Cell-Cell Adhesion Mediated by Binding of Membrane-anchored Ligand LERK-2 to the EPH-related Receptor Human Embryonal Kinase 2 Promotes Tyrosine Kinase Activity*

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Human embryonal kinase 2 (HEK2) is a protein-tyrosine kinase that is a member of the EPH family of receptors. Transcripts for HEK2 have a wide tissue distribution. Recently, a still growing family of ligands, which we have named LERKs, for ligands of the eph-related kinases, has been isolated. In order to analyze functional effects between the LERKs and the HEK2 receptor, we expressed HEK2 cDNA in an interleukin-3-dependent progenitor cell line 32D that grows as single cells in culture. Within the group of LERKs, LERK-2 and -5 were shown to bind to HEK2. Membrane-bound and soluble forms of LERK-2 were demonstrated to signal through HEK2 as judged by receptor phosphorylation. Coincubation of HEK2 and LERK-2 expressing cells induced cell-cell adhesion and formation of cell aggregates. This interaction could be inhibited by preincubation of HEK2 expressing cells with soluble LERK-2. Coexpression of HEK2 and LERK-2 in 32D cells showed reduced kinase activity and autophosphorylation of HEK2 compared with the juxtacrine stimulation, which seems to be due to a reduced sensitivity of the receptor.

Signal transduction from the extracellular environment across the cell membrane governs cellular growth and differentiation. These signals are often transduced by receptor tyrosine kinases (RTKs)†, ‡—Defects in RTK genes, their overexpression, or autocrine ligand production may play a critical role in the induction and progression of certain neoplasms (3). Based on structural considerations RTKs have been divided into 14 sub-families (2). One of these is the EPH family of receptors, which constitutes the largest known family of RTKs, named for the prototype member EPH of this family (2). To date, cDNA sequences for nine distinct human EPH family members have been reported: EPH (4), ECK (5), HEK (6), HEK2 (7), HTK (8), ERK (9, 10) and HEK7, -8, and -11 (11). Despite the large number of RTKs in the EPH family, all of the molecules were identified as orphan receptors without known ligands. This situation has hindered understanding the biochemical roles of this family of RTKs.

The first ligand to be identified was B61 (12, 13). B61, a tumor necrosis factor α-induced gene product from cultured human umbilical vein endothelial cells was found to bind the EPH receptor family member ECK. We have also found that B61 is a ligand for HEK and elk and have named it LERK-1 (14). Subsequently, six additional ligands for the EPH family of RTKs were identified which we have named LERK-2 through LERK-7 (14–18). These ligands have an amino acid sequence identity ranging between 27 and 59% and are membrane-bound. The LERKs can be subdivided into two groups based on their mechanism of membrane attachment. LERK-1, LERK-3, LERK-4, LERK-6, and LERK-7 are anchored by glycosylphosphatidylinositol linkage while LERK-2 and LERK-5 are type 1 transmembrane proteins.

Here we describe that within the group of LERK proteins, LERK-2 and LERK-5 bind to HEK2 expressed in 32D-myeloid progenitor cell line, whereas LERK-1, LERK-3, LERK-4, and LERK-7 do not. In addition, soluble forms of LERK-2 stimulate autophosphorylation of HEK2 so that membrane attachment does not seem to be required for activation of the HEK2 receptor kinase. Furthermore, we provide evidence for stable cell-cell contacts mediated by the interaction of LERK-2 and HEK2 expressing cells and subsequent juxtacrine stimulation of receptor autophosphorylation.

MATERIALS AND METHODS

Reagents—Rabbit polynonal antisera to HEK2 were generated by immunizing animals with a peptide corresponding to amino acid residues 897–998 (7). Monoclonal anti-phosphotyrosine antibodies 4G10 were purchased from Transduction Laboratories. Sepharose-protein A beads, horseradish peroxidase-conjugated goat anti-mouse, and goat anti-rabbit antibodies were from Sigma. The mammalian expression vectors pRcCMV and pCEP4 were supplied by Invitrogen. Gentamicin sulphate (G418) was obtained from Life Technologies, Inc., hygromycin B was from Boehringer Mannheim.

Generation of a Kinase-negative Mutant of HEK2 and Construction of Expression Vectors with HEK2 Receptors or LERK—The HEK2 cDNA, containing the complete open reading frame (nucleotide position 4–3086) (7), was inserted into the HindIII/XbaI site of the mammalian expression vector pRcCMV. A K665R kinase-deficient HEK2 receptor was generated by polymerase chain reaction-mediated site-directed mutagenesis using the following mutagenic oligonucleotide 5'-TGCCGTTGGTTCAGTGAATACAGCCTGGCGCCCGAGGAGGTGTTTGTGGCGCATCCGGAGC-3', corresponding to nucleotides 1968–2025 of the published cDNA sequence (7). The mutated full-length receptor cDNA was cloned into the pCEP4 vector using HindIII and BamHI restriction enzymes.
The LERK-2 cDNA, nucleotide position 308-1348 (14), was cloned into the HindIII and BamHI cloning sites of the mammalian expression vector pCEP4. All constructs were verified by DNA sequencing.

Cell Line, Culture Conditions, and Transfection—32D cells are an immature murine myeloid cell line that is absolutely dependent on exogenously supplied interleukin-3 for maintenance of its growth and survival in culture. These cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, antibiotics (streptomycin, penicillin), and 5% conditioned medium derived from the interleukin-3 producing cell line WEHI-3B. Cells were subjected to electropolution (250 V, 960 microfarad) in RPMI 1640 containing 25 μg of linearized plasmid DNA. Following 2 weeks of selection in 600 μg/ml G418 (pRcCMV) or 2 mg/ml hygromycin B (pCEP4) stable transfec-tants were tested by immunoblotting or Northern blotting.

LERK-2 expressing cells in culture for 2 days and subsequently spun down, and the supernatant was used as conditioned medium for further experiments.

Immunoblotting—Cells were washed twice with ice-cold phosphate-buffered saline (PBS). The cell pellet was resuspended in 500 ml of lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA) freshly supplemented with 10 μg/ml leupeptin per ml, 10 μg/ml phenylmethylsulfonyl fluoride, and 1 mM Na3VO3. Cell lysates were incubated on ice for 15 min with occasional vortexing and then clarified by centrifugation for 10 min at 12,000 × g. The protein concentration was measured using the Pierce BCA protein assay reagents. 25 μg of protein were fractionated per lane on a SDS-7.5% polyacrylamide gel by electrophoresis. After transfer of the proteins onto Immobilon P (Millipore), the filters were preincubated 2 h with 1% bovine serum albumin, 1% gelatin in PBS, washed with PBST (0.05% Tween 20 in PBS), incubated 1 h at 22 °C with primary antibody in reaction buffer (1% bovine serum albumin, 10% fetal calf serum, 0.1% Triton X-100 in PBS), washed three times, and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody. Immunoblots were developed using the ECL system (DuPont NEN) or the diaminobenzidine staining method. In order to reprobe proteins with a second antibody, filters were incubated for 30 min in strip buffer (62.5 mM Tris (pH 6.8), 100 mM β-mercaptoethanol, 2% SDS) at 50 °C.

Immunoprecipitation—Prior to lysis, cells were stimulated with LERK-2 for 30 min at 37 °C. 200 μg of proteins were incubated for 1–2 h at 4 °C with HEK2-specific antibodies coupled to Sepharose-protein A beads. Immunoprecipitates were washed three times with lysis buffer, mixed with SDS-gel sample buffer, heated at 95 °C for 5 min, and subjected to gel electrophoresis.

In Vitro Kinase Assay—Washed immunoprecipitates were suspended in 20 μl of 50 mM Pipes (pH 7.0), 10 mM MnCl2, 5 μM iodoacetamide, DuPont NEN) and incubated at 30 °C for 10 min. Reaction mixtures were mixed with an equal volume of 2 × sample buffer, boiled for 2 min, and fractionated on a 7.5% SDS-polyacrylamide gel. The dried gel was exposed to an x-ray film for 20 min.

Northern Blot Analysis—Total RNA was isolated from 5 × 106 cells using the guanidinium isothiocyanate-CsCl cushion technique. The RNA was fractionated on a 1% formaldehyde agarose gel before being transferred onto a Hybond N+ membrane (Amersham Corp.). Hybridization with polymerase chain reaction-generated radiolabeled probes was performed as described before (7). BlueBlot (K+ of HEK2 cDNA and pCEP4 LEKR-2 cDNA were used as templates in the synthesis of probes. Blots were exposed to an x-ray film for 2 h.

Biotin Assay—Soluble forms of the LEKRs were constructed by fusing the extracellular domains of LEKR-1 (amino acids 1–183), LEKR-2 (amino acids 1–229) (14), LEKR-3 (amino acids 1–220), LEKR-4 (amino acids 1–182) (17), LEKR-5 (amino acids 1–223) (15), and LEKR-7 (amino acids 1–202, GenBank accession number U26403) to the Fc domain of human IgG1 in the mammalian expression vector pDC303 (19). The resulting proteins LEKR-L/Fc, LEKR-2/Fc, LEKR-3/Fc, LEKR-4/Fc, LEKR-5/Fc, and LEKR-7/Fc were expressed and purified as described (17). Binding of purified LEKRs to 32D cells and 32D cells expressing HEK2 were performed at a single concentration (2 μg/ml) utilizing the method described below. Equilibrium binding studies with LEKR-2/Fc were performed on 32D cells expressing HEK2. For both experiments, cells were washed with serum-free RPMI 1640, transferred to 96-well plates, and incubated with LEKR/Fc in binding media (RPMI 1640 containing 2% bovine serum albumin, 20 mM HEPES buffer, and 0.2% sodium azide (pH 7.2)) for 1 h at 4 °C with agitation. Subsequently, cells were washed with cold PBS by centrifugation of the 96-well plate and pipetting off the liquid. They were then incubated with 125I-labeled mouse anti-human IgG antibody prepared in binding medium at saturating concentrations for 1 h at 4 °C. Free and bound cpm were then separated via centrifugation over pthalalate oil tubes. In equilibrium binding assays, nonspecific binding of 125I-antibody was assayed in the absence of LEKR-2/Fc as well as in the presence of LEKR-2/Fc and a 200-fold molar excess of unlabelled mouse anti-human IgG antibody. Free and cell-bound 125I-antibodies were quantified on a Packard Autogamma Counter. Affinity calculations (20) were generated using Delta Graph software.

RESULTS

Expression of HEK2 in the 32D Myeloid Cell Line—To study the effects of ligand binding on HEK2 activation, we inserted the complete HEK2 open reading frame into the pRc/CMV vector, placing the cDNA under the transcriptional control of the cytomegalovirus enhancer-promoter. As a control, we generated a receptor lacking intrinsic protein-tyrosine kinase activity (HEK2/kin–). A single amino acid mutation replaces the conserved lysine, which is proposed to provide an essential role for binding of ATP, by arginine (21). This mutated cDNA sequence was cloned into the cytomegalovirus early promoter-based expression vector pCEP4. Subsequently, 32D cells transfected with these HEK2 expression vectors were cultured for 2 weeks in the presence of a selective drug (G418 for pRc/CMV and hygromycin B for CEP4). Stable transfec-tants were analyzed by Western blotting of whole cell lysates. Rabbit polyclonal antibodies directed against the C terminus of HEK2 (amino acids 897–998) specifically recognized polypeptides of approximately 110 kDa in cells transfected with either HEK2 or mutated HEK2/kin– (data, Fig. 1, lanes 1 and 2). No HEK2-related polypeptides were detected in the parental clone of 32D cells (Fig. 1, lane 3). In all subsequent experiments, cell lines expressing the highest number of receptors (clone 32DH20 for HEK2 and clone HEK2/kin– for the kinase-negative mutant) were used.

Binding of LEKR Proteins to HEK2—The binding characteristics of HEK2 with different LEKRs were analyzed utilizing fusion proteins consisting of the extracellular domain of the LEKRs linked to the Fc domain of human IgG1, resulting in soluble forms of LEKRs, LEKR/Fc. Binding of these molecules was measured using an indirect method in which 32D cells expressing HEK2 were incubated with varying concentrations of LEKR/Fc followed by saturating concentrations of 125I-labeled mouse anti-human IgG antibodies directed against the Fc portion of the molecule. Only LEKR-2 and LEKR-5 were found to bind to HEK2 in this assay (Table I). Further analysis of binding and functional studies were conducted with LEKR-2/Fc. Representative equilibrium binding data are shown in Fig. 2 where Scatchard analysis (20) of the data yielded a single class of binding sites. From an average of three experiments, the affinity constant Kd of LEKR-2/Fc binding to HEK2 was...
HEK2/LERK-2 Interaction Induces Aggregation of Cells

Table I  
LERK/Fc binding to 32D/HEK2 cells

| LERK/Fc | 32D    | 32D/HEK2 |
|---------|--------|----------|
| None    | 1310 ± 40 | 1760 ± 120 |
| LERK-1  | 1350 ± 40 | 1470 ± 140 |
| LERK-2  | 1290 ± 10 | 18900 ± 1870 |
| LERK-3  | 1320 ± 30 | 1500 ± 340 |
| LERK-4  | 1170 ± 50 | 1470 ± 50  |
| LERK-5  | 1120 ± 20 | 18000 ± 2360 |
| LERK-7  | 1440 ± 30 | 1180 ± 130  |

Binding was conducted as described under “Materials and Methods.” Binding to 32D cells was done in duplicate and to 32D/HEK2 cells in triplicate.

1.8 ± 0.3 × 10^9 m⁻¹ with 16,500 ± 5,800 sites/cell.

Autophosphorylation of HEK2 Induced by Soluble LERK-2/Fc Protein—To investigate the ligand dependence of intrinsic HEK2 enzymatic activity, we compared in vivo tyrosine phosphorylation of the receptor in the presence or absence of soluble LERK-2/Fc. HEK2 expressing cells were incubated with increasing concentrations of LERK-2/Fc (50–1000 ng/ml). Immunoprecipitates of HEK2 from lysates of ligand-stimulated cells were fractionated by SDS-polyacrylamide gel electrophoresis and probed with anti-phosphotyrosine antibodies. As shown in Fig. 3 soluble LERK-2/Fc caused dose-dependent phosphorylation of the wild-type HEK2 receptor and reached a maximum at a concentration of 250 ng of LERK-2/Fc per ml in the cell culture medium. Incubation of 32D-HEK2 cells without addition of LERK-2/Fc resulted in a low but detectable level of tyrosine phosphorylation (Fig. 3, lane 1) that might be due to an intrinsic basal level of HEK2 tyrosine phosphorylation, to a stimulatory effect exerted by factors present in the cell culture medium, or to proteins other than LERK-2 expressed on the cell surface of 32D cells. In summary, our results indicate that soluble LERK-2/Fc protein can functionally interact with the HEK2 receptor.

Membrane-bound LERK-2 Induces Phosphorylation of Wild-type HEK2—The ability of membrane-bound LERK-2 to stimulate the HEK2 autophosphorylation was examined as well. For this purpose, we produced 32D-derived cell lines expressing transmembrane forms of LERK-2 or HEK2. Furthermore, LERK-2 and HEK2 coexpressing 32D-derived cells were generated. A Northern blot analysis using specific probes demonstrated equal levels of ligand and receptor transcripts in corresponding cell lines (Fig. 4).

Prior to stimulation LERK-2 expressing cells were washed thoroughly with fresh medium, subsequently mixed at a ratio of 1:1 (6 × 10⁶ cells of each cell type per ml) with HEK2 expressing cells, and incubated for 30 min. Immunoprecipitation of HEK2 from those cells was followed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 5A increased tyrosine phosphorylation is induced by coinubcation of HEK2 and LERK-2 expressing cells (Fig. 5A, lane 7). We found that during the period of stimulation the culture medium of cocultivated cells was devoid of factors such as soluble ligands that could activate receptor phosphorylation.

Membrane-anchored LERK-2 Released to the Cell Culture Medium Activates Kinase Activity of HEK2—Conversion of membrane-anchored growth factors into soluble forms involves a proteolytic system, which acts at or near the cell surface and shows broad proteolytic activity (22). We therefore determined whether LERK-2 is released from 32D cells into the medium and whether it could activate HEK2. As shown in Fig. 5A (lanes 5 and 6), conditioned medium from LERK-2 expressing cells but not from nontransfected cells induces elevated phosphorylation of HEK2 compared with the previously described coinoculation experiments, in which the membrane-bound ligand induced receptor activity (Fig. 5A, lane 7).

Activation of the HEK2 Receptor Is Reduced in HEK2/LERK-2 Coexpressing Cells—We have further analyzed the influence of HEK2/LERK-2 coexpression in 32D cells on the kinase activity of the receptor. This coexpression caused a very low level of autophosphorylation that was at the limit of detection in this analysis (Fig. 5A, lane 8). Prolonged exposure...
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Fig. 5. A, induction of tyrosine phosphorylation of the HEK2 receptor by LERK-2. Cells were co-cultivated for 30 min at 37 °C prior to lysis. Analysis of HEK2 receptor phosphorylation was performed via anti-phosphotyrosine blot (upper panel). The same blot was stripped and reprobed with anti-HEK2 antibody (lower panel). Lanes: 1, 32D cells; 2, 32D cells and LERK-2 expressing 32D cells (32DL6); 3, 32D cells expressing the kinase-deficient HEK2 receptor (HEK2/KIN−); 4, HEK2/KIN+ and 32DL6; 5, 32D cells expressing HEK2 (32DH20); 6, 32DH20 and conditioned medium from 32DL6 cells; 7, 32DH20 and 32DL6; 8, 32D cells coexpressing HEK2 and LERK-2 (H20L12); 9, 32DL6. B, stimulation of the kinase activity of HEK2 by LERK-2. To analyze HEK2 autokinase activity, immunoprecipitates were subjected to in vitro kinase reaction. Lanes: 1, 32D cells; 2, 32D cells and 32DL6; 3, HEK2/KIN−; 4, HEK2/KIN+ and 32DL6; 5, 32DH20; 6, 32DH20 and 32DL6; 7, 32DH20 and conditioned medium from 32DL6; 8, H20L12. C, soluble LERK-2/Fc does not induce tyrosine phosphorylation of HEK2 in HEK2/LERK-2 coexpressing cells. Cells were stimulated for 30 min at 37 °C with 250 ng/ml LERK-2/Fc prior to lysis. Immunoprecipitated HEK2 was blotted with monoclonal antibodies against phosphotyrosine. Lanes: 1, 32DH20; 2, 32DH20 and 32DL6; 3, H20L12; 4, 32DH20 and conditioned medium from H20L12; 5, H20L12 and soluble LERK-2/Fc; 6, 32DH20 and soluble LERK-2/Fc. M, size marker is indicated in kDa.

Discussion

The EPH-related kinases constitute the largest known family of orphan receptor tyrosine kinases with several members being expressed in the developing and adult nervous system. The recent characterization of a family of ligands that we call LERKs is a first step in trying to understand what cellular processes might be regulated by the EPH-related receptor proteins. In this article we have demonstrated that within this family of ligands only the type 1 transmembrane members, LERK-2 and LERK-5, bind to HEK2. Moreover, LERK-2 was shown to stimulate the kinase activity of the HEK2 receptor. In previous indirect binding assays LERK-2 protein expressed on CV-1 transfectants was shown to display also high affinity binding with a \( K_d = 1.08 \times 10^{-9} \text{ M} \) to elk/Fc (14). During the preparation of this manuscript, a study was published demonstrating that LERK-2 binds to CEK5 and CEK10, two EPH-related receptors from chicken tissues (23). The affinity constants for LERK-2 binding to these receptors are \( K_d = 2.3 \times 10^{-9} \text{ M} \) for CEK10 and \( K_d = 2.3 \times 10^{-9} \text{ M} \) for CEK5. Taken together, LERK-2 shows extensive cross-binding to EPH-related RTKs, as demonstrated for other members of the LERK-family (15), indicating functional redundancy.

Previous studies have proposed that membrane attachment of ligands is required to induce functional responses to EPH-
related receptors and that “shed” ligands do not (23–25). However, our results demonstrate that transmembrane as well as soluble forms of LERK-2 (LERK-2/Fc and a “shed” form of LERK-2) are able to induce phosphorylation of HEK2. In addition, Bartley et al. (12) was able to induce phosphorylation of the EPH family receptor, ECK, with a “shed” form of B61 (LERK-1) (12). Clearly, additional experimentation is required to determine the biochemical nature of these ligands. Still, membrane attachment facilitates ligand clustering to promote activation of EPH-related receptors (26).

HEK2/LERK-2 coexpressing cells and coincubated HEK2- and LERK-2 expressing cells were compared with respect to receptor phosphorylation. Interestingly, in the case of coexpressing cells, we observed reduced autophosphorylation of HEK2. This effect might be due to steric hindrance that results in an inappropriate interaction of the ligand to its receptor. Alternatively, the ligand could be modified in a way that reduces its ability to induce receptor phosphorylation. To elucidate this phenomenon, we performed in vitro kinase assays and found that kinase activity with immunoprecipitated HEK2 receptors from coexpressing cells is reduced compared with HEK2- and LERK-2 coincubated cells. Since conformational aspects are essential for receptor activation in vivo and have been shown to be of less importance in kinase assays, we speculate that steric hindrance is not the cause for reduced receptor activation. This was further supported by identical binding affinities in single and coexpressing cells. In addition, we demonstrated that the nature of the ligand from both cell types is very similar as judged by their migration in denaturing gels. Thus, we suggest that the intracellular portion of the LERK-2 inhibits receptor activation by reducing its kinase activity. A negative regulatory role of the intracellular portion of LERK-2 has been postulated in the case of coexpression with chimeras consisting of chicken EPH-related receptors and TrkB as well (23). A functional role for the cytoplasmic domain of LERK-2 is also suggested because of the high amino acid sequence identity between the cytoplasmic domains of LERK-2 and LERK-5 (19).

Cell-cell adhesion and juxtacrine activation that is mediated by interaction of a membrane-attached ligand with its receptor has been demonstrated for different cell-surface receptors (27–30). We asked the question whether the expression of the HEK2 receptor and LERK-2 in 32D cells induces the ability of 32D cells to adhere. Several lines of evidence support the conclusion that adhesion of these two transfectants is mediated by binding to membrane LERK-2 via HEK2 receptors. Cell adhesion mediated by membrane-attached ligands might support cell home to tissue locations that express a certain factor. Especially members of the EPH family containing cell adhesion domains in their extracellular portion are expressed in embryonic development of multicellular organisms at high levels. For example, the subcellular localization of Nuk receptor is concentrated at sites of cell-cell contact (31). High levels of the Nuk protein are found within initial axon outgrowth and associated nerve fibers. Our observations on cell adhesion using the 32D test system and on signaling through an EPH-related receptor could help to study those developmental processes in more detail.

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