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Molecular cloning of a ligand for the EPH-related receptor protein-tyrosine kinase Htk

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ABSTRACT **Htk is a receptor protein-tyrosine kinase that is related to the EPH subfamily of tyrosine kinases. The receptor has a wide tissue distribution including expression in several myeloid hematopoietic cell lines. Using an Htk–Fc fusion protein, a protein ligand for this receptor was expression cloned from the murine kidney mesangial cell line SV40MES 13. The Htk ligand cDNA encodes a transmembrane protein of 336 amino acids. Binding competition experiments demonstrated a K_d of 535 pM for binding of Htk–Fc to the Htk ligand. Incubation of 3T3 cells expressing Htk with COS-7 cells expressing the ligand resulted in tyrosine phosphorylation of Htk. The ligand, like its receptor, is widely expressed and may function in a variety of tissues. However, we localized hematopoietic expression of Htk to the monocytic lineage, suggesting that the ligand may play a role in differentiation and/or proliferation of these cells.**

Receptor protein-tyrosine kinases play important roles in cellular proliferation and morphogenesis in a wide variety of cell types (1, 2). On the basis of predicted structural homologies and sequence conservation, receptor protein-tyrosine kinases have been assigned to several subclasses (2). Of these, the EPH subfamily constitutes the largest thus far discovered, containing at least 12 members. Htk is a member of this subfamily and is most closely related to the ELK and HEK receptors (3). The biological functions of this family of receptors have yet to be discovered but both developmental (4, 5) and central nervous system functions have been postulated (6). Very recently, several ligands for EPH-related receptors have been identified and/or cloned (6–10). B61 was identified as a ligand for the receptor ECK (7), LERK-2 was cloned as a ligand for the ELK receptor (6, 8), ELF-1 as a ligand for MEK and SEK (9), and EHK-1 ligand as a ligand for the EHK-1 receptor (6). However, thus far, this has not led to the demonstration of any biological effects in response to receptor activation.

Unlike most of the family members, Htk does not appear to be expressed in the central nervous system. However, as with other members of the EPH-related receptor family, Htk and its murine homolog myk-1 demonstrate expression in primary epithelia and epithelial cell-derived cell lines (3, 11). In addition, Htk has a wide tissue distribution and includes several myeloid hematopoietic cell lines and human $CD34^+$ cells (3). Interestingly, we isolated the murine homolog of Htk from $AA4^+$ cells from the murine fetal liver (unpublished data). The monoclonal antibody AA4.1 recognizes ≈1% of midgestation fetal liver cells, and this fraction contains nearly all the hematopoietic potential of this major developmental hematopoietic organ (12). Collectively, these data suggest a potentially important hematopoietic role for this receptor.

To assist in defining the functional role of Htk, we sought to clone its cognate ligand. In these studies, we report the cloning of the Htk ligand from the kidney cell line SV40MES 13.¶ Initial results show that the ligand is widely expressed but also indicate that it may play an important role in monocyte development and/or proliferation.

EXPERIMENTAL PROCEDURES

Identification of a Ligand Source. Htk–Fc fusion protein was produced as described (3). Cell lines were screened for Htk–Fc binding by fluorescence-activated cell sorter (FACS) analysis as described (13).

Binding Competition Experiments. Monolayers of COS-7 cells (5×10^5 cells per 10-cm dish) were transiently transfected with murine Htk ligand using the DEAE-dextran transfection method (14). Transfected COS-7 cells (COS-7t) used at 2.5×10^4 cells per binding point or SV40MES 13 cells (5×10^6 cells per binding point) were assayed for steady-state binding of ^{125}I-labeled Htk–Fc in the presence of various amounts of unlabeled Htk–Fc as described (15). The binding data were analyzed to determine the affinity and number of sites per cell as described (16). Htk–Fc fusion protein was iodinated by the lactoperoxidase method as described (17).

Tyrosine Phosphorylation Experiments. One million 3T3 cells stably transfected with Htk (3T3-T) (3) or nontransfected 3T3 cells (3T3) were coincubated with 1×10^6 Htk ligand-transfected COS-7 cells, mock-transfected COS-7 cells, or 3×10^6 SV40MES 13 cells at 37°C for 30 min. Transfected and mock-transfected NIH 3T3 cells were also incubated with anti-human Htk antibody IC2-C2 (10 μg/ml). Cells were lysed in Nonidet-40 lysis buffer (1% Nonidet-40/1 mM EDTA/200 mM NaCl/50 mM Tris·Cl, pH 8.0/2 mM phenylmethylsulfonyl fluoride/2.5 mM Na_3VO_4) immunoprecipitated with anti-human Htk rabbit polyclonal antiserum (3) and analyzed on SDS/4–12% polyacrylamide gradient gels. Gels were then transferred to nitrocellulose and Western blotted using the anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY).

3T3 cells were stably transfected with Htk receptor expressed in the expression vector pRIS by the calcium phosphate method as described (18). Htk ligand was transiently transfected into COS-7 cells by the DEAE-dextran method (14). The murine monoclonal anti-human Htk antibody IC2-C2 was derived by a standard hybridoma preparation. Htk–Fc fusion protein was used as immunogen. Specificity of the antibody was demonstrated by FACS analysis using stably transfected 3T3 cell lines (data not shown).

Cloning of Htk Ligand. Murine Htk ligand was cloned from the SV40MES 13 cell line. An SV40MES 13 cDNA expression library was constructed in pRK5B (19) and screened by transfecting pools of 2000 cDNAs into COS-7 cells and measuring the binding of the Htk–Fc fusion protein to the transfected cells by

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Abbreviations: FACS, fluorescence-activated cell sorter; gD, glycoprotein D.

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¶The sequences reported in this paper have been deposited in the GenBank data base [accession nos. L38847 (murine) and L38734 (human)].

slide autoradiography as described (20). Five positive pools were identified in the first 50 pools screened. Two positive pools of cDNAs were subdivided into smaller pools and reanalyzed for Htk–Fc binding until single cDNA clones were identified. Two independent clones were sequenced to confirm the coding region. The human Htk ligand cDNA was cloned from human fetal brain and human fetal lung (Clontech) libraries by using an 800-bp fragment from the 5′ end of the mouse cDNA as probe. DNA sequencing was performed with the *Taq* dye deoxynucleotide terminator cycle sequencing kit on an automated Applied Biosystems DNA sequencer (model 373A). Both strands of individual clones were sequenced in their entirety.

Expression and Purification of the Soluble Htk Ligand. A herpes glycoprotein D (gD)–Htk ligand fusion was constructed by cloning the reading frame encoding the mature Htk ligand (beginning at amino acid 32) C-terminal to the first 49 amino acids of the herpes gD in the expression vector pRK5B. The gD–Htk ligand fusion construct was transiently transfected in COS-7 cells by using DEAE-dextran. At 48 hr posttransfection, plates were washed with PBS and conditioned for 18–24 hr in PS24 serum-free medium with human tumor necrosis factor (20 ng/ml). Conditioned medium was purified by affinity chromatography using an anti-gD monoclonal antibody (5B6) affinity column. Purified fusion protein was dialyzed against PBS and concentrated to a final concentration of ≈14 ng/μl.

Northern Blot Analysis. Northern blots were obtained from Clontech and contain 2 μg of poly(A)-selected RNA per lane from the tissues indicated. Mouse blots were hybridized in 50% formamide at 42°C to ^{32}P-labeled murine Htk ligand cDNA and washed under stringent conditions (final wash, 0.2× SSC/0.2% SDS at 60°C). Human tissue blots were hybridized in 35% formamide at 42°C and washed under the stringent conditions described above.

Hematopoietic Cell Isolations. Blood or umbilical cord blood was collected in heparin (10 units/ml)/0.5 mM EDTA in PBS. CD34$^+$-enriched cell populations were produced by using the Ceprate LC cell isolation system (CellPro, Bothell, WA). Htk$^+$-enriched populations were produced similarly but with biotinylated anti-human Htk monoclonal antibody IC2-2C2. Enriched populations were washed once with Hanks' balanced salt solution/fetal bovine serum and then restained with either HPCA-2-PE (Becton Dickinson) or anti-CD14-PE (Pharmingen) and fluorescein isothiocyanate conjugated IC2-2C2 for 30 min. Isotype controls at the appropriate concentration were used to determine specificity of staining.

RESULTS

Given the high level of expression of Htk in kidney, heart, and lung, we focused our efforts to locate a source of Htk ligand on cell lines from these organs. We identified a source of the ligand by using a human Htk receptor extracellular domain–Fc fusion protein produced as described (3). Initially, this fusion protein was used to screen a series of kidney cell lines by FACS analysis for their capacity to bind the extracellular domain of Htk. Cell lines specifically binding the fusion protein would be indicative of a membrane-bound or membrane-associated source of the Htk ligand. Screening of some 15 kidney cell lines resulted in the discovery of specific binding to one murine cell line termed SV40MES 13 (21).

Binding competition studies with iodinated and unlabeled human Htk–Fc demonstrated the K_d for fusion protein binding to SV40MES 13 was 2.7 ± 0.2 nM with ≈6500 sites per cell (Fig. 1*A*). Importantly, SV40MES 13 cells were able to activate phosphorylation of the receptor in a coincubation assay (Fig. 2). However, conditioned medium from the SV40MES 13 cell line was unable to activate tyrosine phosphorylation, further supporting the concept of a membrane-bound ligand. Consequently, a cDNA expression library from the SV40MES 13 cell line was constructed in the plasmid vector pRK5B (19). We transfected 50 pools of ≈2000 cDNAs each into COS-7 cells and screened these cells for the capacity to bind radiolabeled Htk–Fc. Five positive pools resulted from this initial screen; two of these pools were subdivided in successive rounds of screening until we reached the level of individual clones.

Binding competition experiments using COS-7 cells transfected with one of the positive clones (clone 7) demonstrated a K_d of 535 ± 12 pM for Htk–Fc binding with ≈1.5 × 10^6 sites per cell (Fig. 1*B*). To confirm that clone 7 encodes a ligand for the Htk receptor, we tested whether COS-7 cells transfected with clone 7 would induce phosphorylation of Htk receptor (Fig. 2*A*). The transiently transfected COS-7 cells were incubated with a NIH 3T3 cell line that stably expressed the Htk receptor (3). Upon coincubation, both clone 7 transfected COS-7 cells and SV40MES 13 cells induced phosphorylation of Htk. The specificity of this response was confirmed by using a monoclonal antibody to Htk (IC2-C2) that also induces phosphorylation (Fig. 2*A*). Interestingly, conditioned medium from COS-7 cells transiently transfected with clone 7 induced phosphorylation of Htk. The activity of the conditioned medium could be enhanced by tumor necrosis factor treatment of the transfected COS-7 cells (data not shown). To obtain purified soluble ligand, we constructed a herpes gD–clone 7 fusion as described in *Experimental Procedures.* The purified material phosphorylated Htk in a dose-dependent manner (Fig. 2*B*).

The sequence of the 4342-bp cDNA clone 7 revealed an open reading frame of 336 amino acids (Fig. 3). This putative protein has an N-terminal signal peptide of 30 amino acids followed by an extracellular domain of 197 amino acids containing two potential N-linked glycosylation sites. The extracellular domain is followed by a transmembrane domain of 26 amino acids and a cytoplasmic domain of 83 amino acids. The predicted molecular mass of the protein after signal peptide cleavage is 34 kDa, with an estimated pI of 8.9. The

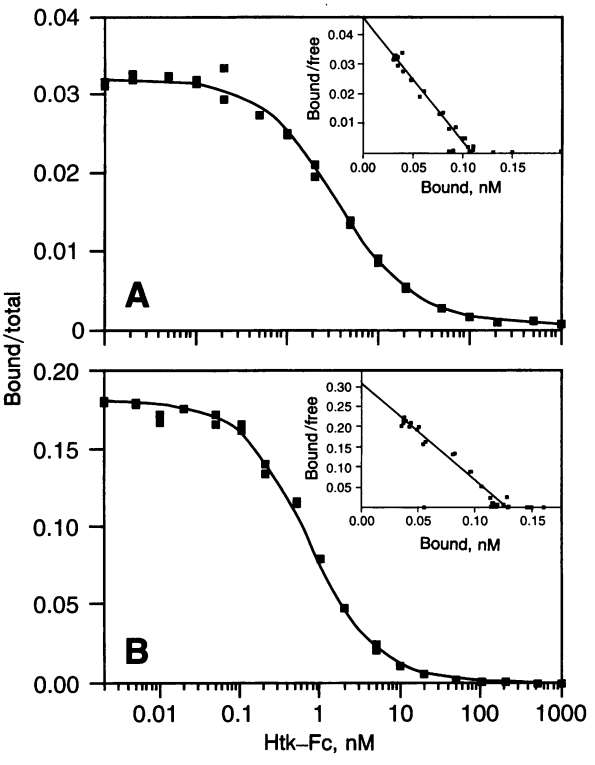

FIG. 1. Binding competition curves of unlabeled Htk–Fc to the SV40MES 13 cell line (*A*) and recombinant Htk ligand expressed in COS-7 cells (*B*). (*Insets*) Scatchard representations of each binding curve revealed K_d values of 3 and 0.5 nM, respectively.

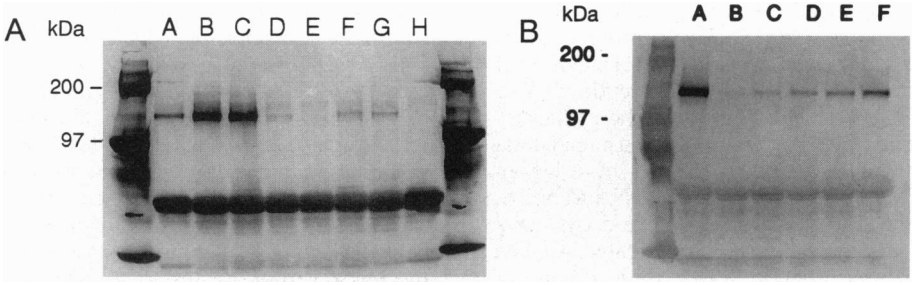

FIG. 2. (*A*) Induction of tyrosine phosphorylation of the Htk receptor by the Htk ligand. Analysis of Htk receptor tyrosine phosphorylation was performed via anti-phosphotyrosine blot of Htk immunoprecipitates in NIH 3T3 cells transfected with the full-length Htk receptor (3T3-T). Htk ligand was transiently transfected into COS-7 cells (COS-7T). Specificity of response was demonstrated by using mock-transfected 3T3 cells. Lanes: A, IC2-C2 antibody (agonist antibody to Htk) and 3T3-T cells; B, SV40MES 13 and 3T3-T cells; C, COS-7 T and 3T3-T cells; D, COS-7 and 3T3-T cells; E, IC2-C2 antibody and 3T3 cells; F, SV40MES 13 and 3T3 cells; G, COS-7 and 3T3 cells; H, COS-7 T and 3T3 cells. (*B*) Induction of tyrosine phosphorylation of the Htk receptor by purified soluble Htk ligand. Analysis of Htk receptor phosphorylation was performed using 3T3-T cells as described above (*A*). Lanes: A, anti-Htk rabbit polyclonal antibody (1:50 dilution); B, no treatment; C, 60 ng of soluble Htk ligand (Htk-L) per ml; D, 210 ng of Htk-L per ml; E, 240 ng of Htk-L per ml; F, 600 ng of Htk-L per ml.

sequence derived from clone 7 was confirmed by sequencing another independent clone of 4700 bp that gave the identical coding sequence. The successful use of human Htk to clone the ligand from a murine source suggested that the mouse and human ligands would be highly homologous at the amino acid level. A human Htk ligand was cloned using the murine ligand cDNA as probe and found to be 96% identical to the murine ligand (Fig. 3). This is consistent with the homology between human Htk and its mouse homologue myk-1 (11), which are 91% identical at the amino acid level.

Very recently, four other ligands for EPH-related family members have been cloned (6–10). Although all the ligands are apparently membrane associated, they appear to fall into two groups. B61 (6–8), ELF-1 (9), and EHK-1 ligand (6) constitute one category of ligands that are presumed to be glycosylphosphatidyl inositol (GPI)-linked membrane proteins. These ligands are closely related to each other and share ≈50% homology. Alternatively, Htk ligand and LERK-2 (ELK ligand) (6, 7) are transmembrane proteins that are 56% homologous and have very highly conserved intracellular domains (75% homology) (Fig. 3). Htk ligand shares four conserved cysteine residues with the B61-like ligands but the overall homology is much lower and is only 23% for B61 (Fig. 3) and 24% for ELF-1.

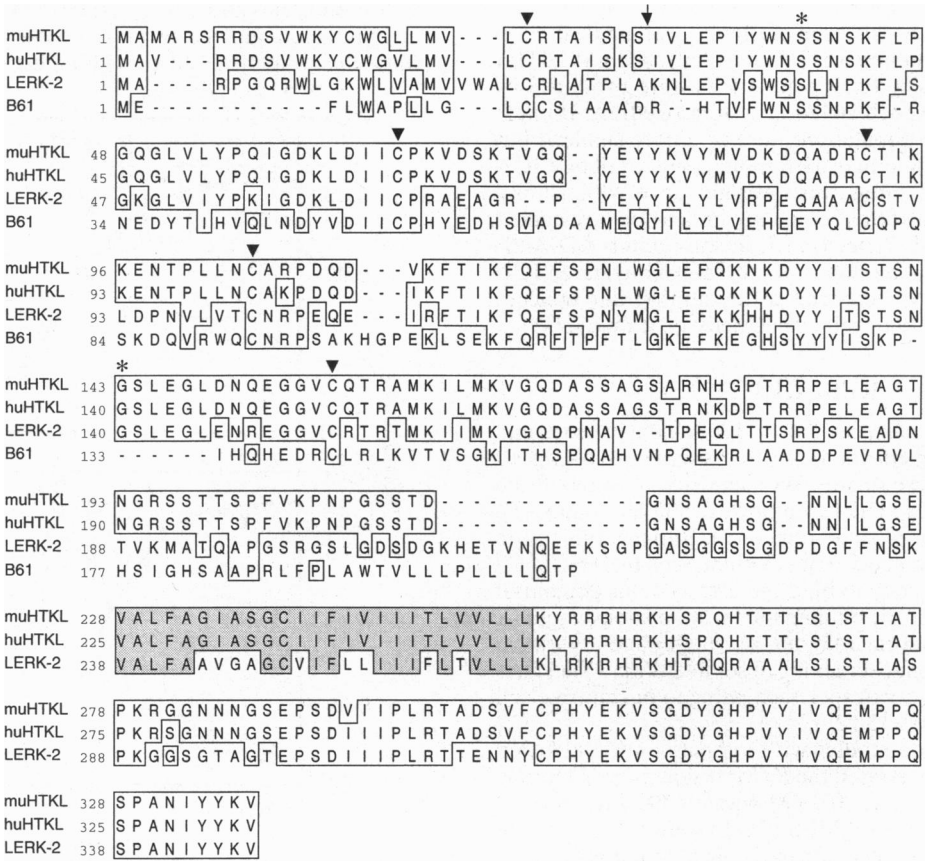

FIG. 3. Amino acid sequence of the murine (muHTKL) and human (huHTKL) Htk ligand and comparison with B61 and LERK-2. Identical residues are boxed. The predicted signal peptide cleavage site (22) for the Htk ligands is designated by an arrow and the amino acids of the putative transmembrane region (23) are shaded. Potential N-linked glycosylation sites are indicated by asterisks. Conserved cysteine residues are indicated by arrowheads.

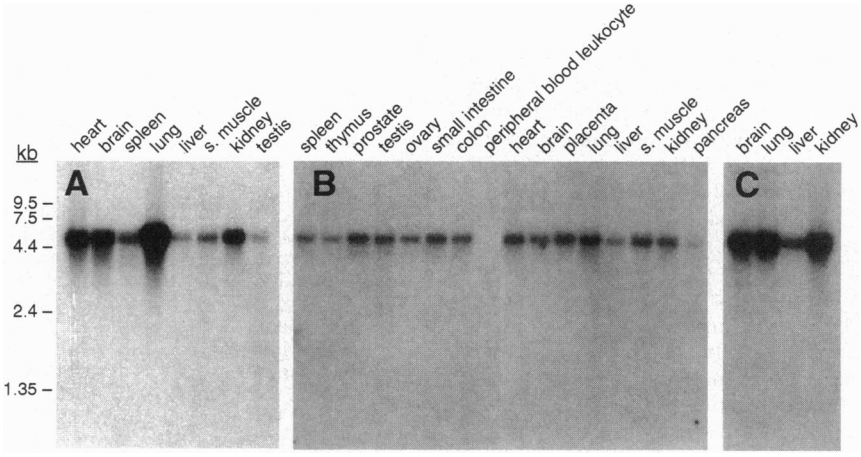

FIG. 4. Northern blot analysis of Htk ligand in mouse adult tissues (*A*), human adult tissues (*B*), and human fetal tissues (*C*). Northern blots were obtained from Clontech and contain 2 μg of poly(A)-selected RNA per lane from the tissues indicated.

Northern blot analysis of mouse and human Htk ligand mRNA in adult and fetal tissues showed widespread tissue expression. There appears to be only one transcript at ≈5.2 kb (Fig. 4). The expression is high in fetal tissues and correlates with the expression profile of the cognate receptor, which is also expressed at its highest levels in the developing embryo (3). We cloned the Htk ligand from a cell line derived from the mesangial cells of the glomerulus. Consequently, we investigated the expression of ligand and receptor in the kidney. *In situ* hybridization demonstrated coexpression of both receptor and ligand in the glomerulus (data not shown). Embryonic expression of both ligand and receptor was also evident in the developing nephrons of the midgestation mouse embryo (data not shown). These data suggest a role for the pair in kidney development and/or function.

Since Htk is expressed in hematopoietic progenitor populations, we further investigated its expression and potential function in this system by using monoclonal antibodies raised against the extracellular domain of the receptor. FACS analysis revealed expression of Htk on a subset of CD34 cells ($CD34^{lo}$) from human cord blood (Fig. 5*A*). Cytospin analysis of these cells suggested that these cells were of the monocytic lineage (data not shown). Further analysis of this population and human peripheral blood illustrated dual staining of cells for Htk and the monocyte marker CD14 (Fig. 5*B*). The presence of Htk on cells from the monocytic lineage suggested that the Htk ligand may also be present in the hematopoietic system. Analysis of fetal liver stromal cell lines using Htk–Fc demonstrated binding to the 7-4 stromal cell line, indicating the presence of Htk ligand on this line (Fig. 5*C*). Northern blot analysis of 7-4 also demonstrated the expression of Htk ligand (data not shown). Interestingly, the 7-4 stromal cell line is capable of producing multilineage expansion of hematopoietic stem cells (27). Collectively, these data suggest a hematopoietic role for the receptor–ligand pair in monocyte maturation and/or proliferation.

DISCUSSION

Signal transduction through receptor tyrosine kinases has been widely demonstrated to be an essential function for the development of multicellular organisms. In these studies, we have cloned the ligand for Htk, a receptor protein-tyrosine kinase from the EPH subfamily of receptor kinases. This subfamily is the largest subgroup of structurally related receptor kinases currently identified. The biological significance of this family is unclear since the functions of these molecules have yet to be defined. However, initial observations suggest that their primary role may not be proliferative (4, 7, 24).

The Htk ligand is closely related to LERK-2 and to a lesser extent the B61-like ligands. As with ligands for the nerve growth factor receptor family, these ligands appear to be capable of binding to more than one member of the EPH family. For example, B61 appears to bind to at least EHK-1, ECK, and ELK receptors (6–8). Furthermore, given the sequence homology between Htk ligand and LERK-2, it is probable that Htk ligand may also bind to other receptors in this family, such as ELK. This speculation is supported by *in situ* studies, which indicate both coexpression and disparate expression patterns of ligand and receptor (W.M., unpublished data).

The striking conservation of the intracellular residues between Htk ligand and LERK-2 is intriguing and clearly suggests a functional role for the C terminus of these molecules.

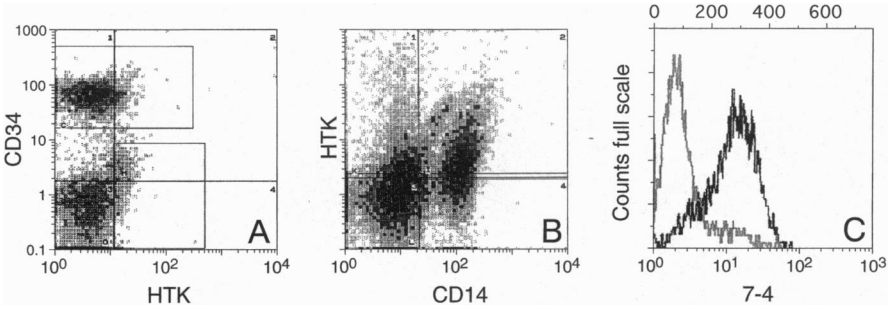

FIG. 5. (*A*) Human cord blood cells were enriched for $CD34^+$ cells and subsequently stained for Htk and CD34 expression. (*B*) Human cord blood cells were enriched for Htk cells and subsequently stained for Htk and CD14 expression. (*C*) Fetal liver stromal cell line 7-4 was incubated with Htk–Fc fusion protein and binding was detected as described. Flk2–Fc fusion protein was used as a control to demonstrate the specificity of binding. Gray line denotes Flk2–Fc; black line denotes Htk–Fc.

It has been suggested that the C-terminal valine residue may be critical for the potential production of a soluble form of LERK-2 (8). Interestingly, tumor necrosis factor treatment of COS-7 cells transfected with full-length Htk ligand results in the production of soluble Htk ligand that phosphorylates the Htk receptor. However, the phosphorylation obtained with soluble material is not as potent as with membrane-bound ligand. This is in keeping with the proposal that membrane attachment of this family of ligands facilitates ligand clustering to promote receptor activation (6).

The widespread expression pattern of both Htk ligand and receptor, and the potential complexity for ligand–receptor interactions within the EPH family, makes defining the functional roles of this pair a complex problem. This will probably require functional analysis of these molecules in each of the various tissues in which they are expressed. Moreover, it is conceivable that specialized functions may have evolved for each cognate pair from the subfamily. For example, the expression of Htk ligand on hematopoietic stromal cells and the presence of receptor on cells of the monocytic lineage suggest a role in monocyte development and/or proliferation. This is extremely intriguing given the recent finding that mice deficient in both granulocyte–macrophage colony-stimulating factor and monocyte colony-stimulating factor still have circulating monocytes and tissue macrophages (25). In addition, the transmembrane nature of both Htk ligand and receptor, together with high levels of expression in the developing embryo, suggests potential roles in organs or tissues requiring cell–cell contact for development. Such a system would be the developing mammary gland where myk-1 is expressed at high levels (11) and where development is dependent on cell–cell contact (26).

It is clear that unraveling the potential complexities of interactions between ligands and receptors for the EPH-related receptor tyrosine kinases is going to be a major task. However, cloning of the Htk ligand allows for a systematic investigation into the functional roles of the Htk ligand and receptor pair. Perhaps as with the nerve growth factor receptor family, gene "knockout" experiments in mice will be the most appropriate method of discerning their functions.

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