Cloning and Characterization of HTK, a Novel Transmembrane Tyrosine Kinase of the EPH Subfamily*

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Using a polymerase chain reaction based strategy, we identified a novel transmembrane tyrosine kinase in CD34+ human bone marrow cells and a human hepatocellular carcinoma cell line, Hep3B. This protein, hepatoma transmembrane kinase or Htk, shares amino acid similarity with the EPH subfamily of tyrosine kinases. The HTK gene is located on human chromosome 7. The predicted 987-amino acid sequence of Htk includes a transmembrane region and signal sequence. In the predicted extracellular domain, a cysteine-rich region and tandem fibronectin type III repeats are present while a single uninterrupted catalytic domain is present in the intracellular domain. These features are consistent with other members of the Eph subfamily. Antibodies raised against Htk extracellular domain immunoprecipitated a 120-kDa protein from either in vitro translated HTK or Hep3B cells which localized primarily to the Hep3B membrane subcellular fraction. Purified in vitro translated Htk was enzymatically active and autophosphorylated on tyrosine in kinase assays. Furthermore, antibodies against Htk ECD were agonistic, inducing Htk tyrosine phosphorylation in transfected NIH3T3 cells. Northern blot analysis demonstrated a single HTK transcript abundantly present in placenta and in a range of primary tissues and malignant cell lines. HTK appears to be expressed in fetal but not adult brain and in primitive and myeloid but not lymphoid hematopoietic cells. The novel transmembrane protein, Htk, may function as a receptor with an expression pattern suggesting a role in events mediating differentiation and development.

Tyrosine kinases mediate critical events in regulating the proliferation and differentiation of eukaryotic cells. The subset of transmembrane tyrosine kinases often serve as receptors for growth factors and have been implicated in developmental processes as well as in malignant transformation (1-4). Among the transmembrane tyrosine kinases there are several subfamilies which may be distinguished according to specific features of their extracellular and/or intracellular domains (3, 4).

The presence of a cysteine-rich extracellular region is characteristic of subfamilies typified by the EGF receptor, the insulineceptor or, the orphan receptor, Eph, respectively. The EGF receptor subfamily typically has two or three such cysteine-rich domains while the insulin receptor subfamily has a single short cysteine-rich region on one of the subunits generated from a proteolytically cleaved precursor protein. EPH and related genes typically encode proteins with a single cysteine-rich domain and two fibronectin type III repeats in the extracellular region on a single, non-cleaved protein (5-14). In addition this subfamily usually encodes a single kinase domain which, unlike the platelet-derived growth factor receptor or kit subfamily, is not interrupted by a kinase insert domain. The gene reported here appears to encode a protein with features typical of the Eph subfamily. Patterns of expression of certain members of this subfamily suggest functional importance in mediating developmental events, particularly in the nervous system (7, 10, 11, 15-18). Additionally, overexpression of the EPH gene can induce tumorigenicity (19).

Given the importance of the transmembrane tyrosine kinases in the regulation of a spectrum of cellular events, we undertook a strategy to identify novel members of the tyrosine kinase family focusing on sequences encoding transmembrane proteins. Reported here is a novel member of the EPH subfamily which has tissue restricted expression and functional characteristics of a tyrosine kinase.

**MATERIALS AND METHODS**

**DNA Amplification and Cloning—** Light density human bone marrow mononuclear cells, obtained from normal volunteers using Deaconess Hospital Institutional Review Board approved protocols and with voluntary written informed consent, were separated by anti-CD34 antibody (AMAC, Westbrook, ME) and immunomagnetic beads (Dynal, Oslo, Norway). Flow cytometric analysis using fluorescein isothiocyanate conjugated anti-CD34 antibody (AMAC) confirmed >95% CD34 positivity of isolated cells. The hepatoma cell line, Hep3B (provided by Dr. M. Goldberg, Brigham and Women's Hospital, Boston, MA), was cultured in α-medium (Life Technologies Inc.) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (Life Technologies Inc.) at 37 °C in a 5% CO2 incubator. Total RNA extracted from CD34+ bone marrow mononuclear or Hep3B cells was reverse transcribed with random primers and the Moloney murine leukemia virus reverse transcriptase following the conditions of the manufacturer (Life Technologies Inc.) in a 20-μl reaction. PCR was performed on the reverse transcriptase product reaction in a 100-μl reaction containing 50 mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM MgCl2, 20 μg/ml gelatin, 0.2 mM dNTPs, 2.5 units of Taq polymerase (Perkin-Elmer), and 50 pmol each of tyrosine kinase-specific degenerate primers (PTK1, 5′-TGGGATCCAC/CGNGAC/CT/TNGGC-3′; PTK2, 5′-TGGGATCCAC/CGNGAC/CT/TNGGC-3′; PTK3, 5′-CTGGATTCC-CA/GAT/A/CC/GT/ACCCAG/CACA/GTC-3′; PTK2B, 5′-CTGGATTCC-CA/GAT/TCC/GT/ACCAT/AACA/GTC-3′) derived from consensus regions among known tyrosine kinases as previously reported by others (20-22). The PCR cyle was 1.5 min at 95 °C, 2 min at 57 °C, and 3 min at 63 °C repeated 36 times. The reaction product was electrophoresed—electrophoresis; ECD, extracellular domain.
soma
cell was exposed to a 2% low-melting agarose gel, purified on an Elutip-D column (Schleicher & Schuell) digested with EcoRI and BamHI, and subcloned into µC 

Recombinants were sequenced by the Sanger dideoxy method and evaluated by the FASTA nucleic acid sequence analysis program. A clone which appeared novel by databank homology search under the BLASTp parameters was selected. The 214 bp Hind III fragment, which encoded the ligand-binding region of HTK, was subcloned into pUC19. Recombinants were sequenced by random priming and used to screen an oligo(dT)-primed AgtLO AG-3'). PCR was performed with 250 ng of DNA and 50 pmol each of the 5' and 3' primers, 50 mM KCl, 1.5 mM MgCl2, 20 pg/ml gelatin, 0.2 mM dNTPs, and 2.5 units of Taq DNA polymerase in a final volume of 100 µl. Cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s were repeated 30 times. A portion of each sample (15 µl) was electrophoresed through a 1.2% agarose, 2.2 M formaldehyde gel and transferred to a nylon membrane (Schleicher & Schuell) digested with EcoRI and BamHI, and subcloned into the EcoRI site of pBluescript (Stratagene). The largest insert, a 3969-bp cDNA, was sonicated to an average size of 800–2000 bp and cloned into the Smal site of M13. Overlapping clones were sequenced using the Taq Dye Primer Cycle Method (CABI) on the Catalyst 800 Molecular Biology Lab Station (ABI). Sequencing reactions were then analyzed on the ABI 373A Automated DNA Sequencer.

Chromosome Mapping—Somatic cell hybrid DNAs from a panel of 25 human-hamster cell lines (Bios, New Haven, CT) were used for chromosomal localization by PCR. Two sets of primers from the 5'-untranslated region of HTK were chosen. The 5' primers were Hptk{5-8} (5'-GGATGGCCTCTAGCGGCTG-3') and Hptk5-9 (5'-GAATCCCTGCA-CGAAAGTTGA-3'). The 3' primers were Hptk5-10 (5'-AATGATAGAGC-GCGACACGTG-3') and Hptk5-12 (5'-AGATGACGAGCAAGCAGCG- AGCGAGCGATTGCAAGAGGATGAATCG-3'). These primers were chosen on the basis of homology to the human chromosomal material present in each of the somatic cell hybrid DNAs.

Northern Blotting—Poly(A)-selected RNA was electrophoresed through a 1.2% agarose, 2.2 M formaldehyde gel and transferred to a nitrocellulose membrane. The gel was prepared or commercially obtained, hybridized in 50% formamide at 42°C to a 32P-labeled HTK cDNA probe and washed with stringent conditions (final wash: 0.1 x SSC, 0.2% SDS at 65°C for 30 min) and 0.1 x SSC, 0.1% SDS for 30 min. A probe for the 3'-end restriction site (5'-CCGGATATCATGGACTACAAG-GGCAAGTATGCAAGAGGATGACG-3') was ligated into the NcoI site (base 88) of HTK in the EcoRV-digested Bluescript (Stratagene, La Jolla, CA) vector.

Antibodies—An HTK extracellular domain-human IgG, Fc fusion gene was constructed and fusion protein produced as previously described (23). Rabbit polyclonal antiserum was raised using the fusion protein. The HTK-specific antiserum was affinity purified and its specificity was assessed by flow cytometric analysis of NIH3T3 cells transfected with full-length HTK or vector alone using a 1:200 dilution of preimmune serum or anti-HTK-IgG-Fc fusion. Significant peak shifts were observed in several HTK expressing clones as compared to either preimmune serum or vector control. In vitro Transcription and Translation—Transcription was performed on 2 pmol of linearized HTK or FLAG-HTK containing plasmid at 37°C for 1 h in a 50-µl volume containing 10 mU dihydrothorietol, 2.5 µg of bovine serum albumin, 0.25 mM each dNTP, 0.5 mM [35S]methionine (14212 cpm) labeling. Sample buffer containing SDS and β-mercaptoethanol was added before boiling and 10% SDS-PAGE.

HTK Expression in NIH3T3 Cells—A 4038-bp ClaI-XbaI cDNA fragment containing 32 bp of linker sequence, 37 bp of pBluescript (Stratagene) polylinker, and the entire 3966-bp HTK cDNA was subcloned into the expression vector pcRII (Invitrogen, Inc.) under the control of the Rous sarcoma virus long terminal repeat promoter. NIH3T3 cells maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum were co-transfected with pHIS-HTK and pNeo (an SV40 based vector containing the neomycin resistance marker) by the calcium phosphate method as described (24). Neomycin-resistant cells were selected 48 h after transfection with G418 (Life Technologies Inc.) at 400 µg/ml. Fourteen days later individual resistant colonies were isolated, expanded, and analyzed by flow cytometry for HTK expression using rabbit polyclonal antiserum.

Immunoprecipitation—Cells (Hep3B, control NIH3T3, or HTK transfected NIH3T3) or in vitro translated protein (Hk or FLAG-Hk) were used for immunoprecipitation with either serum (preimmune or anti-HTK) or monoclonal antibody, was added and rocked overnight at 4°C before 100 µl of protein-A-Sepharose CL-4B was added and the solution rocked at 4°C for an additional 2 h. Immunoprecipitates were washed, suspended in SDS-PAGE loading buffer (10% glycerol, 5% 2-mercaptoethanol, 2.5% SDS, and 62.5 mM Tris-HCl, pH 6.8), heated to 95°C for 5 min, and analyzed by 7.5% SDS-PAGE.

Cell Fractionation—Hep3B cells (1 x 10⁶) were labeled with 200 µCi [35S]methionine in an minimal essential medium with 10% dialyzed fetal calf serum overnight. The cells were washed twice with cold phosphate-buffered saline, scraped into 1 ml of cold buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM EGTA, 0.25 mM sucrose, 0.01% leupeptin, 4 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin), and disrupted by sonication for 30 s. Whole homogenates were centrifuged at 12,000 x g for 15 min, the nuclear pellets isolated, and the decanted supernatant centrifuged at 100,000 x g for 40 min at 4°C to pellet membranes. The resultant supernatant served as the cytosolic fraction.

Nuclear and membrane fractions were washed and dissolved in buffer containing 0.5% Nonidet P-40 prior to immunoprecipitation.

Protein Kinase Assay—Immunoprecipitates were washed once with kinase buffer (25 mM Hepes, pH 7.4, 1 mM dihydrothioietol, 10 mM MgCl2, 10 mM MnCl2), and resuspended in 40 µl of kinase buffer containing either unlabelled ATP or 10 µCi of [32P]ATP (3000 Ci/mmol). After a 1-min preincubation at 37°C, the assays were initiated by adding 40 µl of 2 x sample buffer and boiling the samples for 5 min prior to electrophoresis on 8.0% SDS-PAGE gel. The dried gel was covered with 4 sheets of aluminum foil to block [32P]labeled protein autoradiography and the gel was placed under film for 5 h to overnight.

Western Blotting and Phosphorytoayse Assay—Proteins were electrophoretically transferred to a 0.2-µm nitrocellulose (Bio-Rad) or a 0.45-µm polyvinylidene difluoride (Millipore) membrane in a buffer containing 25 mM Tris-HCl (pH 7.5), 192 mM glycine, and 20% methanol at 100 mA for 2 h. Filters were washed in TBS (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) blocked in TBS-T (100 mM Tris-HCl, pH 7.6; 5% Tween 20) containing 5% dry milk (Carnation) for 2–5 h. Filters were washed four times for 5 min each in TBS and incubated overnight with anti-phosphotyrosine antibody (1:5000 dilution in TBS). Filters were washed four times for 5 min each in TBS and incubated for 2 h with the horseradish peroxidase-labeled anti-mouse secondary antibody (Amersham) at a 1:5000 dilution in PBS. After washing four times, the enhanced chemiluminescence system (ECL, Amersham) was used for detection for 10 s to 2 min.

Antibody-induced Phosphorylation Assay—Cells were plated at a density of 5 x 10⁵ cells/well in a 6-well plate and, after 24 h, were serum starved for 1 h prior to adding preimmune or immune serum at a 1:150 dilution for 30 min. Cells were then washed in phosphate-buffered saline and lysed in either 2 x sample buffer or Nonidet P-40 lysis buffer as described above. Either crude lysates or immunoprecipitated cell lysates were then separated via 4–12% gradient SDS-PAGE and analyzed by anti-phosphotyrosine immunoblot as described above.

RESULTS

The method of Wilks (21), which utilizes consensus sequences within related tyrosine kinases (20) to clone fragments of novel tyrosine kinase-related genes, was adapted using the primers described under "Materials and Methods." Following reverse transcriptase PCR amplification of mRNA from the human hepatoma cell line, Hep3B, approximately 140 recombinants were characterized by nucleic acid sequencing. Comparative databank analysis revealed four novel sequences of which one, termed HpTK5, was identical in sequence to a frag-
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The nucleic acid sequence of full-length HTK cDNA with deduced amino acid sequence indicated above the cDNA sequence. The amino acid predicted to be the cleavage site for the signal peptide is indicated by an arrow. Cysteine residues conserved among Elk subfamily members are circled and the transmembrane region is overlined. GenBank accession number for HTK is UO7695.
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FIG. 2. Amino acid sequence alignment of Htk, Hek2, and Elk. Identical residues are enclosed within line boundaries. The signal peptide cleavage site is indicated by an arrow. The transmembrane region is shaded. The fibronectin type III repeats are overlined and the catalytic domain is bracketed. The Cys residues in the ECD are indicated by inverted triangles.

ment similarly isolated from CD34+ human bone marrow mononuclear cells. The 214-bp insert of HpTK5 was radiolabeled and used to screen 1.2 x 10^6 clones of a λgt10 Hep3B cDNA library. Approximately 100 positive clones were detected, 17 of which were further analyzed. A single full-length 3969-bp cDNA was isolated and sequenced (Fig. 1).

The full-length clone, named hepatoma transmembrane kinase or HTK, included an open reading frame extending from nucleotide 90 to 3050 predicted to encode a 987-amino acid protein of 108,270 daltons. The putative initiation codon is
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**Fig. 3.** HTK is on human chromosome 7. The ethidium bromide-stained gel of DNA from human-hamster somatic cell hybrids amplified by PCR using HTK specific primers is shown in A. The human chromosomal content of the cell lines corresponding to the gel lane numbers is shown in B. Lane numbers in A are enlarged for those where an amplified product was apparent and the correlation of these positive lanes with a human chromosome is shaded in B. Cell line scoring has been simplified with "+" indicating some chromosomal material present, but not all + lines contain the entire indicated chromosome. Lane 26 is a human only (full genome) control and lane 27 is a hamster only control.

The predicted protein sequence includes a transmembrane region (amino acids 538–563) which divides Htk into extracellular (ECD) and intracellular domains. The ECD of 538 amino acids includes a signal peptide of 15 amino acids (38) and a cysteine-rich box containing 20 Cys residues shared among other members of the EPH gene subfamily (Fig. 2) (5–9, 12). In addition, there are two fibronectin type III repeats spanning amino acids 321–425 and 435–526. Similar repeats are found in all other EPH-related proteins as well as the tyrosine kinase, AXL, which is not in the EPH subfamily (26). Asn at positions 208, 340, and 431 are possible sites for N-glycosylation.

The putative intracellular domain contains a kinase consensus region from position 613 to 881 (20). This kinase region includes a putative ATP-binding consensus (Gly-X-Gly-X-Gly) in subdomain I at positions 622–627 (Gly-Ala-Gly-Glu-Phe-Gly) (20, 27–29). A Lys at position 647 (subdomain II) corresponds to an invariant Lys among tyrosine kinases thought to be critical for the phosphotransfer reaction. Signature regions indicative of substrate specificity suggest that Htk is a tyrosine rather than a serine/threonine kinase. These include the Asp-Leu-Ala-Ala-Arg-Asn sequence at positions 740–745 in subdomain VI and the Pro-Ile-Arg-Trp-Thr-Ala-Pro-Glu...
Fetal Tissues

Fig. 4. Northern blot analysis of HTK expression in human fetal (A) or adult tissue (B). Northern blots were obtained from Clontech (Palo Alto, CA) and contain 2 μg/lane of poly(A)-selected RNA from the tissues indicated. The data shown are from overnight autoradiography after hybridization with a 32P-labeled HTK cDNA probe.

at positions 783–790 in subdomain VIII (20, 27–29). Tyr at positions 601, 619, or 741 are possible substrates for tyrosine kinase activity (30–32). The 'I+Ile-Asp-Pro-Phe-Thr-Tyr-Glu-Asp-Pro sequence (amino acids 595–604) immediately downstream of the membrane spanning domain, has been suggested by others (13) to possibly play a role in Eph subfamily members within the intracellular domains, respectively, and 45 and 42% identity within the ECD, respectively.

Primers derived from the unique 3′-untranslated sequence of HTK were used to identify its human chromosomal localization. The 3′-untranslated region characteristically contains few, if any, intervening sequences (35, 36) and has a high degree of diversity among members of gene families (37) making it preferred in this type of analysis. Two independent sets of primers, which were specific for human HTK, were used to amplify DNA from a panel of human-hamster hybrid cell lines. Both sets of primers gave results that were consistent with human chromosome 7 only (single set shown in Fig. 3). Human chromosome 7 also includes the genes for the EGF receptor, hepatocyte growth factor receptor, hepatocyte growth factor, platelet-derived growth factor, and interleukin-6. Karyotypic abnormalities involving this chromosome are common among human leukemias, particularly in aggressive myeloid leukemias that occur following radiation, alkylating agent chemotherapy, or a pre-existing myelodysplastic condition (41).

Northern blot analysis of human fetal tissues revealed a single transcript of ~4 kilobases in heart, lung, liver, and kidney, with a lesser signal detectable in brain (Fig. 4A). In adult human tissue, no signal was detectable in brain, while placenta had a particularly intense signal followed by kidney, liver, lung, and pancreas. Skeletal muscle and heart were of lower signal intensity (Fig. 4B). Cell lines derived from liver, breast, colon, lung, melanocyte, or cervix had detectable signal of appropriate size. Message was present in select cell lines of hematopoietic origin. K562 (a primitive myeloid cell with multipotential), THP-1 (a monocyticoid cell), CMK and SO (both of megakaryocytic origin) were all positive for HTK message, but lymphoid (H9, Jurkat, JH-1, Raji, Ramos) or other select myeloid cells (KG-1 or KMT2) had no detectable transcript by Northern analysis (Fig. 5).

An Htk ECD-IgGFc fusion protein was expressed, purified, and used to generate rabbit antiserum which immunoprecipitated a 120-kDa protein from Hep3B cells (Fig. 6C). The specificity of the antiserum was confirmed by immunoprecipitation of in vitro translated HTK RNA and HTK transfected NIH3T3 cells (Figs. 6A and 9). To determine the functional capacity of Htk, in vitro translated Htk was immunoprecipitated, exposed to kinase conditions (Fig. 6A), and immunoblotted using a phosphotyrosine-specific monoclonal antibody (Fig. 6B). These data indicate that Htk is phosphorylated on tyrosine. However, the presence of other bands consistently appearing in the 32P-labeled immunoprecipitation (Fig. 6A, lane 3, is representative) suggested that the Htk protein was only partially purified and
SDS-PAGE either with or without immunoprecipitation. The electrophoresed gel was immuno-blotted with anti-phosphotyrosine antibody. Enhanced tyrosine phosphorylation of Htk was observed following exposure to polyclonal antiserum (Fig. 9) suggesting an agonist-like effect of antibody binding.

**DISCUSSION**

Transmembrane tyrosine kinases constitute an important class of proteins transducing signals that modulate growth and differentiation. Various subfamilies of the transmembrane tyrosine kinases have been defined based on structural features. The predicted amino acid sequence of HTK most closely resembles that of the subfamily originally defined by EPH. The EPH subfamily, Eph, Elk, Eek, Hek, Hek2, Sek, Ehk-1, Ehk-2, Cek-4 to -10, Tyro 1, 4, 5, and 6, appears to be the largest subfamily of transmembrane tyrosine kinases (5–15, 18, 40). The pattern of expression of the EPH subfamily is suggestive of a role in differentiation and development (7, 10, 11, 15–18). In particular, the emergence of neural elements corresponds with the expression of certain EPH-related genes.

No ligands for the members of the EPH-encoding transmembrane proteins have yet been identified. A chimeric protein, utilizing the intracellular portion of Elk and EGF receptor ECD, served to induce growth in cells exposed to EGF, suggesting that proliferative signals may be transduced via activation of the tyrosine kinase function (15). The tyrosine kinase activity of Htk, a novel member of the EPH subfamily, supports the hypothesis that Htk is a signal transducing molecule. Interaction of Htk with an antibody directed against its ECD induces phosphorylation. This provides further support that Htk may serve as a receptor for a ligand that triggers kinase activation. Details of the signaling pathway of Htk may be further explored using antisera as a surrogate ligand.

Differential expression of the Htk transcript in fetal versus adult brain suggests that Htk may share with other EPH subfamily members, a role in events related to neural development. However, unlike some members of the EPH subfamily which are exclusively expressed in neurons (40), Htk is widely expressed in other tissues. In particular, Htk is expressed in hematopoietic cells including CD34+ hematopoietic progenitor cells. The presence of the Htk message in early hematopoietic cells and cell lines of myeloid lineage, but not in cell lines derived from lymphoid cells, suggests that Htk may have lineage restricted expression. The localization of Htk on human chromosome 7 is also of interest regarding the hematopoietic system, as karyotypic abnormalities of chromosome 7 are frequently seen in acute leukemias (41). The expression of Htk in primary hematopoietic cells and further chromosomal mapping of Htk in normal and malignant cells are under investigation. Structural analysis of Htk and identification of its ligand will be required to further define its biological role.

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