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A novel human tyrosine kinase gene inducible in T cells by interleukin 2

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We have cloned a novel human protein tyrosine kinase gene specific to T cells by the polymerase chain reaction method. This gene encodes a 620 amino-acid polypeptide including a catalytic domain for tyrosine kinase, an SH2 domain and an SH3 domain, seemingly belonging to the *src* family. However, characteristics of a long unique N-terminal stretch and lack of a myristylation site at the N-terminus and of a kinase regulatory tyrosine residue in the C-terminus classify this molecule into a new subfamily comprising recently cloned mouse *tec*, *itk/tsk* and human *atk/bpk* genes. This gene was transcriptionally induced in normal T cells by interleukin 2 stimulation. These results suggest the crucial roles of this gene in T cell proliferation and differentiation.

Protein tyrosine kinase; Molecular cloning; Interleukin 2; T cell

1. INTRODUCTION

Extracellular stimuli responsible for cell activation and proliferation exert their function through interaction with cell surface receptors which generate intracellular signaling pathways. One group of receptors is characterized by protein tyrosine kinase domains in the cytoplasmic regions of receptor molecules. This group includes receptors for growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and colony-stimulating factor-1 (CSF-1) (reviewed in [1]). The second group is a cytokine receptor family in which association of receptor subunits yields functional receptors. No enzymatic activity has been reported in these subunits, but tyrosine kinase molecules are suggested to be associated with the intracytoplasmic regions of the receptor subunits. Receptors for cytokines such as interleukin 2 (IL-2), IL-3, IL-5, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) belong to the second group family (reviewed in [2]).

In both receptor groups, signal pathways induced by extracellular interaction between ligands and receptors include protein phosphorylation that is detected as one of the earliest biochemical reactions; in particular, immediate tyrosine phosphorylation following the extracellular interaction, highlights the significance of activation of tyrosine kinases associated with receptors in signal pathways.

In the course of our studies of tyrosine phosphorylation induced by IL-2, we observed that IL-2-dependent tyrosine phosphorylation was induced even in cells lacking a cytoplasmic tyrosine kinase, $p56^{lck}$ [3–5], that had been reported to be directly associated with the IL-2-receptor β subunit [6]. We thereby wished to explore a tyrosine kinase molecule involved in the IL-2-induced signaling pathway. The polymerase chain reaction (PCR) with primers encoding peptides common to protein kinases was utilized for cloning tyrosine kinase genes expressed specifically in T cells. We found a novel tyrosine kinase, related to but distinct from the previously identified *src* family, expression of which is restricted to T cell and natural killer cell lineages. During analysis of this gene, we learned that mouse and human tyrosine kinase genes highly homologous to our clone have been cloned from T cells and B cells, respectively, indicating the establishment of a novel tyrosine kinase family.

We report here properties of a cloned novel tyrosine kinase gene.

2. MATERIAL AND METHODS

2.1. *Cells*

MT-1, MT-2, HUT 102, TL-Mor, ILT-Mat, TL-Su and TCL-Kan are human T cell lines carrying human T-cell leukemia virus type-I (HTLV-I). MTβ-1 is a stable transformant of MT-1 with the IL-2 receptor β chain gene, resulting in the generation of the functional high-affinity IL-2 receptor with the endogenous IL-2 receptor α and γ chains [3]. Jurkat and MOLT-4 are human acute lymphocytic leukemia T cell lines. YTC3 is a natural killer cell line. Raji, LCL-Kan and LCL-Ter28 are Epstein-Barr virus transformed B cell lines. BALL-1,

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Abbreviations: IL-2, interleukin 2; IL-2R, interleukin 2 receptor; SH2, Src homology 2; SH3, Src homology 3; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HTLV-I, human T-cell leukemia virus type-I.

Daudi and Ramos are B-cell lymphoma cell lines These cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics at 37°C under 7% CO_2 in air. For the IL-2-dependent cell line, ILT-Mat, IL-2 was added up to 500 pM. Human peripheral blood lymphocytes (PBL) were prepared by standard procedures of Ficoll-Conray gradient centrifugation and cultured with phytohemagglutinin (PHA) (1 mg/ml of medium; Difco) with recombinant IL-2 (1 nM; Ajinomoto) in RPMI 1640 with 10% fetal calf serum.

2.2. *RNA preparation*

Total cellular RNA was isolated from each cell line by extraction with guanidinium thiocyanate and centrifugation through a 5.7 M CsCl cushion [7]. Poly(A)$^+$RNA of PHA-PBL was enriched from total RNA with an oligo-(dT) column.

2.3. *cDNA cloning*

First cDNA strand was generated from PHA-PBL mRNA by the standard method The first strand was subjected to PCR amplification of 25 serial cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C with a pair of the degenerate primers for regions common to protein tyrosine kinases. After size fractionation, the PCR products were subcloned into pUC19 and sequenced by the dideoxynucleotide chain termination method with Sequenase ver.2.0 (USB).

An as yet uncharacterized tyrosine kinase related fragment, clone pTK-11, was used as a probe to obtain a full-length cDNA clone from a PHA-PBL cDNA library in λZAP II (Stratagene) under high-stringent conditions. A clone carrying the longest insert (4.4 kbp), named pN2 was fully sequenced on both strands.

2.4. *RNA blot analysis*

Total RNA was size-fractionated by electrophoresis on a formaldehyde–agarose gel and blotted to Hybond-N membranes (Amersham). Membranes were UV cross-linked and subjected to hybridization. A 4.4-kbp fragment specific for the cloned protein kinase gene and a 1.2-kbp *Pst*I fragment from pGAD-28 for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were used as probes [7]. Probes were labeled with random primers, [α-^{32}P]dCTP (Amersham) and T7 polymerase (Pharmacia). Hybridization was performed for 20 h at 42°C in 50% formamide, 5 × Denhardt's solution, 5 × SSC, 0.1% SDS, 10% dextran sulfate, 20 mM Tris-HCl (pH 7.5) and 200 μg/ml sonicated and denatured salmon sperm DNA. Membranes were washed in 2 × SSC and 0.1% SDS three times at room temperature and at 65°C with the same solution Radioactivity was analyzed with a bioimage analyzer BAS 2000 (Fuji Film).

3. RESULTS AND DISCUSSION

Isolation of novel tyrosine kinase genes in T cells was carried out by PCR with primers of degenerate oligonucleotides corresponding to two regions of the catalytic domain conserved in protein kinases. The degenerate primers are 17-mer sense and antisense oligonucleotide primers derived from K(I/V)(A/G)DFG in the VII and DVWS(F/Y)G in the IX, respectively, among the 11 highly homologous regions (I-XI) of the conserved catalytic domain of protein kinases ranging over about 250 amino acids [8]. The first cDNA strand reverse-transcribed from PHA-stimulated PBL mRNA was used in PCR amplification. PCR products were digested with *Bgl*I and *Hae*II to eliminate the fragment from the *fyn* and *lck* genes dominantly expressed in T cells [9–11], separated on an acrylamide gel and then 140–160 base pairs (bp) fragments were recovered, which correspond with the expected length. The fragments were cloned and sequenced. Among 24 clones, a half had nucleotide sequences identical to previously identified genes such as protein kinaseC-β (PKCβ) [12], basic-fibroblast growth factor(bFGF)-receptor [13], PDGF-receptor β [14], and another half encoded polypeptides related to protein kinases but distinct from published sequences. Ten out of the 12 unidentified clones were the same, having homology to yeast polymyxin B resistance gene [15]. One of the remaining two resembled serine/threonine kinases and the other seemed to stem from an unidentified tyrosine kinase. Thus we focused our attention on the unidentified putative tyrosine kinase clone. Its whole cDNA was isolated from a human PHA-PBL cDNA library using the cloned fragment as a probe. Out of fifteen clones containing identical sequences, the longest clone, pN2, was further analyzed. The pN2 clone carried a 4,384-bp insert, which contained an open reading frame encoding 620 amino-acid polypeptide with a calculated molecular weight of 71,827 (Fig. 1A).

The deduced amino acid sequence predicted a possible catalytic domain for protein kinase with a stretch of 250 amino acids at residues 363–612. This included several characteristics to protein kinases like an ATP-binding site, GSGQFG at positions 370–375, with a K residue at position 391, the highly conserved DFG motif, at positions 500–502, involved in ATP binding, and the SPE/APE motif, at positions 526–528, representing the most typical sequence of protein kinases. In addition to these sequences common to protein kinases, several other motifs specific for tyrosine kinases were included; DLAARN in subdomain VI and PVKWASPE in subdomain VIII. Moreover, a putative autophosphorylation site corresponding to the tyrosine residue at position 416 in c-*src* was present at position 512 in this molecule [8]. These characters presumably indicated that the clone encodes a novel tyrosine kinase.

The putative tyrosine kinase also showed homology to Src homology 2 (SH2) and Src homology 3 (SH3) domains (reviewed in [16]), which are seen in tyrosine kinases and other members of cytoplasmic signal transducing molecules. The SH2 and SH3 domains in

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Fig. 1. (A) Deduced amino acid sequence of human *itk/tsk* (h-Itk) in comparison with mouse *itk* (m-Itk), human *atk* (h-Atk) and mouse *tec* type I (m-Tec1). Mouse *tsk* (not shown) has 6 amino-acid deletion (TLVYLQ at position 82–87 of mouse *itk*) and 1 amino-acid replacement (A to R at position 158 of mouse *itk*) compared with mouse *itk*. Residues found in human *itk/tsk* that are identical to others are shaded. Gaps (indicated by dashes) are introduced to optimize the alignment. The SH2, SH3 and kinase catalytic domains described in the text are indicated by dashed underlines. PCR primers used for cloning are indicated by arrows. The tyrosine residue corresponding to the putative autophosphorylation site is indicated by an asterisk. The nucleotide sequence data of the gene will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number D13720

A

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h-Itk   MNNFILLEEQ-LIKKSQQKRRTSPSNFKVRFFVLT--KASLA-Y-FEDRHGKKRTLK-GSI--ELSRI  60
m-Itk   MNNFILLEEQ-LIKKSQQKRRTSPSNFKVRFFVLT--KASLA-Y-FEDRHGKKRTLK-GSI--ELSRI  60
h-Atk   MAAVIL-ESIFL-KRSQQKKKTSPLNFKKRLFLLTVHKLSYYEYDFE-R-GR-RGSKKGSIDVEK--I  61
m-Tec1  MMV------------SF------P----V--------KIN-----F---H---------S-----S--  14

h-Itk   KCVEIV---K--------------SD----ISI----PCHYKYPFQ--V---V-HDNYLLYVFAPDRE  97
m-Itk   KCVEIV---K--------------SD----ISI----PCHYKYPFQTLVYLQVVHDNYLLYVFAPDCE 103
h-Atk   TCVETVVPEKNPPPERQIPRRGEESSEMEQISIIERFP--Y--PFQ--V---VY-DEGPLYVFSPTEE 119
m-Tec1  -------------------------------------P-------Q----------------------  16

h-Itk   SRQRWVLALKEETRNNNS-LVPKYHPNFWMDGKWRCCSQLEKLATGCAQYDPTKNA--S--------- 153
m-Itk   SRQRWVLTLKEETRNNNS-LVSKYHPNFWMDGRWRCCSQLEKPAVGCAPYDPSKNA--S--------- 159
h-Atk   LRKRWIHQLKNVIRYN-SDLVQKYHPCFWIDGQYLCCSQTAKNAMGC-QILENRNG--SLKPGSSHRK 183
m-Tec1  SRDRWVKKLKEEIKNNNN-IMIKYHPKFWADGSYQCCRQTEKLAPGCEKY----NLFES---------  70

h-Itk   -K--KPLPPTPE-DN-RR--PLW-E-P-EE----T-----VVIALYDYQ-T-N--DPQELA--LR--R 194
m-Itk   -K--KPLPPTPE-DN-RR--SFQ-E-P-EE----T-----LVIALYDYQ-T-N--DPQELA--LR--C 200
h-Atk   TK--KPLPPTPEEDQ-ILKKPLPPE-P-AAAPVSTSELKKVV-ALYDYM-PMNANDLQ-L----R--K 237
m-Tec1  -SIRKTLPPAPE-IKKRR--PPP-PIPPEE--ENT--EEIVV-AMYDFQAT-------E-AHDLRLER 120
                                                [------------------SH3-----

h-Itk   NEEYCLLDSSEI---HWWRVQDRNGHEGYVPSSYLV-EK--SPNNLETYEWYNKSISRDKAEKLLLDT 256
m-Itk   DEEYYLLDSSEI---HWWRVQDKNGHEGYAPSSYLV-EK--SPNNLETYEWYNKSISRDKAEKLLLDT 262
h-Atk   GDEYFILEESNL---PWWRARDKNGQEGYIPSNY-VTEAEDSI---EMYEWYSKHMTRSQAEQLLKQE 298
m-Tec1  GQEYIIL---EKNDLHWWRARDKYGW--YCR------------N---T----NRS----KAEQLLR-T 159
        ---------------------------------]                [-----------------

h-Itk   G-KEGAFMVRDSRTAGTYTVSVFTKAVVSENNPC--IKHYHIKETNDNPKR-YYVAEKYVFDS-IPLL 319
m-Itk   G-KEGAFMVRDSRTPGTYTVSVFTKAIISEN-PC--IKHYHIKETNDSPKR-YYVAEKYVFDS-IPLL 324
h-Atk   G-KEGGFIVRDSSKAGKYTVSVFAK---STGDPQGVIRHYVVCST---PQSQYYLAEKHLF-STIPEL 358
m-Tec1  EDKEGGFMVRDSSQPGLYTVSLYTKFGG-EGSSG--FRHYHIKETATSPKK-YYLAEKHAFGS-IPEI 222
        ------------------------------------------SH2-----------------------

h-Itk   INYHQHNGGGLVTRLRYPVCF-GRQ-K-APVTAGLRYGKWVIDPSELTFVQEIGSGQFGLVHLGYWLN 384
m-Itk   IQYHQYNGGGLVTRLRYPVCS-WRQ-K-APVTAGLRYGKWVIQPSELTFVQEIGSGQFGLVHLGYWLN 389
h-Atk   INYHQHNSAGLISRLKYPVSQ---QNKNAPSTAGLGYGSWEIDPKDLTFLKELGTGQFGVVKYGKWRG 423
m-Tec1  IEYHKHNAAGLVTRLRYPVSTKG---KNAPTTAGFSYDKWEINPSELTFMRELGSGLFGVVRLGKWRA 287
        ------------------]                           [---------------------

h-Itk   K-DKVAIKTIREGAMSEED-FIEEAEVMMKLSHPKLVQLYGVCLEQAPICLVF---EFMEHGCLSDYL 447
m-Itk   K-DKVAIKTIQEGAMSEED-FIEEAEVMMKLSHPKLVQLYGVCLEQAPICLVF---EFMERGCLSDYL 452
h-Atk   QYD-VAIKMIKEGSMSE-DEFIEEAKVMMNLSHEKLVQLYGVCTKQRPI---FIITEYMANGCLLNYL 486
m-Tec1  Q-YKVAIKAIREGAMCEED-FIEEAKVMMKLTHPKLVQLYGVCTQQKPIYIVT---EFMERGCLLNFL 350
        --------------------------------------------------------------------

h-Itk   RTQR-GLFAAET--LLGMCLDVCEGMAYLEEACVIHRDLAARNCLVGEN-Q-VIKVSDFGMTRFVLDD 510
m-Itk   RSQR-GLFAAET--LLGMCLDVCEGMAYLEKACVIHRDLAARNCLVGEN-Q-VIKVSDFGMTRFVLDD 515
h-Atk   REMR-HRFQ--TQQLLEMCKDVCEAMEYLESKQFLHRDLAARNCLV--NDQGVVKVSDFGLSRYVLDD 549
m-Tec1  R-QRQGHFSRDM--LLSMCQDVCEGMEYLERNSFIHRDLAARNCLVNEA-G-VVKVSDFGMARYVLDD 413
        ----------Kinase catalytic domain-----------------------------------
         *
h-Itk   QYTSSTGTKFPVKWASP-EVF--S-FSRYSSKSDVWSFGVLMWEVFSEGKIPYE-NRS-NSEVVEDIS 572
m-Itk   QYTSSTGTKFPVKWASP-EVF--S-FSRYSSKSDVWSFGVLMWEVFSEGKIPYE-NRS-NSEVVEDIS 577
h-Atk   EYTSSVGSKFPVRW-SPPEVLMYSKFS--S-KSDIWAFGVLMWEIYSLGKMPYE--RFTNSETAEHIA 611
m-Tec1  QYTSSSGAKFPVKWCPP-EVF--N-YSRFSSKSDVWSFGVLMWEIFTEGRMPFEKNT--NYEVVTMV- 474
        --------------------------------------------------------------------

h-Itk   T-GFRLYKPRLASTHV-YQIMNHCWKERPED-RPAFSRLLRQ---LA---EIAES-GL           620
m-Itk   T-GFRLYKPRLASCHV-YQIMNHCWKEKPED-RPPFSQLLSQ---LA---EIAEA-GL           625
h-Atk   Q-GLRLYRPHLASEKV-YTIMYSCWHEKA-DERPTFKILLSN--ILDVMDE--ES              659
m-Tec1  TRGHRLHRPKLA-TKYLYEVMLRCWQERPEG-RPSFEDLLRTIDELV---ECEETFGR           527
        ---------------------------------------------]
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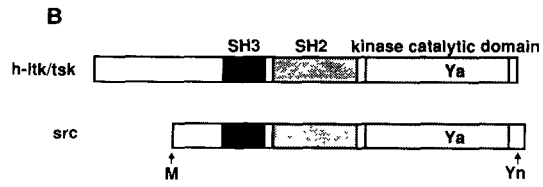

Fig. 1. (B) Schematic comparison of human *itk/tsk* with a *src* family protein tyrosine kinase. M, Ya and Yn indicate the myristylation, the putative autophosphorylation and the negative regulatory site, respectively, as described in the text.

the cloned tyrosine kinase were at positions 239–338 and at positions to adjacent to the SH2 domain, respectively. Number as well as the order of the SH2 and SH3 domains, together with the kinase catalytic region in the clone was the same as those of non-receptor *src* family tyrosine kinases (Fig. 1B). No typical hydrophobic region was seen, indicating that it is present in the cytoplasm. A computer search confirmed that the clone is novel, having high similarity to the members of the *src* family, Drosophila *src*28C [17], human *lck*, human *fgr* [18], human *fyn* and mouse *tec* type I [19]. These results strongly lend support to the fact that our clone encodes a cytoplasmic tyrosine kinase.

It should be noted that our clone is close to the mouse *tec* type I gene preferentially expressed in liver because it codes for a cytoplasmic tyrosine kinase that is related to but significantly distinct from the *src* family. Both clones lack a tyrosine residues which is known to be a negative regulatory site of the *src*-family tyrosine kinases at the C-terminal [20–22] and have rather shorter C-terminals, suggesting different mechanisms by which phosphate transfer activities are regulated. Other distinct figures are found in amino acid sequences upstream of the SH3 domain which are longer than those of the *src* family tyrosine kinases, and do not have a myristylation site at their amino ends which enable the *src* members to be attached to the inner surface of the cell membrane (Fig. 1B). This may suggest differences in subcellular localization as well as signaling roles. Very recently, having prepared this report, we learned that two novel kinases have been cloned; *itk/tsk* isolated from mouse T cells [23,24], and *atk/bpk* from human B cells [25,26], both of which have the SH3, SH2 and tyrosine kinase domains in order and lack the tyrosine residue in the C-terminals and the myristylation site at the amino ends. Surprisingly, striking similarity between these molecules and our clone was observed with amino acid homology of 93% to mouse *itk/tsk*, 53% to human *atk* and 50% to mouse *tec* type I. Thus our clone is believed to be a human counterpart of the mouse *itk/tsk* gene. Due to these similarities in sequence and structural organization, these clones, human and mouse *itk/tsk*, mouse *tec* type I and human *atk/bpk*, seem to establish a new subfamily among non-receptor type tyrosine kinases.

Expression of the human *itk/tsk* gene was examined by RNA blot analyses with a variety of human cell lines. Under high stringent hybridization conditions, the human *itk* cDNA probe identified a single RNA transcript of 4.4-kb in restricted cell lines, mainly in T cell lines (Fig. 2). Low to moderate expression of human *itk/tsk* mRNA was seen in T cell lines Jurkat and MOLT-4, and in an HTLV-I-infected T cell line, MT-1 and relatively high in HTLV-I-producing T cell lines, TL-Su, ILT-Mat, MT-2, HUT 102, TCL-Kan and TL-Mor. Moderate level of expression was also seen in a natural killer cell line YTC3 but not detected in B cell lines, Raji, BALL-1, Ramos, LCL-Kan, LCL-Ter28 and Daudi. Expression of human *itk/tsk* mRNA was not detected in other hematopoietic cell lines, a promonocytic cell line THP-1, an erythroleukemic cell line K562, a promyelocytic cell line HL-60 and an eosinocytic cell line Eol 3, and also totally undetectable in a non-hematopoietic cell, an epithelioid cell line HeLa, fibroblastoid cell lines WI26 and WI38, a neuroblastoma cell line SK-N-SH, glioma cell lines Hs683 and Onda 11, a fibrosarcoma cell line HT-1080, an epidermoid carcinoma KB and a hepatocellular carcinoma Hep G2 (data not shown). These results demonstrate that the human *itk/tsk* gene expression is restricted in T and natural killer cell lines.

We next examined whether expression of the human *itk/tsk* gene is enhanced upon stimulation leading to T cell activation. Low level expression of *itk/tsk* mRNA in unstimulated PBL was converted to the level as high as in HTLV-I-expressing T-cell lines after PHA plus IL-2 stimulation (Fig. 3A). IL-2 alone was enough for the significant induction in MTβ-1, as shown with the mouse *itk/tsk* [23], and this level was observed to be maximum 6 h after stimulation (Fig. 3B). This may imply a possible role of *itk/tsk* in T cell activation and

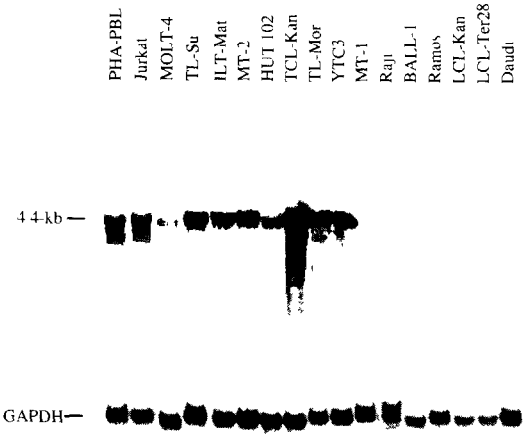

Fig. 2. RNA blot analysis of human *itk/tsk* mRNA in various cells. The cDNA from pN2 was used to detect a 4.4-kb message. Total RNA (20 μg) was electrophoresed in a denatured 2% agarose-formaldehyde gel and transferred to membranes. After hybridization with the human *itk/tsk* probe, they were rehybridized with the GAPDH probe as control. A prolonged exposure revealed a faint 4.4-kb signal in MT-1

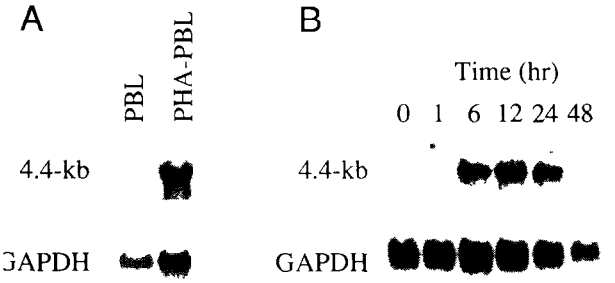

Fig. 3. Induction of human *itk/tsk* mRNA by IL-2 (A) Total RNA from PBL was extracted before and after the stimulation with PHA. Two days after PHA stimulation, IL-2 was added at a concentration of 1 nM and cultured for another 2 days. (B) MTβ-1 cells were stimulated by 2 nM IL-2. Cells were harvested after 0, 1, 6, 12, 24 and 48 h. Total RNA (30 μg) was electrophoresed for each lane

proliferation. Indeed, expression of the mouse *itk/tsk* gene was shown to be developmentally regulated [24]. In this context, the recently cloned human B-cell specific clone, *atk/bpk*, was interestingly demonstrated to be involved in X-linked agammaglobulinemia, suggesting its crucial role in B-cell ontogeny. Similarly, since our human *itk/tsk* clone is a member of the same subfamily of tyrosine kinases, we cannot ignore the possibility that the *itk/tsk* product plays a pivotal role in T cell development and that its impairment results in T cell or T and B cell combined immunodeficiency as observed in human severe combined immunodeficiency (SCID) [27].

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