Ligand for EPH-related Kinase (LERK) 7 Is the Preferred High Affinity Ligand for the HEK Receptor

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HEK is a member of the EPH-like receptor tyrosine kinase family, which appear to have roles in development and oncogenesis. Recently, we purified a soluble HEK ligand which is also a ligand (AL1) for the HEK-related receptor EHK1. Promiscuity appears to be a characteristic feature of interactions between the EPH-like receptors and their ligands, termed ligands for EPH-related kinases (LERKs). This prompted us to analyze the interactions between the HEK exodomain and fusion proteins comprising candidate LERKs and the Fc portion of human IgG (Fc) or a FLAG-peptide tag by surface plasmon resonance, size exclusion high performance liquid chromatography, sedimentation equilibrium, and transphosphorylation. Our results indicate that AL1/LERK7 is the preferred high-affinity ligand for HEK, forming a stable 1:1 complex with a dissociation constant of 12 nM. As expected the apparent affinities of bivalent fusion proteins of LERKs and the Fc portion of human IgG, had significantly reduced dissociation rates compared with their monovalent, FLAG-tagged derivatives. High-avidity binding of monovalent ligands can be achieved by antibody-mediated cross-linking of monovalent ligands and with LERK7 results in specific phosphorylation of the receptor. By extrapolation, our findings indicate that some of the reported LERK-receptor interactions are a consequence of the use of bivalent ligand or receptor constructs and may be functionally irrelevant.

The EPH-like branch of receptor tyrosine kinases (RTKs) has more than 28 members described in several vertebrate species (1–3). All were identified prior to the characterization of their cognate ligands by methods independent of a biological assay or specific physiological activity (2). As a consequence little is known about their specific functions. However, expression patterns of several EPH-like RTKs in embryogenesis, in particular in the nervous system, suggests a role in development (4, 5). Overexpression of some family members including HEK, EPH, ERK, and ECK in tumor-derived cell lines, tumor specimens, and transfected cells implicate these receptors in oncogenesis (6–10).

Recently we identified HEK on the cell surface of a pre-B acute lymphoblastic leukemia cell line, LK63, using the IIIA4 monoclonal antibody (mAb) (7). Immunofluorescence studies with IIIA4 revealed expression of HEK in blood samples from patients with acute leukemia, but not in normal adult tissues or blood cells (7, 11). In embryos, the expression patterns of the murine and chicken HEK homologues MEK 4 and CEK 4, and their recently identified ligand ELF1 (12) and RAGS (13), respectively, suggest a role in the development of the retinotectal projection map. We isolated a soluble HEK ligand from human placenta-conditioned medium using a biosensor-based affinity detection approach (14). The HEK ligand was identified by sequence homology as a soluble form of AL-1 (15), a member of the ligands for EPH-Related Kinases (LERKs) family (16, 17) and for consistency with other members will be referred to as LERK 7. This family of transmembrane or membrane-associated proteins were isolated as potential ligands for EPH-like RTKs through their interactions with recombinant EPH receptor family exodomains (15, 18–21). In most cases, bivalent Fc fusion proteins of either the receptor or the ligand were used for detecting potential binding partners. A requirement for membrane association of the ligand and the ability of more than one ligand to bind the same receptor with comparable affinity appeared to be characteristic of many of these ligands (21–25). However, soluble ligands for both ECK (26) and HEK (14) have been isolated from biological sources using receptor affinity-based protocols. Functional assays with the natural ligands leave some ambiguity about the absolute requirement for membrane association of the ligands for biological function (9, 15, 26, 27). In addition to binding LERK7, a bivalent HEK fusion construct was shown to bind with significant affinity to LERK 1, 2 (18), LERK 3, 4 (28), and LERK 5 (24).

Here we report the results of studies on the interaction between HEK and bivalent Fc-fusion proteins of LERKs 1 to 5 and LERK 7 or monovalent LERK3 and LERK7 produced as Flag-epitope tagged proteins. We use BIACore technology, SE-HPLC, sedimentation equilibrium centrifugation, and functional assays which show that LERK 7 is the principal HEK
and LERK 7—ligand interactions.

MATERIALS AND METHODS

Production of LERK 3 and LERK 7 (AL-1) Expression Constructs

The 5′-LERK7 oligonucleotide (GTAGTCTGACGACGCCGCGCT-
CCAAGGC) was based on the N-terminal amino acid sequence (QPDP-
SKA) of the mature protein, with a 5′-tag sequence and XhoI site
preceding the coding nucleotides. The 5′-LERK oligonucleotide (GTA-
GCTGATGATCCTCTTCGCCCGCGGATG) was based on the pre-
dicted C terminus of the glycosylphosphatidylinositol-linked form (PSR-
GEN), the coding bases were followed by a stop signal, an XhoI site, and
a spacer sequence. The polymerase chain reaction was performed using
an aliquot of a placentale cDNA library (kindly provided by Dr. Tracy
Wilson, Walter & Eliza Hall Institute) and Taq EXTEND (Boehringer
Mannheim). A 490-base pair product was detected on a 1.4% TAE,
ag NCAA, and the DNA excised and used to clone the C-terminal
sequence (PKLEKSI) followed by a stop signal, an XhoI site, and
a tag sequence.

Expression and Purification of Recombinant Receptor and Ligand Proteins

sHEK—Recombinant soluble HEK protein (sHEK) was purified from
culture supernatants generated from a Chinese hamster ovary (CHO)
cell transfectant and tested for conformational integrity using the BIA-
core™ with the sensor chip-immobilized anti-HEK mAb IIIA4 as de-
scribed previously (14).

Transfection of cells with LERK 3 and LERK 7 DNA—Purified LERK
7-pEFSBos or LERK 3-pEFSBos DNA was transfected into CHO cells.
Briefly, 2 × 10⁶ cells were suspended in 500 µl of PBS and 10 µg of
LERK-pEFSBos DNA and 1 µg of pSV2neo DNA added. After mixing
and transfer to a 0.4-cm electroporation cuvette (Bio-Rad), the cells
were electroporated at 270 V and 960 microfarads and the cells centri-
fuged through an fetal calf serum underlayer. Transfectant clones were
selected previously (14).

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were electroporated at 270 V and 960 microfarads and the cells centri-
fuged through an fetal calf serum underlayer. Transfectant clones were
selected in medium containing 600 µg/ml G418. Individual clones were
isolated and samples (5 µl) of CHO cell supernatants from confluent
cultures were dotted onto a nitrocellulose membrane, air-dried, and
re-hydrated in blocking buffer (5% skim milk powder, 0.1% Tween 20 Mr.
PBS) prior to incubation with M2 anti-FLAG antibody at 1:1000 dilu-
tion. The immobilization of sHEK onto the sensor chip surface was
performed essentially as described (14), LERK-FLAG (47 µg/ml in 20
µM sodium acetate, pH 4.5) was coupled at 5 µl/min onto 0-nitro-
hydroxycinnamide-N-ethyl-N′-(diethylamino)propylcarbodiimide (0.2 M)
fused to the LERK7-FLAG construct by comparison with the native LERK 7 molecule.

The LERK 7 cDNA was derived in a similar fashion and from the
same source using a sense oligonucleotide based on the N-terminal
sequence (NHAVAYYW) preceded by an XhoI site and a spacer (GTAG)
sequence (GTAGTCTGACGACGCCGCGCTGG) based on the N-
terminal sequence (PKLEKSI) followed by a stop signal, an XhoI site, and
a tag sequence.
Kinetics of the HEK-LERK Interaction

equilibrium responses according to,
\[
R_{eq}/C = K_d R_{max} - K_d R_{eq}
\]  
where \(R_{eq}\) and \(R_{max}\) are the equilibrium and maximum response levels, respectively (32). In addition to the analysis of ligand binding to sensor chip-immobilized sHEK, the interaction between LERK 3 and LERK 7 with sHEK was studied in solution. A constant ligand concentration was incubated with increasing concentrations of the soluble receptor. The free ligand concentration (\(F_{LERK}\)), estimated from the BIAcore response of a known LERK sample (32) was used to calculate the concentration of bound receptor (\(B_{LERK}\)) or ligand (\(B_{LERK}\)), and free sHEK (\(F_{HEK}\)) using the initial receptor concentration (\(T_{LERK}\)) and assuming in this case a single site interaction: 
\[
[F_{HEK}] = [T_{LERK}] - [B_{HEK}B_{LERK}], \text{ where } [B_{HEK}B_{LERK}] = [B_{HEK}] = [B_{LERK}].
\]
Thus the dissociation constant, \(K_d\) was estimated from,
\[
K_d = \frac{[F_{HEK}][F_{LERK}]}{[F_{HEK}]^2 - [F_{LERK}]} = \frac{[F_{HEK}]}{[F_{LERK}]} \quad \text{(Eq. 6)}
\]
according to Ward et al. (31), and Scatchard transformation yielded equation 7.

\[
\frac{[B_{LERK}]}{[F_{LERK}]} = \frac{1}{K_d} [F_{HEK}]
\]  

Immunoprecipitation and trans-Phosphorylation Assays

LERK3 cells (33) were maintained in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% fetal calf serum. The cells were passaged into fresh medium 24 h before the experiments. Following three washes with PBS, 0.5-ml samples of \(2 \times 10^7\) LERK3 cells/ml were incubated with 5 \(\mu\)g/ml LERK3-FLAG, LERK7-FLAG, or with preformed complexes (30 min at room temperature) of either LERK3-FLAG (2 \(\mu\)g) or LERK7-FLAG (2 \(\mu\)g) and anti-FLAG mAb (2 \(\mu\)g) for 1 h on ice. The cell suspension was warmed to \(37^\circ\)C for 10 min, then washed (ice-cold PBS) and the cell pellet lysed in 750 \(\mu\)l of TBS, pH 7.5, containing 1% Triton X-100, 1 mM orthovanadate, 4 mM sodium fluoride, and 1 \(\mu\)M chymostatin, leupeptin, antipain, and pepstatin. HEK was immunoprecipitated from the lysates using IIIA4-Trisacryl beads (Sepracor/IBF, Villeneuve la Garenne, France) as described previously (7). The immunoprecipitates were subjected to 7.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Amersham, Australia). The Western blots were probed using an anti-phosphotyrosine mAb (PY20, ICN Immuno-Biologicals, Costa Mesa, CA) and developed using the ECL Western blot analysis kit (Amersham, Australia). The membranes were stripped and re-probed with a rabbit polyclonal antiserum raised against sHEK (rabbit anti-HEK).

RESULTS

Binding of Various LERK-Fc Fusion Proteins to Sensor Chip-immobilized sHEK—Receptor-ligand interactions within the EPH-type RTK family have been studied mainly by a modified indirect Scatchard analysis of human IgG-Fc fusion proteins of ligands or receptors binding to receptor or ligand-transfected cells, respectively (18, 20, 22, 24, 27, 34). To evaluate if BIAcore
analysis also detected the interaction of various LERKs with HEK we compared the binding of bivalent, Fc-fusion proteins of LERK (1 to 5) and AL1/ LERK 7 (Fig. 1C) to sensor chip-immobilized sHEK. Each ligand construct was injected at concentrations between 0.1 and 10 μg/ml (approximately 0.8–80 nM) across the sensor chip. A sample containing 10 μg/ml of the recombinant human Fc fragment was used as a control. The relative binding response units of various samples at 10 μg/ml are illustrated in Fig. 2, indicating comparable responses for LERK 3, 4, 5, and 7 which were considerably greater than the responses of LERK 1 and LERK 2. Apparent dissociation constants derived from equilibrium responses (Equation 4) at the four highest concentrations suggested a decreasing order of nanomolar affinities AL1/ LERK 7 > LERK 3 > LERK 4 > LERK 5 (data not shown). The interactions with LERKs 1 and 2 did not reach equilibrium responses in our experiments and hence precluded estimation of dissociation constants. Only background binding was seen with the control recombinant Fc construct on its own.

**Interaction Kinetics of LERK 3-Fc and LERK 7-Fc Binding to sHEK**—The similar binding of the LERK 3, LERK 4, and LERK 7-Fc fusion proteins to sHEK (Fig. 2) prompted us to study the binding kinetics of two of the proposed HEK ligands, LERK 3 (28) and AL1/ LERK 7 (14) in more detail. sHEK-BIAcore signals to serial dilutions of bivalent, LERK 3 or AL1/ LERK 7-Fc fusion proteins are shown in Fig. 3 and were used to derive the kinetics of the interactions. As suggested (32), the early parts of the dissociation phase (550–650 s, Fig. 2) were chosen for analysis. Examination of the raw data on the basis of a pseudo-first order reaction revealed a correlation-concentrated change (not shown) in the apparent dissociation rate constants from 0.1 to 1.1 × 10^{-3} s^{-1} for LERK 3 (5–80.0 nM) and from 7.2 to 8.6 × 10^{-4} s^{-1} for AL1/ LERK 7 (5–80.0 nM) and an increasingly inferior fit (χ2) to the assumed dissociation model (Table I). Apparent association rate constants of k_{a} a = 1.3 × 10^{5} m^{-1} s^{-1} for LERK 3-Fc and of k_{a} a = 9.6 × 10^{5} m^{-1} s^{-1} for LERK 7-Fc (Table I) were estimated from the rate change of the BIAcore signal (Equations 2 and 3) with an improved fit to a single-component association model (Table I). Again, an early part of the association phase was selected for the analysis. Nonlinear least square analysis performed on the same regions of the sensorgrams gave similar values (data not shown). Estimation of apparent equilibrium responses from the association data (Equation 3) and their analysis in a Scatchard format (Equation 5) yielded curvilinear plots (not shown) suggesting deviation of the LERK-Fc/HEK interaction from linear, single component kinetics.

The deconvolution of BIAcore raw data on the basis of a two-component dissociation (Equation 4) improved the correlation with the kinetic model substantially and a comparison of the two approaches by t test unambiguously favored the two component model (not shown). Dissociation rate constants (Table I) for LERK 3-Fc of 0.05 s^{-1} (fast) and 5.7 × 10^{-4} s^{-1} (slow) and for LERK 7-Fc of 0.04 s^{-1} (fast) and 5.7 × 10^{-4} s^{-1} (slow) were estimated. With the association rate constants suggested above, apparent low and high affinity constants were derived (kd. Table I) for LERK 3-Fc (K_{D1} = 3.8 × 10^{-7} M, K_{D2} = 4.4 × 10^{-9} M) some 5–10-fold lower than the corresponding affinity constants for LERK 7-Fc (K_{D1} = 4.1 × 10^{-9} M, K_{D2} = 5.9 × 10^{-10} M). Although this evaluation undoubtedly harbors some ambiguity with respect to the association kinetics, the identical treatment of the data for both LERK-Fc constructs justifies the use of these dissociation constants in a qualitative comparison of the LERK-Fc interactions with sHEK.

To address ambiguities which may affect the kinetic analysis of BIAcore data, including rebinding of dissociating ligand and heterogeneity of the receptor derivatized sensor surface (35), we examined the LERK-Fc interactions with sHEK in solution and estimated affinities by Scatchard analysis of free and receptor-bound ligand at equilibrium (31). Sensor chip-immobilized sHEK was used to monitor the abundance of free ligand in samples of 2 μM LERK 3-Fc (Fig. 4A) or LERK 7-Fc (Fig. 4B) which had been incubated for 16 h at 15 °C with increasing concentrations of sHEK. Concentrations of free LERK-Fc ([P\text{LERK}]) were estimated by linear regression from responses of samples with a defined LERK-Fc concentration, whereas the concentration of bound ligand ([P\text{LERK}]) was derived as difference between total and free ligand under the premise of single component interactions. Although the slope of the Scatchard plots (Fig. 4A and B) gave no direct indication for a deviation from first-order kinetics, negative estimates for [B\text{LERK}]/ [P\text{LERK}] at low sHEK concentrations suggested an apparent [F\text{LERK}] exceeding the total ligand concentration ([T\text{LERK}]) and thus reflect artificially high responses in these samples. BIAcore signals, in general, depend on concentration, size, and affinity of the interacting ligand (36). Thus, responses exceeding the signal for [T\text{LERK}] suggest either an increased size (due to LERK-Fc proteins containing a single bound sHEK) or increased affinity of the interacting LERK-Fc proteins and indicate a receptor concentration-dependent deviation from first-
order kinetics. The apparent affinity derived from this experiment for the LERK 3-Fc/HEK interaction with a $K_D = 3.6 \times 10^{-3} \text{M}$ (Fig. 4A) was significantly lower than the affinity of the LERK7-Fc/HEK interaction ($K_D = 5.7 \times 10^{-9} \text{M}$, Fig. 3B). Interaction Kinetics of LERK3-FLAG and LERK7-FLAG Binding to sHEK—The above discussed kinetic analyses indicated that reactions of both LERK-Fc proteins with sensor chip-immobilized sHEK apparently involved low- and high-affinity components. To evaluate if the bivalency of Fc-ligand constructs was a likely cause for the observed heterogeneity of the kinetics we performed binding experiments with monovalent forms of LERK 3 and LERK 7. Corresponding FLAG peptide-tagged fusion proteins were expressed in CHO cells and purified to homogeneity from culture supernatants of selected clones by anti-FLAG affinity chromatography and ion exchange HPLC. The identity of the recombinant ligand proteins was confirmed by N-terminal amino acid sequence analysis.

A qualitative comparison of the BIACore data, illustrating binding of increasing amounts of LERK3-FLAG (Fig. 5A) and LERK7-FLAG (Fig. 5B) to a HEK sensor chip, reveals marked differences in the kinetics of the two interactions. The LERK3-sHEK interaction is characterized by extremely fast on- and off-rates and comparable responses of LERK 3 or LERK 7 binding to sHEK were found only at approximately 30-fold higher LERK 3 concentrations in the applied sample. Kinetic analysis of the association and dissociation phases using a single component model yielded apparent association and dissociation rate constants of $k_a = 4.8 \pm 0.13 \times 10^{9} \text{M}^{-1} \text{s}^{-1}$ and $k_d = 6.1 \pm 0.8 \times 10^{-3} \text{s}^{-1}$ for LERK 7 and $k_a = 3.7 \pm 0.9 \times 10^{9} \text{M}^{-1} \text{s}^{-1}$ and $k_d = 0.26 \pm 0.06 \text{s}^{-1}$ for LERK 3. Apparent dissociation constants $K_D = 1.2 \times 10^{-8} \text{M}$ for LERK7-FLAG and $K_D = 5.9$ M. Lackmann, L. Kravets, T. Bucci, and A. Boyd, unpublished data.

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**Fig. 4.** Solution equilibrium constants of the LERK 3-Fc and LERK 7-Fc interaction with sHEK. Samples (35 μl) containing LERK 3-Fc (panel A) or LERK 7-Fc (panel B) at a constant concentration (2 nM), in equilibrium with increasing amounts of sHEK after a 12-h incubation at 15 °C, were injected across a sHEK sensor surface. The concentration of free ligand in these samples was derived by correlation of responses to known LERK-Fc concentrations and enabled estimation of solution equilibrium affinity constants by Scatchard analysis (see “Materials and Methods”).

**Fig. 5.** Binding curves for the interaction of monovalent LERK-FLAG fusion proteins with immobilized sHEK. Homogeneous preparations of CHO cell-derived LERK3-FLAG (panel A) or LERK7-FLAG (panel B) at increasing concentrations (8.22, 16.44, 32.88, 65.75, 131.5, 263, and 526 nM LERK3-FLAG, 1.25, 2.5, 5, 10, 20, 40, and 80 nM LERK7-FLAG) were injected across a sHEK-derivatized sensor surface and BIACore data for the association and dissociation phases (as detailed for Fig. 2) used to estimate corresponding kinetic rate constants on the basis of a one to one interaction model (see “Materials and Methods”). Panel C, free sHEK was estimated in samples of increasing sHEK with a constant LERK7-FLAG concentration at equilibrium as outlined in the legend to Fig. 4 and used to calculate the equilibrium dissociation constant $K_D$ by Scatchard analysis (see “Materials and Methods”).
(±0.4) × 10^{-7} m for LERK3-FLAG were estimated, which compare well with the estimates of the low-affinity components of the corresponding LERK-Fc constructs (see Table I). Analysis of the raw data revealed good fits to linear, “one to one” interactions, yielding $\chi^2$ values of 0.64 ± 0.18 and 0.47 ± 0.06 for the LERK 7 and LERK 3 reactions, respectively. The apparent equilibrium affinity constant for LERK 7 was substantiated by Scatchard analysis of the “in-solution” interaction (31), yielding an identical dissociation constant of $K_D = 1.2 \times 10^{-8}$ M (Fig. 5C). On the other hand, the affinity of the LERK3-FLAG interaction was too low to obtain reliable data by in-solution analysis.

Cross-linking of LERK-FLAG Proteins with Anti-FLAG MAb Alters Interaction with sHEK—We next addressed the notion that the differences observed in the binding of either FLAG-tagged and Fc-tagged LERKs to sensor chip-immobilized HEK were due to increased avidity of the divalent, Fc-tagged ligands. To qualitatively examine this effect in situ, we assembled bivalent ligand-mAb complexes before or during BIAcore experiments by cross-linking FLAG-tagged LERK 7 (Fig. 6, A and B) and LERK-3 (Fig. 6, C and D) with the anti-FLAG mAb, M2.

The interactions of preformed LERK-FLAG-M2 mAb complexes (Fig. 6, B and D, graph e) with a sHEK sensor chip resulted in 3–5-fold increased BIACore responses and markedly reduced off-rates of the ligand-antibody complexes compared with the non-complexed LERK-FLAG proteins (Fig. 6, A and C, graph c), reflecting the increased size, and indicating an altered avidity, of the interacting complexes. To confirm this notion, we injected FLAG peptide, competing with the LERK-FLAG proteins for M2 mAb-binding sites, into the dissociation phase of LERK-FLAG-M2 mAb complex (Fig. 6B, graph f, “2”). A dramatically increased off-rate in this experiment confirmed that the suggested increase in avidity was dependent upon M2 mAb-mediated cross-linking of the LERK-FLAG proteins. Furthermore, injection of the M2 mAb into dissociating LERK-FLAG proteins at the end of the first injection cycle (”1”) resulted in a pronounced rise of the BIACore signals, likely due to binding of newly formed ligand-mAb complexes (Fig. 6, B and D, graph d). The increase of the responses above the levels observed with the monovalent ligands in the first part of the sensorgram (between “1” and “2”), presumably reflects the increased size of the interacting ligand-mAb complexes. On the other hand, amplitude and slope of the response curve are also determined by the abundance and avidity of the ligand available for complex formation at the time of mAb injection. Since injection of equimolar amounts of LERK 3-Fc or LERK 7-Fc are expected to yield the same ligand concentrations at the end of the first injection cycles, differences in the amplitude of the response following mAb injection “2” (compare graph d in Fig. 6, B and D) must portray primarily the different affinities of the LERK-FLAG-M2 mAb complexes. In support of this notion, the dissociation curves and the response levels of pre-formed (graph c) and in situ formed (graph d) ligand-mAb complexes at the end of the second injection cycle (after 1090 s) were found identical (Fig. 6D) or very similar (Fig. 6B).

Taken together, this strictly qualitative analysis demonstrates that M2 mAb cross-linked LERK-FLAG dimers bind sHEK with increased avidity due to decreased dissociation rates. The resulting response curves are qualitatively very similar to the sensorgrams of the corresponding LERK-Fc fusion proteins (Fig. 3), suggesting that avidity plays a major role in the interaction kinetics of these ligand constructs.

Analysis of LERK-FLAG Binding to sHEK by SE-HPLC and SDS-PAGE—A key issue in interpreting the biological response to ligand binding is the stoichiometry of receptor-ligand complexes. Since kinetic considerations above strongly implied a one to one interaction we further examined receptor-ligand complexes by SE-HPLC, SDS-PAGE, and equilibrium sedimentation. To facilitate complete conversion of the monomeric ligands into ligand-receptor complexes, solutions of LERK3-FLAG or LERK7-FLAG were incubated for 2–12 h with a 10-fold molar excess of the receptor exodomain prior to analysis by micropreparative SE-HPLC in a physiological buffer. The absorption of eluting proteins was monitored at 215 nm and proteins in manually collected fractions analyzed by SDS-PAGE and silver staining (Fig. 7). The chromatogram and corresponding SDS-PAGE profile (Fig. 7A) indicate complete conversion of monomeric LERK7-FLAG into a sHEK:LERK7-FLAG complex which elutes as a shoulder (lane 1) on the ascending part of the major HEK peak (Fig. 7A, “1”, lane 2). No free LERK7-FLAG is detected at the corresponding elution position (Fig. 7A, “1”), confirmed by SDS-PAGE/silver staining of this sample (Fig. 7A, lane 3).

By contrast, SE-HPLC and SDS-PAGE profiles of the LERK3-FLAG containing sample (Fig. 7B) do not reveal a stable, high-molecular weight LERK 3-FLAG-sHEK complex. A protein peak eluting between sHEK (Fig. 7B, “3”) and LERK3-
FLAG and containing both, ligand and receptor (Fig. 7B, lane 2), as well as some apparently non-complexed LERK3-FLAG in the adjacent fraction corresponding to its original elution position (Fig. 7B, lane 3) suggest only a weak interaction between the two components.

However, the addition of cross-linking M2 antibody to the receptor/ligand-FLAG mixture results in the formation of a high-molecular weight complex, eluting as a shoulder in front of the elution position of the LERK3-FLAG-M2 complex (Fig. 7C, lane 4). SDS-PAGE and silver staining suggests that this fraction contains M2-mAb, sHEK, and LERK3-FLAG (Fig. 7C, lanes 1 and 2), whereas no free LERK3-FLAG is detected in the fraction corresponding to free ligand (lane 4).

Equilibrium Sedimentation Analysis—Equilibrium sedimentation was used to analyze the receptor-ligand complexes in detail and substantiate our findings from BIAcore and SE-HPLC experiments, inferring a stable one to one interaction between sHEK and LERK7-FLAG, but not LERK3-FLAG. To achieve a complete conversion of sHEK into a receptor-ligand complex suitable for sedimentation analysis, a 10-fold molar excess of LERK 7 was incubated with the soluble receptor for 1 h and the complex purified by SE-HPLC (inset to Fig. 8A). Rechromatography of this material confirmed a stable interaction and yielded a purified receptor-ligand complex (Fig. 8A) suitable for analysis by analytical ultracentrifugation. The linear dependence of the logarithm of the concentration upon the square of the radial distances for ligand, receptor, and ligand-receptor complexes is shown in Fig. 8B and suggests homogeneity with respect to the molecular weights. By contrast, we were unable to isolate a stable LERK 3-sHEK complex under identical conditions (not shown). Results illustrated in Fig. 8B and Table II reveal molecular sizes of 27,300 and 67,900 daltons for LERK7-FLAG and sHEK, respectively, in very good agreement with the apparent molecular masses derived from SDS-PAGE (LERK 7, 28,000, sHEK, 68,000, Fig. 7) and SE-HPLC (14). Furthermore, an unambiguous molecular mass assignment of 89,400 daltons for the receptor-ligand complex...
The values for $m (1 - \nu)$ were derived from sedimentation equilibrium data (Fig. 8B). The values of $\nu$ were estimated as described previously (31) from sedimentation equilibrium data, assuming a value of 0.61 ml/g for bound carbohydrate and using values of 0.7267 ml/g and 0.728 ml/g calculated from the amino acid composition of sHEK and LERK 7, respectively. The value of $\nu$ for the complex was calculated with the assumption of zero volume change on association and equimolar ratios of the components.

Molecular weight values are calculated from the measured values of $m (1 - \nu)$ and the assigned values of $\nu$.

Table II

| Species          | $m (1 - \nu)^a$ | $\nu^b$ | Molecular weight |
|------------------|-----------------|---------|-----------------|
| sHEK             | 19.110          | 0.715   | 67,900          |
| LERK 7           | 7.695           | 0.715   | 27,300          |
| sHEK + LERK 7    | 25.170          | 0.715   | 88,400          |

*a The values for $m (1 - \nu)$ were derived from sedimentation equilibrium data (Fig. 8B).

*b The values of $\nu$ were estimated as described previously (31) from sedimentation equilibrium data, assuming a value of 0.61 ml/g for bound carbohydrate and using values of 0.7267 ml/g and 0.728 ml/g calculated from the amino acid composition of sHEK and LERK 7, respectively. The value of $\nu$ for the complex was calculated with the assumption of zero volume change on association and equimolar ratios of the components.

Induction of HEK Phosphorylation in Ligand-treated Cell Cultures—In addition to the kinetic analysis of the LERK-sHEK interaction we compared the ability of either LERK 3 or LERK 7 to mediate transphosphorylation of HEK in LK63 cells which have been shown to express the receptor constitutively (7). LK63 cell cultures were incubated with buffer or solutions containing LERK3-FLAG, LERK7-FLAG, or pre-formed complexes of these ligands with anti-FLAG mAb, M2. In the latter samples the concentrations of LERK-FLAG proteins and M2 mAb were adjusted to provide divalent ligand constructs by occupancy of both binding domains of the mAb with ligand-FLAG construct. The HEK receptor was then immunoprecipitated from the cells and analyzed by Western blot analysis. The results in Fig. 9 illustrate the analysis with PY20 antiphosphotyrosine antibody (panel A) followed by reprobing the stripped blots with rabbit anti-HEK antibody. Phosphotyrosine analysis shows no significant differences between control (lane 2), LERK3-FLAG (lane 4), or LERK7-FLAG (lane 3)-treated samples. In contrast, incubation of cells with LERK3-FLAG-M2 complex induced a small but significant increase (lane 2) and with LERK7-FLAG-M2 complex (lane 1) gave a dramatic increase in phosphotyrosine content of HEK. Corresponding bands on the rabbit anti-HEK probed blots (panel B), show no significant difference in total HEK protein between the experimental groups.

Discussion

The apparent “cross-talk” between various members of the LERK family and the EPH-like receptor HEK (18, 22, 24, 28) including LERK 7 (14), prompted us to study receptor-ligand interactions between HEK and its proposed ligands, employing BIACore and other analytical technologies. In our experiments we sought to (a) define the highest affinity ligand for the HEK RTK; (b) define the kinetics and stoichiometry of the receptor-ligand complex formation; (c) determine the biological response to ligands either in monomeric or dimeric forms.

Most of the studies of EPH-like receptors and their ligands carried out to date have been performed with divalent (Fc fusion) constructs of either ligand or receptor (Refs. 4 and 37). We compared the binding of different LERK-Fc fusion proteins to HEK sensor chips and confirmed the suggested (18, 28) cross-reactivity of all the tested LERK-Fc constructs with HEK (Fig. 2). In accord with these reports, the interaction between HEK and Fc constructs of LERKs 1 and 2 was distinctively weaker than binding of LERKs 3 and 4, which yielded in our experiments similar BIACore responses to LERK 7. On the other hand, while the previously published affinities of LERKs 1, 2, and LERK 5-Fc for HEK are very similar (18, 43, and 23 nM, respectively (18, 24)), we could estimate apparent dissociation constants only from equilibrium responses of LERKs 3, 4, 5, and LERK 7-Fc (K$_d$ values of 5, 6, 24, and 3 nM, respectively, data not shown), whereas binding of LERKs 1 and 2 was too weak for a kinetic analysis. In addition, biphasic binding was reported previously only for the interaction between HEK-Fc with LERK 2, where a low affinity constant of 430 nM was found (18). Our comparative analysis of the association and dissociation phases of two candidate HEK ligands, LERK 3 (28) and LERK 7 (14), indicated a concentration-dependent increase of the apparent dissociation rate constants (not shown) and an increasingly poor fit to the assumed one-component dissociation model (Table I).

This significant deviation of the divalent LERK-Fc kinetics from a linear, single component interaction, suggesting a high-affinity interaction at low and a low-affinity interaction at high ligand concentrations confirms earlier studies by Hogg et al. (38) and Posner et al. (39) which demonstrate that kinetic models based on a one to one stoichiometry do not adequately describe the dissociation of bivalent solutes from surface-bound receptors.

The use of different approaches for the kinetic analysis of HEK/LERK-Fc interactions could explain the differences between the published data and our findings. A direct evaluation of kinetic data from BIACore progress curves is likely to be more sensitive to changes in kinetic rate constants than "indirect Scatchard analysis" which relies on the use of labeled mouse anti-human IgG antibodies to detect receptor-Fc fusion proteins bound to ligand-transfected cells (15, 18, 22, 24, 28, 37). Competitive binding experiments of the LERK-Fc/HEK interaction in solution (Fig. 4), which are not affected by immobilization artifacts and/or rebinding of dissociating ligand (31, 40) but rely on an "indirect" estimation of bound ligand or receptor (see "Materials and Methods"), gave no direct indication for biphasic kinetics from the slope of the Scatchard plots but yielded negative [B$_{LERK}$/[F$_{LERK}$] values at low sHEK concentrations, thus indicating artifically high responses in these samples. The interaction of bivalent LERK 7-Fc, containing only a single bound sHEK, via the remaining free LERK 7 moiety to the sHEK sensor surface, is a likely explanation for this artifact and confirms the concentration-dependent bivalency of the LERK-Fc/HEK interaction.

The comparative evaluation of all our binding data suggests that the bivalent, high-affinity interaction of two covalently linked binding domains of the ligand/Fc fusion protein with two adjacent, sensor chip-immobilized receptor molecules will compete at saturating ligand/Fc concentrations with a low-affinity monovalent interaction of a single binding domain with a single receptor molecule. Similar effects have been described for the analysis of mAb-antigen interactions (40–45) and for the interaction of dimeric interleukin (IL) 6 with the sensor chip-immobilized IL-6 receptor-exodomain (31, 46).
In other studies of EPH receptor-LERK interactions, the effect of solute bivalency has not been addressed. The necessity of ligand clustering for efficient receptor activation (15, 22, 24) seemed to warrant the use of bivalent receptor or ligand constructs. Such constructs were also used most recently in whole embryo in situ staining to confirm kinetic experiments performed with the same receptor-Fc constructs (37). On the other hand, it remains to be demonstrated that the interaction between membrane-bound ligands (or receptors) and Fc-tethered, bivalent receptors (or ligands) is a suitable system to study kinetics of physiological interactions of membrane-bound ligands and receptors (4). Our experiments indicate that the artificial bivalency of the ligand constructs obscures an unambiguous analysis of the reaction kinetics. In agreement with a report on the kinetics of the cell adhesion molecule CD 2 and its ligands (Figs. 5 and 6). Due to an extremely fast off-rate, the interaction of monovalent LERK3-FLAG with the immobilized receptor is very weak (Fig. 5F), an observation confirmed in solution which indicated an unstable, transient LERK3-FLAG-sHEK complex (Figs. 6 and 7). By contrast, binding of LERK7-FLAG to sHEK was characterized by a 40 times lower off-rate and resulted in a stable receptor-transient LERK3-FLAG complex from solution (Figs. 7 and 8) and to facilitate its characterization by equilibrium sedimentation analysis (Fig. 8F). The demonstration of a 1:1 stoichiometry confirms our findings from BIAcore and SE-HPLC experiments, indicating that HEK has a single binding site for LERK7 and explaining the necessity of ligand cross-linking for receptor activation and transphosphorylation demonstrated in this study (Fig. 9) and reported by others (21, 22, 48).

We attempted to produce a LERK 7-dependent cell line by transfection of DCPC-1 and Ba/F3 cells with HEK at several receptor densities. The inability of monomeric or complexed LERK 7 to rescue transfected cells after intermittent or complete IL-3 removal suggests that the HEK cytoplasmic domain does not mediate signals to induce proliferation or that cytosolic components for HEK-specific signaling are absent from these cells. On the other hand, this finding could also suggest that EPH-type RTKs regulate cell movement rather than cell growth.

In summary, our results clearly identify LERK 7 as the best candidate for a physiological HEK ligand. Despite very similar apparent affinity constants for the LERK 3 and LERK 7-Fc fusion proteins the interaction between their monovalent analogues and sHEK differ substantially by a markedly higher dissociation rate of LERK3-FLAG protein. Cross-linking of the dissociating ligands with anti-FLAG mAb decreases the dissociation rates and results in similar interaction kinetics for both ligands. Our results could suggest, that the reported interactions between some of the LERKs and HEK are influenced by the choice of the ligand construct. Extrapolating our observations to the in vitro situation, it seems likely that LERK 3 would function as an effective ligand only at very high receptor and ligand densities on opposing cell membranes, whereas a stable LERK7-HEK complex will persist at much lower receptor and ligand numbers. The demonstration of ligand and receptor gradients of HEK and AL1 homologues during neural development (13, 49) would support a notion whereby the hierarchy of ligand affinities and characteristic ligand gradients would provide a subtle regulation of the migratory behavior of HEK-positive cells.

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