs encode proteins of 333 and 336 amino acids, respectively, with a 97% amino acid identity; LERK-5 has an amino acid identity of 27–59% with the other reported LERKs. LERK-5 is a ligand for both elk and hek and induces receptor phosphorylation. It is expressed in adult lung and kidney and the fetal tissues heart, lung, kidney, and brain. In addition, Southern blot analysis of DNA from interspecific backcross mice indicated that LERK-5 (*Eplg5*) maps to the proximal region of mouse chromosome 8.

Key words: ligand, receptor tyrosine kinase, eph-family.

INTRODUCTION

One of the major mechanisms of cell growth and regulation is intracellular phosphorylation of proteins mediated by the interaction of receptor tyrosine kinases (RTKs) with their ligand(s). The RTKs have been grouped into families of proteins based on structural similarities in the extracellular and cytoplasmic domains, and in some cases, based on functional similarities (Fantl *et al.*, 1993; van der Geer *et al.*, 1994). The largest of these families is named after the first member to be identified, the so-called eph-related RTK family (Hirai *et al.*, 1987). There exist more than a dozen members of this family, not counting apparent orthologues, including the receptors elk and hek (Lhoták *et al.*, 1991; Wicks *et al.*, 1992). We recently reported the characterization of four different cDNA clones that encode membrane-bound ligands that bind to both hek and elk (Beckmann *et al.*, 1994; Kozlosky *et al.*, 1995). In addition, several groups have found that some of these ligands as well as a related protein, ELF-1, which we call LERK-6, bind to other eph-related receptors (Bartley *et al.*, 1994; Cheng and Flanagan, 1994; Davis *et al.*, 1994; Shao *et al.*, 1994). Due to their promiscuous binding, we have termed these proteins ligands of the eph-related kinases or LERKs.

LERK-1, LERK-3, and LERK-4 are anchored to the cell surface by glycosyl-phosphatidylinositol (GPI)-linkage, whereas LERK-2 is a type 1 transmembrane protein (Beckmann *et al.*, 1994; Kozlosky *et al.*, 1995). The LERKs share an amino acid identity ranging from 30 to 44%, including four invariant Cys residues. We report here the isolation and characterization of LERK-5. LERK-5 is predicted to be a type 1 transmembrane protein most similar to LERK-2 and is a ligand for elk and hek RTKs.

METHODS AND MATERIALS

Isolation of LERK-5 cDNAs

The expressed sequence tag (EST) (GenBank no. L13819) was isolated by the PCR from cDNA prepared from CCRF-HSB-2 (ATCC CCL 120.1) cells using synthetic oligonucleotide primers corresponding to nucleotides 317–334 (sense) and nucleotides 636–653 (antisense) (Fig. 1A). The nucleotide sequence of the T7 polymerase promoter (5′AATACGACTCACTATAG 3′) was added to the 5′ end of the antisense oli-

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A.

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CACAGCCATGGCTGTGAGAAGGGACTCCGTGTGGAAGTACTGCTGGGGTGTTTTGATGGTTTTATGCAGAACTGCGATTTCCAAATCGAT   90
      M  A  V  R  R  D  S  V  W  K  Y  C  W  G  V  L  M  V  L  C  R  T  A  I  S  K  S  I    28

AGTTTTAGAGCCTATCTATTGGAATTCCTCGAACTCCAAATTTCTACCTGGACAAGGACTGGTACTATACCCACAGATAGGAGACAAATT   180
 V  L  E  P  I  Y  W  N  S  S  N  S  K  F  L  P  G  Q  G  L  V  L  Y  P  Q  I  G  D  K  L    58

GGATATTATTTGCCCCAAAGTGGACTCTAAAACTGTTGGCCAGTATGAATATTATAAAGTTTATATGGTTGATAAAGACCAAGCAGACAG   270
 D  I  I  C  P  K  V  D  S  K  T  V  G  Q  Y  E  Y  Y  K  V  Y  M  V  D  K  D  Q  A  D  R    88

ATGCACTATTAAGAAGGAAAATACCCCTCTCCTCAACTGTGCCAAACCAGACCAAGATATCAAATTCACCATCAAGTTTCAAGAATTCAG   360
 C  T  I  K  K  E  N  T  P  L  L  N  C  A  K  P  D  Q  D  I  K  F  T  I  K  F  Q  E  F  S    118

CCCTAACCTCTGGGGTCTAGAATTTCAGAAGAACAAAGATTATTACATTATATCTACATCAAATGGGTCTTTGGAGGGCCTGGATAACCA   450
 P  N  L  W  G  L  E  F  Q  K  N  K  D  Y  Y  I  I  S  T  S  N  G  S  L  E  G  L  D  N  Q    148

GGAGGGAGGGGTGTGCCAGACAAGAGCCATGAAGATCCTCATGAAAGTTGGACAAGATGCAAGTTCTGCTGGATCAACCAGGAATAAAGA   540
 E  G  G  V  C  Q  T  R  A  M  K  I  L  M  K  V  G  Q  D  A  S  S  A  G  S  T  R  N  K  D    178

TCCAACAAGACGTCCAGAACTAGAAGCTGGTACAAATGGAAGAAGTTCGACAACAAGTCCCTTTGTAAAACCAAATCCAGGTTCTAGCAC   630
 P  T  R  R  P  E  L  E  A  G  T  N  G  R  S  S  T  T  S  P  F  V  K  P  N  P  G  S  S  T    208

AGACGGCAACAGCGCCGGACATTCGGGGAACAACATCCTCGGTTCCGAAGTGGCCTTATTTGCAGGGATTGCTTCAGGATGCATCATCTT   720
 D  G  N  S  A  G  H  S  G  N  N  I  L  G  S  E  V  A  L  F  A  G  I  A  S  G  C  I  I  F    238

CATCGTCATCATCATCACGCTGGTGGTCCTCTTGCTGAAGTACCGGAGGAGACACAGGAAGCACTCGCCGCAGCACACGACCACGCTGTC   810
 I  V  I  I  I  T  L  V  V  L  L  L  K  Y  R  R  R  H  R  K  H  S  P  Q  H  T  T  T  L  S    268

GCTCAGCACACTGGCCACACCCAAGCGCAGCGGCAACAACAACGGCTCAGAGCCCAGTGACATTATCATCCCGCTAAGGACTGCGGACAG   900
 L  S  T  L  A  T  P  K  R  S  G  N  N  N  G  S  E  P  S  D  I  I  I  P  L  R  T  A  D  S    298

CGTCTTCTGCCCTCACTACGAGAAGGTCAGCGGGGACTACGGGCACCCGGTGTACATCGTCCAGGAGATGCCCCCGCAGAGCCCGGCGAA   990
 V  F  C  P  H  Y  E  K  V  S  G  D  Y  G  H  P  V  Y  I  V  Q  E  M  P  P  Q  S  P  A  N    328

CATTTACTACAAGGTCTGAGAGGGACCCTGGTGGTACCTGTGCTTTCCCAGAGGACACCTAATGTCCCGATGCCTCCCTTGAGGGTTTGA  1080
 I  Y  Y  K  V  *                                                                            333

GAGCCCGCGTGCTGGAGAATTGACTGAAGCACAGCACCGGGGGAGAGGGACACTCCTCCTCGGAAGAGCCCGTCGCGCTGGACAGCTTAC  1170

CTAGTCTTGTAGCATTCGGCCTTGGTGAACACACACGCTCCCTGGAAGCTGGAAGACTGTGCAGAAGACGCCCATTCGGACTGCTGTGCC  1260

GCGTCCCACGTCTCCTCCTCGAAGCCATGTGCTGCGGTCACTCAGGCCTCTGCAGAAGCCAAGGGAAGACAGTGGTTTGTGGACGAGAGG  1350

GCTGTGAGCATCCTGGCAGGTGCCCCAGGATGCCACGCCTGGAAGGGCCGGCTTCTGCCTGGGGTGCATTTCCCCCGCAGTGCATACCGG  1440

ACTTGTCACACGGACCTCGGGCTAGTTAAGGTGTGCAAAGATCTCTAGAGTTTAGTCCTTACTGTCTCACTCGTTCTGTTACCCAGGGCT  1530

CTGCAGCACCTCACCTGAGACCTCCACTCCACATCTGCATCACTCATGGAACACTCATGTCTGGAGTCCCCTCCTCCAGCCGCTGGCAAC  1620

AACAGCTTCAGTCCATGGGTAATCCGTTCATAGAAATTGTGTTTGCTAACAAGGTGCCCTTTAGCCAGATGCTAGGCTGTCTGCGAAGAA  1710

GGCTAGGAGTTCATAGAAGGGAGTGGGGCTGGGGAAAGGGCTGGCTGCAATTGCAGCTCACTGCTGCCCGGAATTC               1786
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B.

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mLERK-5 MAMARSRRDSVWKYCWGLLMVLCRTAISRSQVLEPIYWNSSNSKFLPGQGLVLYPQIGDKLDIICPKVDSKTVGQYEYYKVYMVDKDQAD 90
hLERK-5 MAV...RRDSVWKYCWGVLMVLCRTAISKSSIVLEPIYWNSSNSKFLPGQGLVLYPQIGDKLDIICPKVDSKTVGQYEYYKVYMVDKDQAD 87
hLERK-2 MARPGQRWLGKWLVAMVVW.ALCRIQTPLAKNLEPVSWSSLNPKFLSGKGLVIYPKIGDKLDIICPRAE..AGRPYEYYKLYLVRPEQAA 87

mLERK-5 RCTIKKENTPLLNCARPDQDVKFTIKFQEFSPNLWGLEFQKNKDYYIISTSNGSLEGLDNQEGGVCQTRAMKILMKVGQDASSAGSTRNH 180
hLERK-5 RCTIKKENTPLLNCAKPDQDIKFTIKFQEFSPNLWGLEFQKNKDYYIISTSNGSLEGLDNQEGGVCQTRAMKILMKVGQDASSAGSTRNK 177
hLERK-2 ACSTVLDPNVLVTCNRPEQEIRFTIKFQEFSPNYMGLEFKKHHDYYITSTSNGSLEGLENREGGVCRTRTMKIIMKVGQDPN..AVTPEQ 175

mLERK-5 GPTRRPELEAGTNGRSSTTSPFVKPNPGSSTD.....GNSAGHSG..NNLLGSEVALFAGIASGCIIFIVIIITLVVLLLKY 255
hLERK-5 DPTRRPELEAGTNGRSSTTSPFVKPNPGSSTD.....GNSAGHSG..NNILGSEVALFAGIASGCIIFIVIIITLVVLLLKY 252
hLERK-2 LTTSRPSKEADNTVKMATQAPGSRGSLGDSDGKHETVNQEEKSGPGASGGSSGDPDGFFNSKVALFAAVGAGCVIFLLIIIFLTVLLLKL 265

mLERK-5 RRRHRKHSPQHTTTLSLSTLATPKRGGNNNGSEPSDVIIPLRTADSVFCPHYEKVSGDYGHPVYIVQEMPPQSPANIYYKV 336
hLERK-5 RRRHRKHSPQHTTTLSLSTLATPKRSGNNNGSEPSDIIIPLRTADSVFCPHYEKVSGDYGHPVYIVQEMPPQSPANIYYKV 333
hLERK-2 RKRHRKHTQQRAAALSLSTLASPKGGSGTAGTEPSDIIIPLRTTENNYCPHYEKVSGDYGHPVYIVQEMPPQSPANIYYKV 346
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Fig. 1. (A) DNA sequence and predicted amino acid sequence of human LERK-5. The predicted amino terminal amino acid (circled), potential N-linked glycosylation sites (underlined), and transmembrane amino acids (boxed) are indicated. (B) Alignment of human and murine LERK-5 with human LERK-2 (Beckmann *et al.*, 1994). DNA sequences of LERK-5 have been submitted to GenBank under accession numbers U16797 (human) and U16819 (murine).

gonucleotide to aid in the synthesis of riboprobes. Full-length LERK-5 cDNAs were isolated by screening cDNA libraries prepared from human fetal brain (Cat. no. HL3003a, Clontech, Palo Alto, CA), human fibroblast cell line WI-26VA4 (Goodwin *et al.*, 1990) or murine 11.5-day embryo (Cat. no. ML1027a, Clontech) with a ^{32}P-labeled EST probe as described (Morris *et al.*, 1992). Filters were hybridized at 63°C and washed at 63°C in 0.2 × SSC/0.1% SDS prior to autoradiography. DNAs from positive hybridizing phages were purified and cDNA inserts sequenced.

RNA hybridization.

Northern blots (Cat. no. 7759-1, 7760-1, and 7761-1, Clonetech) were probed with antisense riboprobes as previously described (Cosman *et al.*, 1984). The 354 bp EST PCR product was used as a template in the synthesis of probe.

Binding assays

For binding assays of LERK-5, adherent CV-1/EBNA cells in 10 cm dishes were transfected with the mammalian expression plasmid pDC410 containing human LERK-5 cDNA (Kozlosky *et al.*, 1995). After 24 hr, cells were replated into 24-well plates and incubated for an additional 48 hr prior to binding analysis. Binding experiments with purified elk-Fc (rat) and hek-Fc fusion proteins were conducted as previously described (Beckmann *et al.*, 1994; Kozlosky *et al.*, 1995). There is no significant binding of hek-Fc or elk-Fc to mock transfected CV1/EBNA cells.

LERK-5 induced receptor phosphorylation

A soluble form of LERK-5 was constructed by fusing the extracellular domain of human LERK-5 (amino acids 1–223, Fig. 1) to the Fc domain of human IgG1 in the mammalian expression vector pDC303 (Cerretti *et al.*, 1993). The resulting protein, LERK-5/Fc, was expressed and purified as described (Kozlosky *et al.*, 1995). Rat elk (Lhoták *et al.*, 1991) was inserted into the mammalian expression vector, pDC303, and transfected into CV1/ENBA cells as described above. Cells were split from one 10 cm plate to a six-well plate after 24 hr. After two additional days, cells were starved for methionine and cysteine and than radiolabeled for 3 hr with a 1:1 mixture of [^{35}S]methionine and [^{35}S]cysteine (Amersham) at a total concentration of 100 μCi/ml. Stimulations were then performed by adding LERK-2/Fc or LERK-5/Fc at 1 μg/ml for 10 min at 37°C. Cells were then quickly washed twice with cold PBS + 1 mM orthovanadate and lysed in lysis buffer (25 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM Na Fluoride, 1% NP-40, 1 mM DTT, 1 mM orthovanadate, plus protease inhibitors). Cell lysates were immunoprecipitated with a rabbit anti-elk antibody with Protein G Sepharose. The anti-elk antibody was generated by immunizing rabbits with a human elk-Fc fusion protein (Beckmann *et al.*, 1994). After washing three times with RIPA buffer (Cerretti *et al.*, 1988) and once with PBS, samples were put into reducing buffer and analysed by SDS–PAGE. Proteins were then transferred to nitrocellulose and [^{35}S]-labeling detected on a Phophorimager (Molecular Dynamics). The membrane was than immunoblotted with anti-phosphotyrosine 4G10 (UBI) followed by biotin-goat anti mouse (Kirkegaard + Perry), streptavidin–peroxidase (K + P), and then developed using ECL developing reagents (Amersham).

Interspecific backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J × *M. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Eplg5* (LERK-5) locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins *et al.*, 1982). All blots were prepared with Hybond-N$^+$ nylon membrane (Amersham). The probe, a ≈1 kb *Xho*I/*Not*I fragment of human cDNA, was labeled with [α^{32}P] dCTP using a nick translation labeling kit (Boehringer Mannheim); washing was done to a final stringency of 0.8 × SSCP, 0.1% SDS, 65°C. Fragments of 6.2 and 1.5 kb were detected in *Pvu*II digested C57BL/6J DNA and fragments of 9.2 and 1.5 kb were detected in *Pvu*II digested *M. spretus* DNA. In addition, *Sac*I digestion produced fragments of 8.7, 4.6 and 1.6 kb (C57BL/6J) and 5.3, 2.8, and 1.6 kb (*M. spretus*). The presence or absence of the 9.2 kb *Pvu*II and 5.3 and 2.8 *Sac*I *M. spretus*-specific fragments, which cosegregated, was followed in backcross mice. The *Pvu*II and *Sac*I data were combined.

A description of the probes and RFLPs for loci linked to the *Eplg5* including the insulin receptor (*Insr*), plasminogen activator tissue (*Plat*), and fibroblast growth factor receptor 1 (*Fgfr1*) has been reported previously (Ceci *et al.*, 1990; Kuo *et al.*, 1991). Recombination distances were calculated as described (Green, 1981) using the computer program *SPRETUS MADNESS*. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

Identification and characterization of LERK-5

Using a BLAST homology search of the NIH genetic databases with sequences from the recently identified family of ligands for the eph-related RTKs (Beckmann *et al.*, 1994; Kozlosky *et al.*, 1995), we identified an expressed sequence tag (EST) (GenBank accession no. L13819) with homology to LERK-2, a ligand for hek and elk. PCR amplification was used to generate a riboprobe template from the EST, as described in the Method and Materials. Northern analysis indicated high levels of expression in the human fetal brain and adult lung; therefore, cDNA libraries from these tissues were screened. Six clones were isolated that defined a cDNA of 1777 bp (Fig. 1A). A Met codon beginning at nucleotide 8 and a translation termination codon beginning at nucleotide

1007 defined an open reading frame of 333 amino acids, although a longer open reading frame cannot be ruled out at present. The nucleotide sequence adjacent to the predicted initiation Met closely conformed to the Kozak consensus translation initiation rules (Kozak, 1986) and the predicted protein is similar in size to the other members of the ligand family (Bartley *et al.*, 1994; Beckmann *et al.*, 1994; Cheng and Flanagan, 1994; Davis *et al.*, 1994; Shao *et al.*, 1994; Kozlosky *et al.*, 1995).

Hydrophilicity analysis suggested that the predicted LERK-5 protein is a type 1 transmembrane protein similar to LERK-2 (data not shown). The LERK-5 protein is predicted to have a 25 amino acid signal sequence, followed by a 199 amino acid extracellular domain, a 26 amino acid transmembrane domain, and an 83 amino acid cytoplasmic domain. The predicted size of the mature protein is 33,996 Da, but there are two potential N-linked glycosylation sites at Asn36 and Asn139. The nucleotide sequence reported here differs from the EST at nucleotide 575 (G to A), resulting in an amino acid substitution (Asp186 to Asn186). The difference may be the result of cloning artifacts by either group or may reflect an allelic difference.

Comparison of the predicted LERK-5 protein sequence with other LERKs demonstrated that it is most similar to LERK-2 (59% amino acid identity) (Fig. 1*b*). LERK-2 is the only other ligand in this family that is predicted to be a transmembrane protein (Davis *et al.*, 1994; Kozlosky *et al.*, 1995). Interestingly, the cytoplasmic domains of LERK-2 and LERK-5 are the most closely related, encoding 83 amino acid residues and exhibiting 75% amino acid identity. The cytoplasmic domains of these two proteins might play some functional role in post-receptor binding signal transduction in the ligand-expressing cell. Comparison of LERK-5 with the GPI-anchored members of this ligand family reveals only a 27–28% amino acid identity. The only region of significant homology for all members of this family is the putative receptor binding region, including four conserved Cys residues (Fig. 2).

A murine homologue of LERK-5 was isolated from a cDNA library prepared from 11.5 day embryos. DNA sequence analysis of the 2611 bp cDNA (data not shown) revealed an open reading frame of 336 amino acids (Fig. 1*b*). The amino acid sequences of human and murine LERK-5 are highly conserved with an identity of 97%. Interestingly, the only other ligand for a RTK that is so highly conserved through evolution is LERK-2 where the human and rat proteins are 95% identical (Fletcher *et al.*, 1994a). In addition, this evolutionary conservation in the amino acid sequence is also seen for the eph-related receptors (Sajjadi *et al.*, 1991; Wicks *et al.*, 1992; Maisonpierre *et al.*, 1993; Henkemeyer *et al.*, 1994; Zhou *et al.*, 1994), indicating an important functional role for this ligand/receptor system.

Binding characterization of recombinant LERK-5

The binding characteristics of human LERK-5 with elk and hek receptors were analyzed utilizing fusion proteins consisting of the extracellular domain of hek or rat elk linked to the Fc domain of human IgG1, resulting in soluble forms of hek (hek-Fc) and elk (elk-Fc). Binding of these molecules was measured using an indirect method in which CV1/EBNA cells expressing LERK-5 were incubated with varying concentrations of either hek-Fc or elk-Fc followed by saturating concentrations of ^{125}I-labeled mouse anti-human IgG antibodies directed against the Fc portion of the molecule. Representative equilibrium binding data is shown in Fig. 3 where Scatchard analysis (Scatchard, 1949) of the data yielded a single class of binding sites for both molecules. From an average of four experiments, the affinity constant (K_a) of elk-Fc binding was $1.2 \pm 0.3 \times 10^9$/M and the K_a of hek-Fc binding was $4.3 \pm 3.3 \times 10^7$/M. These values are very similar to the affinity constants previously determined (Beckmann *et al.*, 1994) for LERK-2 binding to elk-Fc (1.08×10^9/M) and hek-Fc (2.3×10^7/M). In the latter case, a very low affinity binding component (2.9×10^6/M) of hek-Fc to LERK-2 was also observed which was not reliably detected with LERK-5.

To determine if LERK-5 would induce receptor phosphorylation, a soluble form of LERK-5 was constructed by fusing the extracellular portion of LERK-5 with the Fc portion of human IgG1 resulting in LERK5/Fc. LERK-5/Fc was added to CV1/EBNA cells transfected with a plasmid expressing full length rat elk. Western analysis of elk immunoprecipitates with an anti-phosphotyrosine antibody showed that LERK-5/Fc stimulated elk phosphorylation (Fig. 4, lane 3), as did a soluble form of LERK-2, LERK-2/Fc (Fig. 4, lane 2). Ligand dependent phosphorylated proteins were not detected in CV1/EBNA cells transfected with a control plasmid (data not shown). Similar results were obtained for B61 (LERK-1) phosphorylation of eck (Bartley *et al.*, 1994) and ehk-1 (Davis *et al.*, 1994) and for EFL-3 (LERK-2) phosphorylation of elk (Davis *et al.*, 1994).

Northern analysis

LERK-5 riboprobes were generated and used for Northern analysis of human tissues. A single RNA species of ~5 kb was detected (Fig. 5). Expression of LERK-5 mRNA was observed in several fetal tissues including heart, lung, kidney and brain, but the only significant expression in adult tissues was detected in the lung and kidney. The developmental expression pattern is very similar to the closely-related LERK-2, with the exception of the adult placenta, where LERK-2 expression is quite high (Fletcher *et al.*, 1994a). Lower levels of expression were observed in adult brain, placenta, skeletal muscle and small intestine. Longer exposure of the Northern blot revealed even lower levels of expression in the remaining tissues except for adult pancreas, thymus and peripheral blood leukocytes (data not shown).

Mapping of LERK-5 (Eplg5)

The mouse chromosomal location of *Eplg5* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)

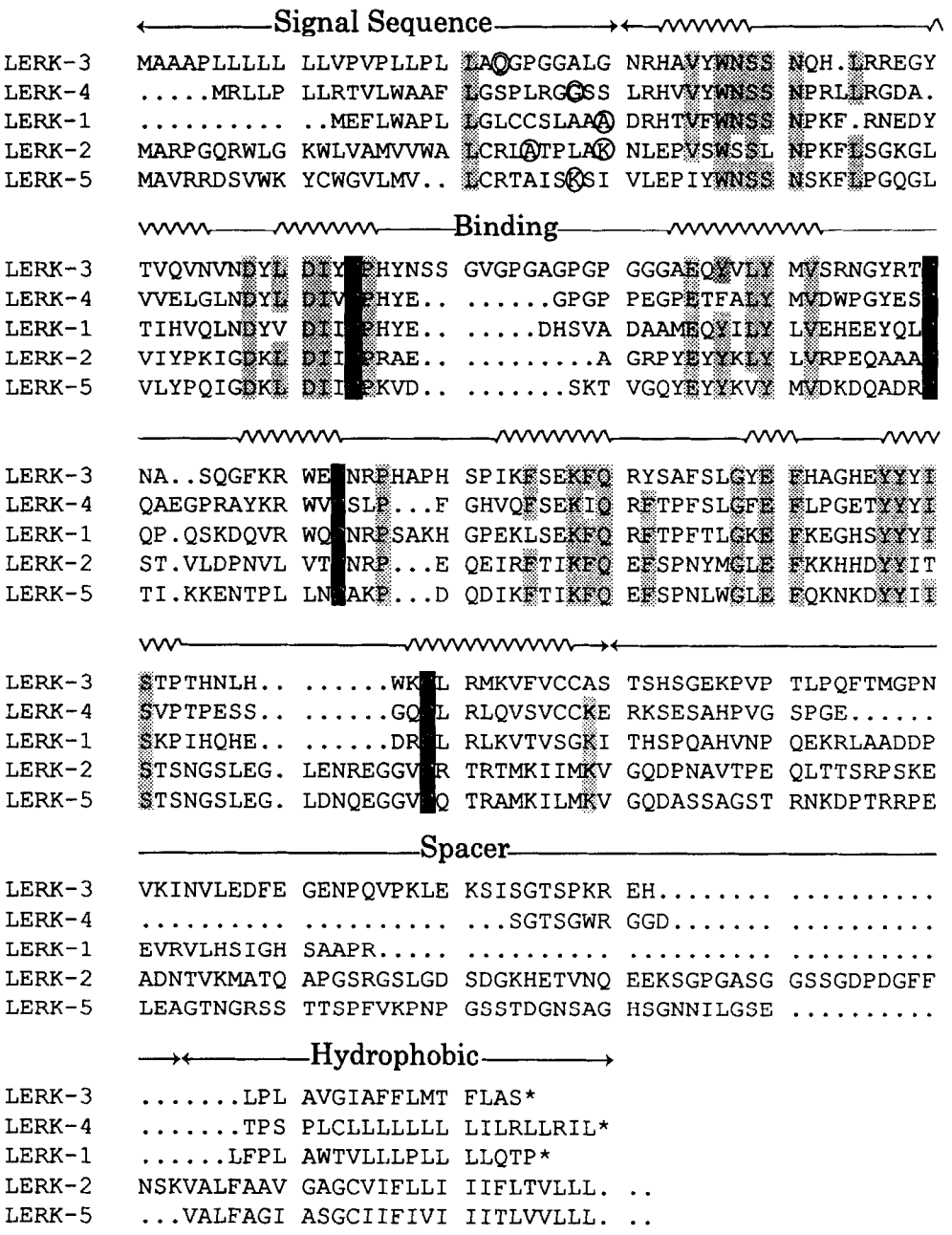

Fig. 2. Alignment of the LERK amino acid sequences. Amino acids are deduced from the DNA sequences of human LERK-1, LERK-2, LERK-3, LERK-4 (Beckmann *et al.*, 1994; Kozlosky *et al.*, 1995), and LERK-5. The sequences of LERK-2 and LERK-5 do not include the 83 amino acid cytoplasmic region. Alignment was done by the GAP program (Devereux *et al.*, 1984) and visual inspection. Shaded amino acids indicate identity of four or more residues, circled amino acids indicate the predicted amino terminus, and boxed amino acids indicate the conserved Cys residues. The jagged line indicates residues with b-extended conformations (Kozlosky *et al.*, 1995).

$F_1 \times$ C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1800 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a human cDNA probe. The 9.2 kb *Pvu*II and 5.3 and 2.8 kb *Sac*I *M. spretus* RFLPs (see Method and Materials) were used to follow the segregation of *Eplg5* locus in backcross mice. The mapping results indicated that *Eplg5* is located in the proximal region of mouse chromosome 8 linked to *Insr*, *Plat* and *Fgfr1*. Although 85 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 6), up to 100 mice were typed for some pairs of markers. Each locus was analysed in pairwise combination for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analysed for each pair of loci and the most likely gene order are: centromere-*Insr*–1/100–*Eplg5*–5/100–*Plat*–2/100–Fgfr1. The recombination fre-

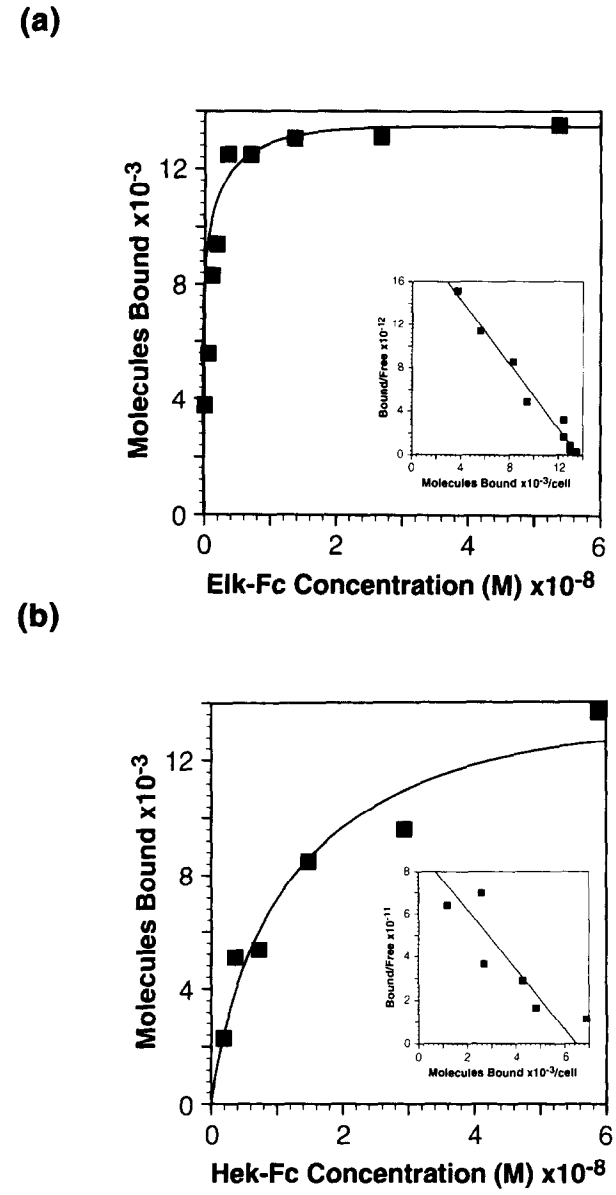

Fig. 3. Binding of rat elk-Fc and hek-Fc to LERK-5. Monolayers of CV1/EBNA cells transfected with an expression plasmid containing human LERK-5 were assayed for elk-Fc (a) or hek-Fc (b) binding as described in Methods and Materials. Inset shows Scatchard representations of specific binding replotted for each curve.

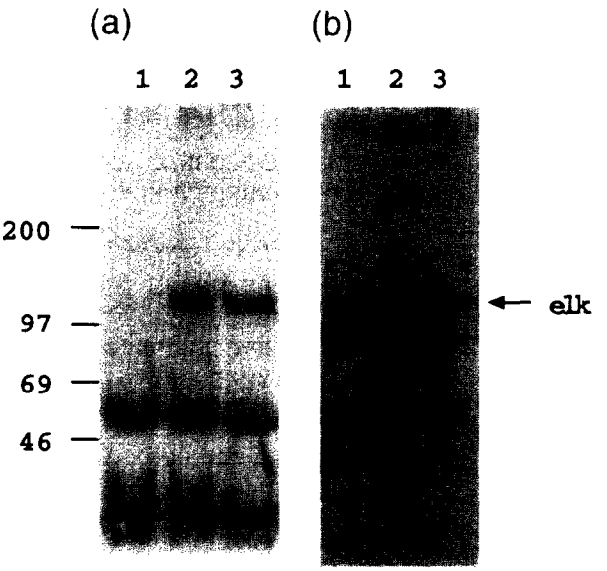

Fig. 4. LERK-5/Fc induced receptor phosphorylation. CV1/EBNA cells expressing rat elk were treated with media only (lane 1), LERK-2/Fc (lane 2), or LERK-5/Fc (lane 3) after radiolabeling with [35S]methionine and [^{35}S]cysteine. Cell lysates were immunoprecipitated with an anti-elk antibody and immunoblotted with anti-phosphotyrosine (a) or exposed on a phosphorimager to detect [^{35}S]-labeled proteins (b).

quencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] are: *Insr*–1.0 ± 1.0–*Eplg5*–5.0 ± 2.2–*Plat*–2.0 ± 1.4–*Fgfr1*.

DISCUSSION

Several members of the eph family of RTKs have been suggested to play an important role in pattern formation during early vertebrate development (Gilardi-Hebenstreit *et al.*, 1992; Nieto *et al.*, 1992; Henkemeyer *et al.*, 1994; Ruiz and Robertson, 1994; Xu *et al.*, 1994; Zhou *et al.*, 1994). Until recently, elucidation of the role of eph-related RTKs in the poorly-defined cell–cell interactions involved in early vertebrate pattern formation was hindered by the lack of well-defined peptide ligands for these RTKs. We report here the most recent member of the rapidly expanding family of ligands for the eph-related RTKs, LERK-5. LERK-5 is a transmembrane protein most similar to LERK-2, another transmembrane protein. Both ligands show high affinity binding to elk ($K_a = 1 \times 10^9$/M) and both will induce phosphorylation of elk (Fig. 4). All of the other known members of this ligand family are anchored to the membrane by GPI-linkage (Kozlosky *et al.*, 1995). Alignment of the amino acid sequences of members of this ligand family reveals that the only region of significant homology between all the LERKs is the putative receptor binding domain (Fig. 2). It is noteworthy that the LERKs exhibit extensive cross-binding to the eph-related RTKs, suggesting some functional redundancy. The similar expression patterns that have been described for the eph-related RTKs and LERKs further suggests functional redundancy. The requirement to develop accurate spatial information during embryonic pattern formation makes it difficult to hypothesize a role for the eph-related RTK/ligand system in such developmental systems. Perhaps the receptors and ligands describe information in the developing embryo through a combinatorial mechanism, such that the eph-related RTKs and/or ligands expressed on a certain tissue segment precisely define that segment spatially and/or temporally. It is further possible that the eph-related RTKs form functional heterodimers, as has been demonstrated for other closely-related RTK families (Wada *et al.*, 1990; Bellot *et al.*, 1991; Schlessinger and Ullrich, 1992; Plowman *et al.*, 1993).

We have compared our interspecific map of chromosome 8 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (compiled by M.T. Davisson, T.H. Roderick, A. L. Hillyard and D.P. Doolittle and provided from

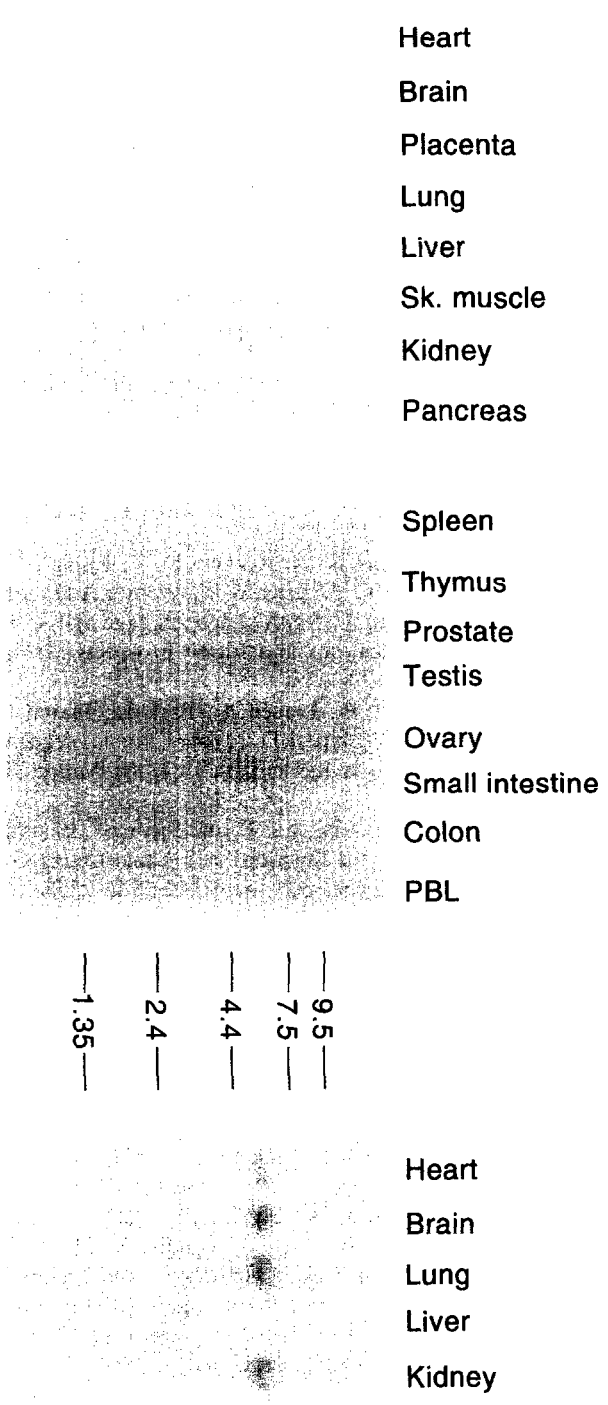

Fig. 5. Northern blot analysis of expression of LERK-5 in various human adult (top and middle panels) and fetal (bottom panel) tissues where each lane contains approximately 2 mg of Poly A+ RNA. The blot was probed as described in Materials and Methods.

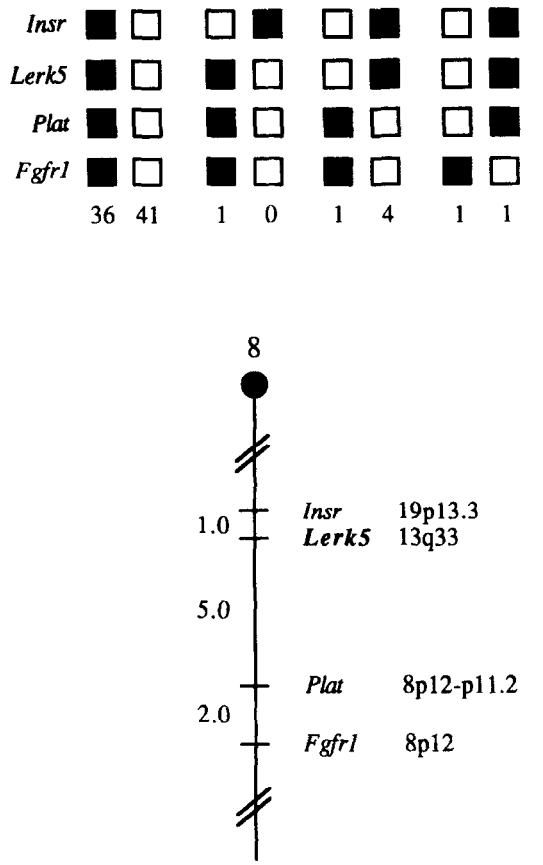

Fig. 6. *Eplg5* (LERK-5) maps in the proximal region of mouse chromosome 8 by interspecific backcross analysis. The segregation patterns of *Eplg5* and flanking genes in 85 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 85 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 8 linkage map showing the location of *Eplg* 5 in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centiMorgans are shown to the left of the chromosome and the positions of loci in human chromosomes are shown to the right. References for the human map positions of loci mapped in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

GBASE, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). *Eplg5*, which is expressed in many tissues, including brain, mapped in a region of the composite map that contains one neurological mouse mutation (data not shown). Mice carrying this dominant mutation, motor neuron degeneration (*Mnd*), display hindlimb weakness and ataxia by 5–11 months; the disease eventually progresses to paralysis of all limbs (Green, 1989; Messer *et al.*, 1992). It would be interesting to determine whether *Eplg5* is altered in *Mnd* mutant mice.

The proximal region of mouse chromosome 8 shares regions of homology with human chromsomes 19p, 13q, and 8p (summarized in Fig. 6 and Copeland *et al.*, 1993). The observation that *Eplg5* maps to 13q33 in humans (GenBank accession no. L13819) confirms and extends the region of homology between mouse 8 and the long arm of human 13.

Although it is accepted that the eph-related RTKs can initiate signal transduction through their catalytic kinase domains, it is also possible that the transmembrane

ligands can mediate post receptor-binding signal transduction in the ligand-expressing cell. This phenomenon has been described for the ligands of the TNFα receptor family (Cayabyab *et al.*, 1994; Pollok *et al.*, 1994). The cytoplasmic domain of LERK-2 is completely conserved between the mouse, rat and human (Fletcher *et al.*, 1994b), and the cytoplasmic domain of LERK-5 is 98% identical between mouse and human. The most homologous region between LERK-5 and LERK-2 is the cytoplasmic domain, having many conserved Ser, Thr and Tyr residues in common. It is difficult to imagine such high sequence conservation in the absence of an important functional role, but experimental evidence for this hypothesis has not been obtained. Such reverse signal transduction by the LERKs, if it exists, might not be restricted to the transmembrane proteins. Antibodies to the GPI-linked proteins Ly-6 (Malek *et al.*, 1986), Thy-1 (Gunter *et al.*, 1984), and CD55 (Davis *et al.*, 1988; Shenoy-Scaria *et al.*, 1992) can induce calcium mobilization or cytokine production in the ligand-expressing cells; thus, it is also possible that the GPI-linked LERKs (LERK-1, LERK-3 and LERK-4) mediate signal transduction in the ligand-expressing cell. With the availability of ligands to the eph-related kinases we can begin to address some of these questions.

After completion of this work, a ligand to the eph family receptor htk was identified that is identical to LERK-5 (Bennett *et al.*, 1995).

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