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MOLECULAR CLONING AND CHARACTERIZATION OF MOUSE *TIE* AND *TEK* RECEPTOR TYROSINE KINASE GENES AND THEIR EXPRESSION IN HEMATOPOIETIC STEM CELLS [+]

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Summary: To identify receptor tyrosine kinases (RTKs) critical to early hematopoiesis, we performed polymerase chain reaction-based cloning from yolk sac and highly enriched bone marrow hematopoietic stem cells (HSCs). Characterization of two novel genes of their full-length cDNA sequences revealed that they were mouse homologues of the endothelial cell RTK genes, *TIE* and *TEK*. They shared a unique structural property of coexistent immunoglobulin-like domain, epidermal growth factor-like repeats, and fibronectin type III repeats in their extracellular domains. Both genes were expressed in a similar fashion in adult tissues and primitive hematopoietic cells, predominantly in the bone marrow HSCs.
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[+]The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank, and DDBJ Nucleotide Sequence Databases under the accession number X73960.

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Abbreviations: HSC, hematopoietic stem cell; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; FNIII, fibronectin type III; Ig, immunoglobulin; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide.

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Receptor tyrosine kinase (RTK)/ligand systems play an important role in the constitutive hematopoiesis, as seen in *c-fms* /M-CSF and *c-kit* /steel factor (1,2). It is possible that unknown RTKs work for the self-renewal and differentiation of hematopoietic stem cells (HSCs).

To identify RTKs critical to early hematopoiesis, we performed polymerase chain reaction (PCR)-based cloning of RTK-related sequences. Characterization of two novel genes of their full-length cDNA sequences revealed that they were mouse homologues of the endothelial cell RTK genes, *TIE* and *TEK* (3-5). This is the first report of the primary structure of mouse *TIE* gene which is expressed in HSCs.

MATERIALS AND METHODS

Preparation of yolk sac and fractionated bone marrow cells: Yolk sac was obtained from day 9.5 BALB/c fetus. Bone marrow cells were stained with allophycocyanin (APC)-conjugated anti-*c-kit* -encoded molecule monoclonal antibody (MoAb;ACK-2), fluorescein isothiocyanate (FITC)-conjugated anti-Ly6A/E MoAb (Sca-1) and biotinylated lineage markers MoAb (Lin; B220, Mac-1, Gr-1, CD4, CD8 and TER119), and were analyzed and sorted on a FACStar^{plus} as previously described (6).

RT-PCR amplification of PTK-related sequences: Total RNA was obtained from yolk sac by acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC) method (7) and from $1x10^4$ Lin$^-$Kit$^+$Sca-1$^+$ mouse HSCs by small-scaled AGPC method (8). The first strand cDNA was synthesized from 10 μg of yolk sac total RNA and from all of the total RNA from HSCs using avian myeloblastosis virus reverse transcriptase (Life Sciences Inc., St. Petersburg, FL) and oligo(dT) primer. The amplification was performed using the oligonucleotides PTK1 and PTK2 as described by Wilks (9). The amplified cDNA with the size around 210 base pairs was subcloned into pBluescript (Stratagene, La Jolla, CA) and inserts were sequenced by the dideoxynucleotide chain termination method, using AutoRead sequence kit and Automated Laser Fluorescent A.L.F. DNA Sequencer (Pharmacia, Uppsala, Sweden).

Screening of cDNA libraries: A mouse fetal liver cDNA library (day 13, in λgt22, BRL) was screened by the [^{32}P]-labeled PCR-amplified YS228 insert. cDNA library of DA-1 cells (IL-3-dependent undifferentiated hematopoietic cell line) in λZAP (Stratagene) was screened by the [^{32}P]-labeled PCR-amplified STK1 insert.

Northern blot analysis: Total RNA was isolated from tissues and cell lines by AGPC method, and poly(A)$^+$RNA was selected using Oligotex-dT30 (Takara Shuzo, Kyoto, Japan). Two microgram of poly(A)$^+$RNA was loaded on a formaldehyde gel, and Northern blotting was done using a Zeta-Probe blotting membrane (Bio-Rad, Richmond, CA) under the manufacturer's instruction. [α-^{32}P]-labeled extracellular portion of the cDNA was hybridized to the blot.

RT-PCR analysis in fractionated bone marrow cells: Fractionated bone marrow cells (Lin$^+$, Lin$^-$, Lin$^-$kit$^-$, Lin$^-$kit$^+$, Lin$^-$kit$^+$Sca-1$^-$, Lin$^-$

kit+Sca-1+) were prepared and total RNA was obtained as described above. Amplification was carried out on random hexamer-primed cDNA samples representing approximately equivalent cell numbers (4,000). Cycling parameters were 1 minute at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C for 40 cycles. Mouse β-actin was used as a reference gene. Primer sequences were:*TIE* sense (nucleotide 1872-1891), 5'-ACCCACTACCAGCTGGATGT-3';*TIE* antisense (nucleotide 2153-2172), 5'-ATCGTGTGCTAGCATTGAGG-3'; *TEK* sense, 5'-CCACTACCTACTAGTGAAG-3';*TEK* antisense, 5'-ATGCCCTTCTCCACCCTCT-3'; and β-actin sense, 5'-TCGTGCGTGACATCAAAGAG-3'; β-actin antisense, 5'-TGGACAGTGAGGCCA GGATG-3'. Ten percent of this reaction mixture was electrophoresed on a 2% agarose gel and Southern blotting was done using a Hybond N+ filter (Amersham Japan, Tokyo). The filter was hybridized with a [α-^{32}P]-labeled DNA fragments of *TIE* and *TEK*.

RESULTS AND DISCUSSION

PCR-based cloning of TIE and TEK cDNAs: Random sequence analyses of PCR-amplified cDNA clones identified two novel RTK genes. One, YS228 cloned from yolk sac, was the putative mouse homologue of the human RTK gene, *TIE* (3), and the other, STK1 from HSCs, was highly homologous to YS228.

Mouse TIE cDNA sequence: Full-length cDNA of mouse *TIE* was isolated from mouse fetal liver cDNA library using the YS228 insert as a probe and its sequence was determined (Fig.1). This gene contained a single open reading frame of 1134 amino acids. The ATG codon at nucleotide 45-47 was in good agreement with the consensus sequence for a translation initiation site (10), and was followed by a hydrophobic sequence characteristic of a signal sequence. The predicted protein sequence showed a similar structure as the human *TIE* protein; namely the extracellular domain contained three EGF-like repeats embedded between two pairs of cysteines residues, which were followed by three FNIII repeats. A computer analysis of second structure revealed only the first pair of cysteines formed an Ig-like domain. The cytoplasmic domain had a typical kinase domain with a short insertion of 14 amino acid long. Five potential N-linked glycosylation sites (NxS/T) and two putative binding sites of p85 N-terminal SH2 domain (11) were identified. The homology with the human *TIE* protein is 92.4% in total, 96.2% in EGF-like domains, 87.4% in FNIII domains , and 99.6% in kinase domain.

Isolation of a TIE-related gene, TEK: Full-length cDNA of STK1 was isolated from DA-1 cDNA library using the STK1 insert as a probe and the sequence was determined. A novel gene, STK1, turned out to be identical to recently reported RTK gene *TEK* or *HYK* (4,12) Human homologue has

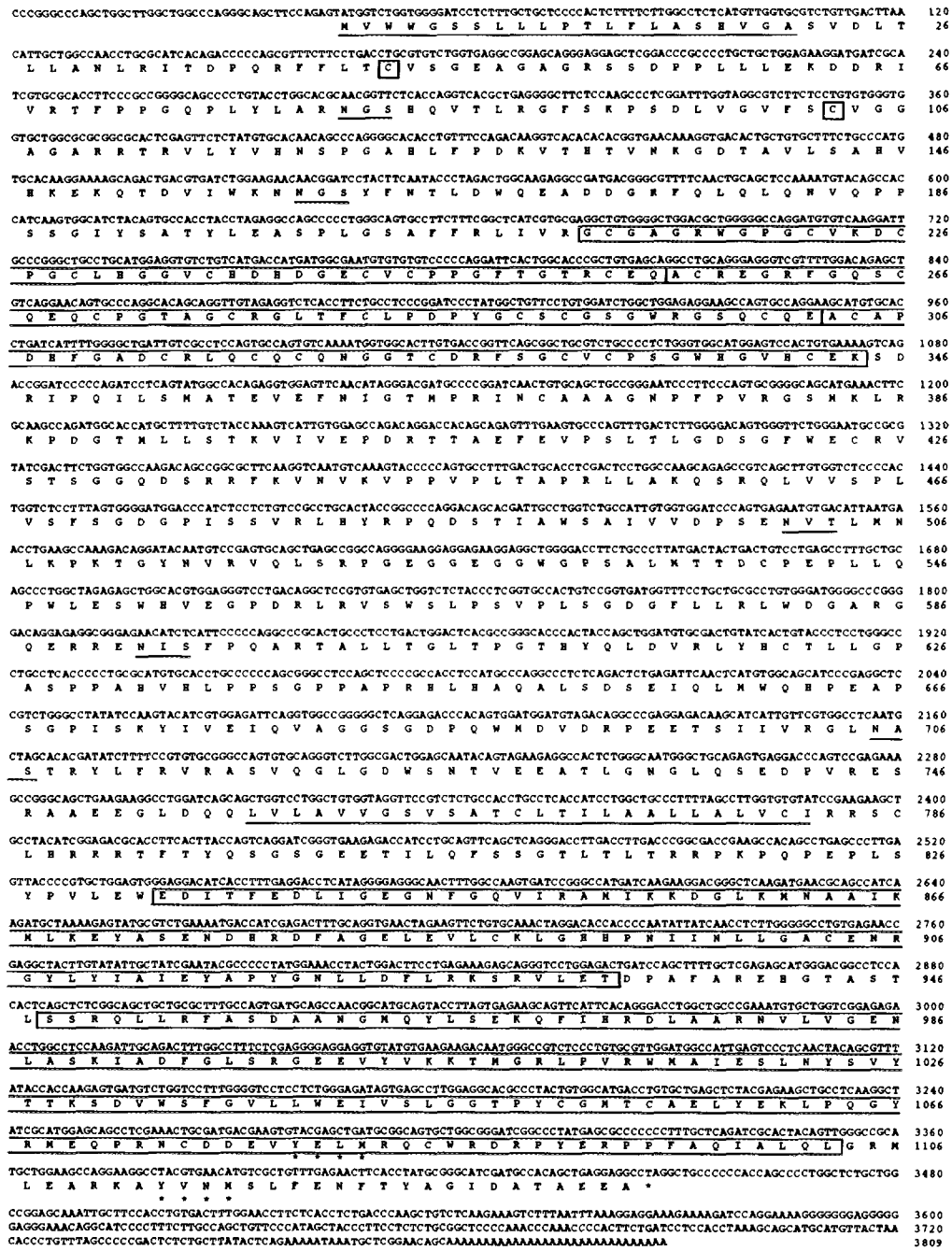

Figure 1. Nucleotide sequence and deduced amino acid sequence of mouse *TIE* cDNA. The predicted signal sequence (SS) and the transmembrane domain (TM) are underlined. Potential N-glycosylation sites are also underlined. Two cysteins of the Ig-like domain (Ig), three EGF-like repeats (EGF1-3), and the kinase domain (TK1-2) are boxed. Two putative binding sites of p85 N-terminal SH2 domain are marked with asterisks. FN1-3: fibronentin type III domain 1-3.

been also reported (5). Although there is three amino acid difference between STK1 and *TEK* (4)(Fig.2), STK1 should be referred to as *TEK*. The predicted protein sequence showed a similar structure as the mouse

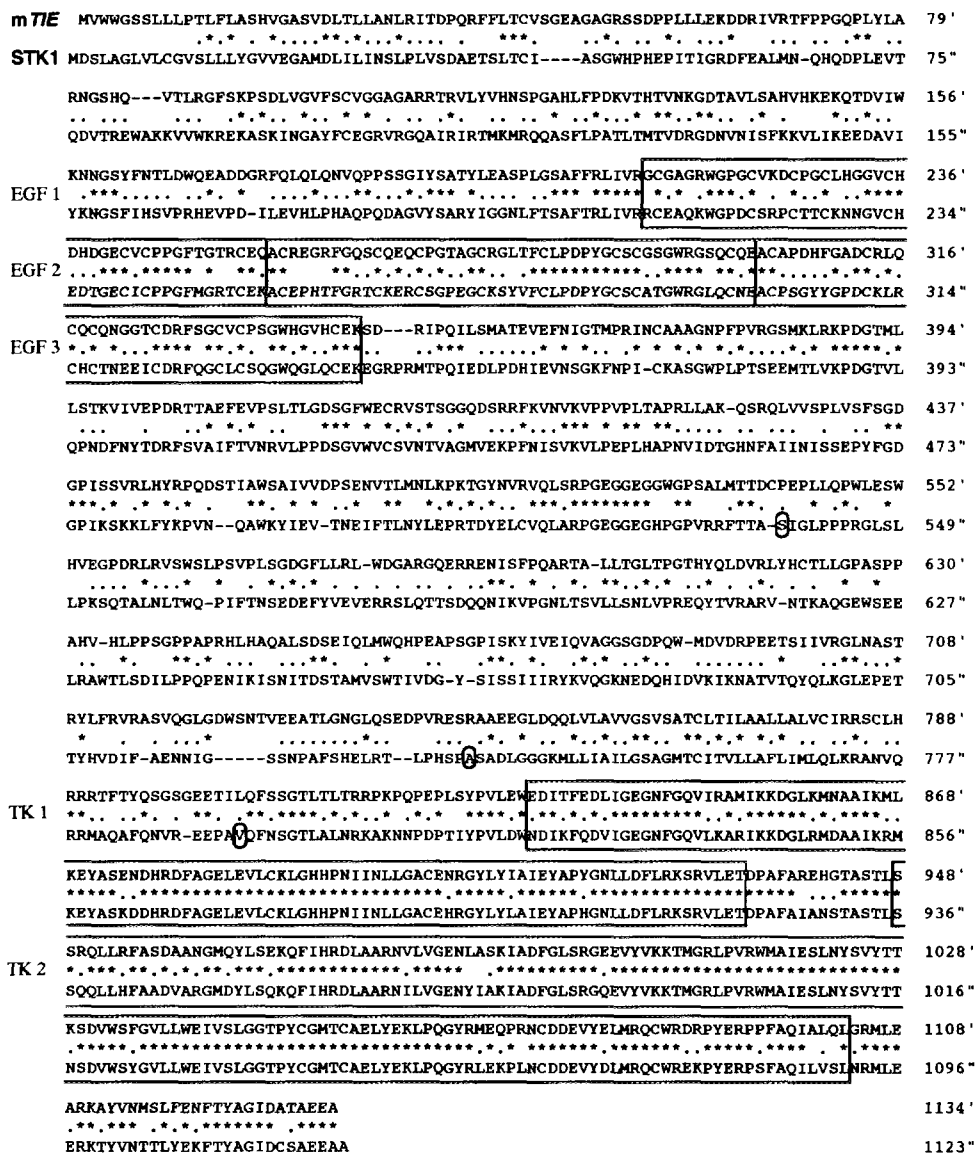

Figure 2. Alignment of deduced amino acid sequence of the mouse *TIE* gene with that of the STK1 cDNA clone. Identical amino acids are indicated with an asterisk and conservative or similar amino acid changes are indicated with a dot. EGF-like repeats and the kinase domain are boxed. Three amino acid difference between STK1 and the reported form of *TEK* (4) are circled (cysteine to serine at amino acid 538, glycine to alanine at 736, and an additional valine at position 793 in STK1).

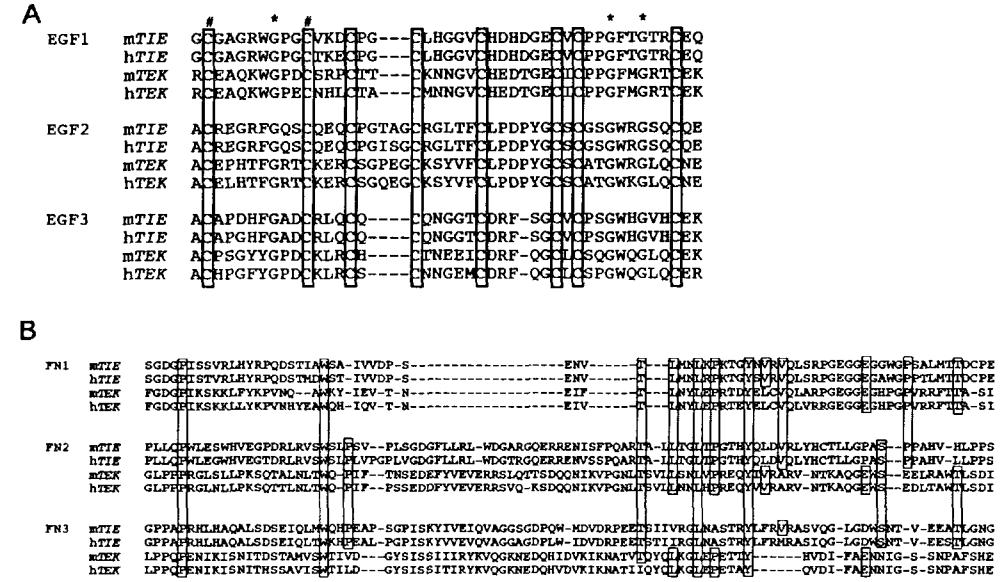

Figure 3. Sequence alignment of (A) three EGF-like domains and (B) three FNIII domains of mouse *TIE* with those of human *TIE* (3), mouse *TEK* (STK1), and human *TEK* (5). (A) Conserved cysteine residues are boxed and unique cysteine residues observed in *TIE* and *TEK* are marked (#). Conserved glysines are marked with asterisks. (B) Conserved amino acid residues are boxed.

TIE protein (FIG.2); namely the extracellular domain contained an Ig-like domain, three EGF-like repeats, and three FNIII repeats. A comparison in EGF-like domains and FNIII domains is shown in Fig.3. Two additional cysteine residues in EGF-like domains were conserved in *TIE* and *TEK*. The amino acid homology between murine *TIE* and *TEK* was 46.2% in total, 30.3% in FNIII domains, while the homology in EGF-like domains and kinase domain is high (55.3% and 84.4 %)(Fig.2). These findings suggest that *TIE* and *TEK* segregate a new subfamily among RTKs.

The expression of mouse TIE and TEK genes: Northern blot analysis showed a closely related expression pattern of mouse *TIE* and *TEK*. In adult tissues, mouse *TIE* and *TEK* were abundantly expressed in lung and heart, moderately in brain, liver, and kidney, and weakly in thymus, spleen, and testis (Fig.4A). Human *TIE* gene and mouse *TEK* gene are reported to be expressed in endothelial cells (3,4,13). Mouse *TIE* gene has been partially isolated and also reported to be expressed in endothelial cells (14).

Here, we also found that mouse *TIE* and *TEK* were expressed in primitive hematopoietic cells. In hematopoietic cell lines and stromal cell lines, undifferentiated hematopoietic cell line, DA-1 cells, expressed *TIE*

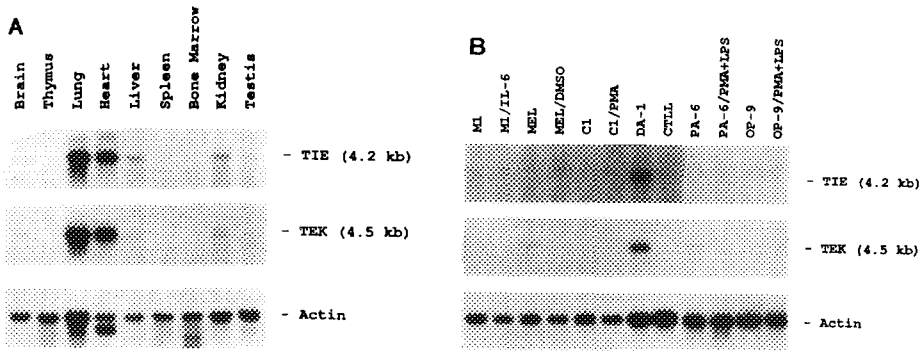

Figure 4. Northern blot analysis of mouse *TIE* and *TEK* mRNA (A) in adult tissues and (B) hematopoietic and stromal cell lines. M1 (myeloid), CTLL (T lymphoblastic), OP-9 (OP mouse-derived stroma). M1/IL-6: M1 cells were differentiated into macrophage lineage by the treatment with human recombinant IL-6 (20 ng/ml) for 3 days. MEL/DMSO: MEL cells were differentiated into erythroid lineage by the treatment with1.5% DMSO for 3 days C1/PMA: C1 cells were differentiated into megakaryocytic lineage by the treatment with 10 ng/ml PMA. PA-6/PMA+LPS, OP-9/PMA+LPS: Each stromal cell line was treated with 5 ng/ml PMA and 1 μg/ml LPS for 3 days. Loading was normalized by probing with a β-actin probe. The blot was exposed for 2 days.

and *TEK* moderately. Only *TEK* was weakly expressed in erythroblastic cell line, MEL cells, and bone marrow-derived stromal cell line, PA-6 cells, and its expression was downregulated when MEL cells were induced erythroid differentiation with DMSO or PA-6 cells were stimulated with PMA and LPS (Fig.4B). Our previous papers and others show that HSCs are included in the cells of $Lin^-Kit^+Sca\text{-}1^+$ fraction (6,15).

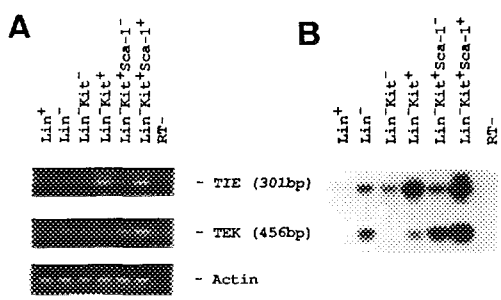

Figure 5. RT-PCR analysis of mouse*TIE* and *TEK* expression in fractionated bone marrow hematopoietic cells. β-actin was used as a reference gene for semiquantitative analysis of *TIE* and *TEK* expression. (A) Ethidium bromide staining. (B) Southern blotting. RT-: Lineage negative cells were processed without reverse transcriptase (RT).

In this study, we performed RT-PCR analyses using fractionated bone marrow cells (Fig.5A,B). Both genes were expressed more abundantly in Lin^- cells than Lin^+ cells, Lin^-Kit^+ than Lin^-Kit^-, and $Lin^-Kit^+Sca\text{-}1^+$ than $Lin^-Kit^+Sca\text{-}1^-$, respectively. These data suggest *TIE* and *TEK* are expressed in more primitive cells and their ligands are expected to act on HSCs. Since HSCs are dependent on stromal cells at the earlier stage of differentiation, it is supposed that their ligands are membrane-bounded forms and are derived from stromal cells as is the case in steel factor (2).

The endothelial cells and hematopoietic cells are thought to originate from the same progenitor cells. It is very interesting that *TIE* and *TEK* are expressed in both endothelial cells and HSCs, and coexpression of both molecules may reflect the same origin of these two cell lineages.

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