

# A rare mRNA variant of the human lymphocyte-specific protein tyrosine kinase *LCK* gene with intron B retention and exon 7 skipping encodes a putative protein with altered SH3-dependent molecular interactions

Solange Nervi<sup>a,b</sup>, Rodolphe Guinamard<sup>b</sup>, Bénédicte Delaval<sup>a</sup>, Patrick Lécine<sup>a</sup>,  
Bernard Vialettes<sup>c</sup>, Philippe Naquet<sup>b</sup>, Jean Imbert<sup>a,\*</sup>

<sup>a</sup> Institut de Cancérologie de Marseille, UMR599 INSERM-Institut Paoli-Calmettes-Université de la Méditerranée,  
27 boulevard Leï Roure, 13009 Marseille, France

<sup>b</sup> Centre d'Immunologie de Marseille-Luminy CNRS-INSERM-Université de la Méditerranée, case 906, 13009 Marseille, France

<sup>c</sup> UPRES-EA 2193, Université de la Méditerranée, CHU Sainte-Marguerite, 270 boulevard de S<sup>c</sup> Marguerite, 13009 Marseille, France

Received 12 January 2005; received in revised form 6 June 2005; accepted 16 June 2005

Available online 16 August 2005

Received by K. Gardiner

## Abstract

A rare mRNA variant of the human lymphocyte-specific protein tyrosine kinase *LCK* gene that retains intron B and excludes exon 7 ( $B^{+7-}$ ) due to alternative splicing of the canonical *LCK* transcripts was identified and characterized.  $LCK B^{+7-}$  mRNA is detected in all tested peripheral blood T lymphocytes total RNA samples but is apparently sequestered in the nucleus. The presence of intron B sequence does not disrupt the reading frame and results in the insertion of 58 aminoacids, containing a proline-rich region just upstream of p56lck SH3 domain. This putative isoform encodes an unstable 516 aminoacids protein ( $Lck^{B^{+7-}}$ ) which can be expressed in transfected COS-7 cells. Furthermore in Jurkat T cell extracts, a recombinant intron B plus SH3 p56lck domain fails to interact with some TCR-induced tyrosine phosphorylated polypeptides and known p56lck partners such as Sam68 and *c-Cbl*. The biological function of this rare messenger remains to be elucidated.

© 2005 Elsevier B.V. All rights reserved.

**Keywords:** *LCK* gene; Intron retention; Exon skipping; mRNA variant; Alternative splicing

**Abbreviations:** A, adenosine; AMV, avian myeloma virus; ATP, adenosine triphosphate; bp, base pair(s); C, cytidine; *c-Cbl*, cellular homolog of CAS-BR-M murine ecotropic retroviral transforming sequence; cdc2, cell division cycle 2; cDNA, DNA complementary to RNA; DMEM, dubelcco's modified Eagles medium; G, guanosine; Grb-2, growth factor receptor-bound protein 2; GST, glutathione-S-transferase; iB, intron B; IU, international unit(s); kb, kilobase(s); kDa, kilodalton(s); *LCK*, lymphocyte-specific protein tyrosine kinase; M, A/C; MAPK, mitogen-activated protein kinase; mRNA(s), messenger RNA(s); N, A/C/G/T; oligo(dT), oligodeoxythymidine-*n*; PBL, peripheral blood lymphocyte(s); PBMC, peripheral blood mononucleate cell(s); PBS, phosphate buffered saline; PCR, polymerase chain reaction; PI2K, phosphatidylinositol 3-kinase; PTK, protein tyrosine kinase; PVDF, polyvinyl difluoride; R, A/G; Ras-GAP, Ras guanosine triphosphatase activating protein; RT, reverse transcriptase; Sam68, src-associated mitotic cell protein; SCID, severe combined immunodeficiency; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SH $n$ , src homology domain *type n*; SLP-76, SH2 domain-containing leukocyte protein 76-kDa; Src, rous sarcoma virus oncogene homolog; T, thymidine; TCR, T cell receptor; Tec, Tec protein tyrosine kinase; Th $n$ , T helper *type n*; Wasp, Wilkott-Aldrich syndrome protein; Y, C/T.

\* Corresponding author. Tel.: +33 491 75 84 04; fax: +33 491 75 55 03.

E-mail address: [imberty@arseille.inserm.fr](mailto:imberty@arseille.inserm.fr) (J. Imbert).

## 1. Introduction

The common pathway for TCR-activated signaling involves a protein tyrosine kinase (PTK) cascade including the Src family member p56lck (Palacios and Weiss, 2004). p56lck is predominantly expressed in T cells and mainly localized in microdomains. It plays a crucial role in early activation events, thymic differentiation, cell cycle progression, Th1/Th2 differentiation (Yamashita et al., 1998), apoptosis and homeostatic proliferation of naïve T cells. Like other Src family kinases, p56lck is organized in domains including a C-terminal kinase domain, Src homology domains (SH4, SH3 and SH2), and a N-terminal region. SH3 domain mediate protein interactions by binding proline-rich aminoacid sequences and is also important for intra- and intermolecular interactions that regulate the catalytic activity, the localization into membrane rafts (Patel et al., 2001), and the recruitment of downstream effectors such as *c-Cbl*, PI3K, Ras-GAP, SLP-76, Cdc2, MAPK, Sam68 (Togni et al., 2004). Markedly in T cells, decreased p56lck protein expression has been observed in a variety of cancers (Majolini et al., 1999) and in autoimmune diseases including type 1 diabetes (Nervi et al., 2000).

The human lymphocyte-specific protein tyrosine kinase *LCK* gene resides at chromosomal locus 1p35-p34.3 and is composed of a segment of approximately 14 kb containing 12 exons. This gene belongs to the large category of human genes that undergo alternative splicing and whose isoforms are involved in regulatory functions (Modrek et al., 2001). Expression of the *LCK* gene is under the control of two structurally distinct promoters separated by a genomic region of 35 kb, approximately, the so-called distal and proximal promoters (Takadera et al., 1989). The promoter alternate usage produces two major mRNAs in human, designated type I when transcribed from the proximal promoter and type II from the distal promoter. Type I and II mRNAs differ only in their non coding 5' end. In each class, several mRNAs are transcribed through the use of alternative transcription initiation sites or by alternative splicing (Rouer and Benarous, 1992). Interestingly, an alternative splicing produces two type II mRNAs. The mRNA IIA is the most abundant in mature T cells whereas the minor mRNA IIB, lacking exon 1' encoding for the N-terminal domain of Lck, is devoid of the coding sequence for the interaction motif with CD4 and CD8 coreceptors (Huse et al., 1998). Another alternative splicing produces an exon 7-less mRNA encoding for an unstable Lck protein isoform. The sole expression of this defective exon7-less transcript was correlated with a signaling deficit downstream of CD3/TCR complex in a young immunodeficient patient (Goldman et al., 1998). However, further analysis evidenced the presence of both transcripts in all tested human cells expressing the *LCK* gene (Nervi et al., 2000; Rouer et al., 1999). It encodes a truncated p56lck protein (Lck $\Delta$ 7) lacking the ATP binding site and with the characteristics of a p56lck cell-signaling regulator (Germani et al., 2003).

During an attempt to elucidate the molecular basis of p56lck deficiency in type 1 diabetes (Nervi et al., 2000), we have identified a rare human *LCK* transcript retaining intron B (iB) and lacking exon 7. It contains an open reading frame encoding for 516 aminoacids (Lck<sup>B+7-</sup>) with the insertion of 58 mostly hydrophobic proline-rich aminoacids just upstream of the SH3 domain and lacking the ATP binding site. GST pull down experiment with recombinant p56lck intron B+SH3 domain evidenced loss of interaction with known partners of p56lck including Sam68 and *c-Cbl*. Lck<sup>B+7-</sup> mRNAs were detected in all tested total RNA samples prepared from various T lymphocyte subsets regardless of the diabetic phenotypes of the individuals. However, the physiological relevance of this transcript isoform is unclear since it appears predominantly sequestered in the nucleus of T lymphocytes.

## 2. Materials and methods

### 2.1. Cells

Peripheral blood mononuclear cells from healthy volunteers or type 1 diabetic patients were purified by Ficoll-Hypaque (Nervi et al., 2000). The human leukemia Jurkat (clone H6.2) (Nunes et al., 1993) and the Lck-deficient Jurkat mutant (JCam1.6) (Penninger et al., 1993) T cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin. The COS-7 cells were grown in DMEM supplemented as described above.

### 2.2. *LCK* mRNA cloning and sequencing

Total RNA was extracted from 10<sup>7</sup> resting PBMC with Trizol reagent (Life Technologies) and converted into cDNA using oligo(dT) primers (Promega) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). For cloning, an aliquot of the cDNA (1/25) was used as template and the complete *LCK* gene coding sequence was amplified by PCR in a 50  $\mu$ l-mixture including 1  $\mu$ l high fidelity *Pfu* Taq polymerase (Promega) and 50  $\mu$ M of two primers matching its 5' and 3' ends, respectively (sense 1F: 5'-ATGGGCTGTGGCTGCAGCTCACACCC-3', reverse 1530R: 5'-TCAAGGCTGAGGCTGGTACTGGCCC-3'). Cycling conditions included an initial denaturation at 94 °C for 2 min followed by 30 cycles at 95 °C for 1 min, 65 °C for 2 min, 74 °C for 4 min, and a final extension step at 74 °C for 5 min. The PCR products were separated by gel electrophoresis and purified using a Nucleospin kit (Macherey–Nagel) and subcloned into the pUC18 shuttle vector. To determine the presence of intron B sequence within *bona fide* LCK mRNAs, full length cDNA were also synthesized from total RNA according to the GeneRacer™ protocol (Invitrogen Life Technologies) including dephosphorylation, decapping and reverse-transcription with AMV reverse

transcriptase using the GeneRacer™ Oligo dT primer. A fragment of the full length LCK cDNA was then amplified by PCR with ThermoZyme™ DNA polymerase (Invitrogen) and two specific primers within exon 2 and 5 respectively (exon 2 sense 5'-TCTGAGGTGCGGGACC-CACTGGTTAACCT-3', exon 5 antisense 5'-CCCGGATGAGGAAGGAGCCGTGAGTGTT-3') according to manufacturer's protocol. Cycling conditions included an initial denaturation at 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. After gel purification, the products were cloned into pCR®4-TOPO® vector (Invitrogen). All products were commercially sequenced (Genome Express, Meylan, France).

### 2.3. RT-PCR screening for intron B

Total RNA was extracted from PBMC or cell lines using the SV total RNA isolation kit (Promega), including a DNase treatment. Reverse transcription was performed with 2 µl cDNA in a 20 µl final volume using Superscript RT (Life Technologies). PCRs were performed with 2 µl cDNA in a 25 µl final volume using Taq DNA polymerase (Gibco BRL). The initial denaturation step (95°C for 2 min) was followed by 33 cycles of amplification: 95 °C, 30 s; 63 °C, 30 s; 72 °C, 40 s) and a final extension step at 72 °C for 5 min. The forward and reverse human *LCK* gene primers respectively annealed within exons 2 (GAAATGGCTCTGAGGTGCGGG) and 4 (GGGCTCCAGGCTGTTCGCTTTGG). The lack of potential genomic contamination was checked by the lack of amplification in samples without reverse transcriptase.

### 2.4. Transient transfections and Western blot analysis

The COS-7 cell line was transiently transfected with 5 or 10 µg of full-length or B<sup>+</sup>7<sup>-</sup> LCK cDNA in 100-mm plate using Fugene 6 transfection reagent (Roche Diagnostics). Cells were harvested 24 or 48 h after transfection. Western blots were performed with transfected cells washed twice in ice cold PBS and lysed in 1% Nonidet P-40, 150 mM NaCl, 20 mM Tris pH 7.4 and a cocktail of proteases and phosphatases inhibitors (Roche Diagnostics). Whole cell lysates were resolved on 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Inc.), blocked and immunoblotted with monoclonal anti-Lck (clone 3A5, Santa Cruz) or polyclonal anti-intronB-specific Lck antibody. Bound antibodies were visualized by ECL (Amersham Pharmacia Biotech).

### 2.5. Preparation of GST-LCK proteins and protein-protein interaction assay

GST fused to Lck SH3 aminoacids 63–122 preceded or not by iB sequence were created by subcloning fragments into pGEX-2T (Pharmacia Biotech) by PCR amplification

using the following primers: 5' SH3 GGGGATCCGA-CAACCTGGTTATCGCTCTG, 3' SH3 ATGAATTCGGG-GCTCCAGGCTGTTCGCTTTGG, 5' iB GGGG-ATCCGGTGACCCCAGGCAGCAGGG, 3' iB ATGAATTCCTGTAAAGACAAGGGGCATCAGGG. Fusion proteins were expressed and purified according to the manufacturer's recommendations. Clarified lysates were incubated with GST fusion protein coupled to GST-Sepharose 4B beads for 2 h at 4 °C. Samples were washed in lysis buffer, separated on 10% SDS-PAGE and immunoblotted as indicated in Fig. 4 legend.

### 2.6. Antibodies

The following antibodies were used: anti-Lck 3A5, -c-Cbl, -Sam68, -Wasp (Santa Cruz Biotechnology, Inc.), anti-phosphotyrosine 4G10 (Upstate) and anti-GST (abcam®). We raised a rabbit polyclonal serum (B76) directed against the 13 first iB aminoacids (GDPRQQLKDKAC).

## 3. Results and discussion

### 3.1. Cloning of a new alternative LCK gene transcript in human T lymphocytes

A full length LCK coding sequence cDNA was amplified by RT-PCR using a pair of 5'- and 3'-end primers (1F/1530R) with a random selection of 3 type 1 diabetic patients and 3 control individuals. After cloning in pUC18, the complete sequence of the cDNA inserts was determined, 1 to 3 clones were sequenced per donor (for a total of 12 clones). The two expected LCK cDNAs corresponding, respectively, to the full length mRNA (1530 nucleotides) and to the previously reported exon 7-less isoform (1380 nucleotides) (Straus and Weiss, 1992) were found. A third isoform with 1551 nucleotides was also detected (GenBank/EMBL accession no. AJ865079) in 5 out of 12 clones, independently of the disease status. Sequence alignment with full length LCK cDNA showed that it contained both an insertion of 174 bps between exons 2 and 3 and missed exon 7 (Fig. 1A). Sequence comparison with Genbank entry X14053 that contains the LCK genomic sequence including exons 1–8 evidenced that the 174 bps fragment was the intron B but with two single nucleotide insertions, a G at position 204 and a C at position 221. However, further search in Genbank database identified a contig (Genbank accession no. AL121991) containing a LCK genomic sequence that perfectly matched the intron B sequence included in the newly identified cDNA. Hence, Genbank entry X14053 either contains two errors or corresponds to an individual with two rare SNPs.

A spurious cloning from either LCK genomic DNA or unspliced RNAs was *a priori* excluded on the basis of three arguments. Firstly, the full length B<sup>+</sup>7<sup>-</sup> LCK cDNA was cloned after oligo dT priming. Secondly, a product contain-

**A**

1	ATGGGCTGTGGCTGCAGCTCACACCCGGAAGATGACTGGATGGAAAACAT	727	gagacgctgaagctggtggagcggtgggggctggacagttcggggaggt
1	ATGGGCTGTGGCTGCAGCTCACACCCGGAAGATGACTGGATGGAAAACAT	809	-----
51	CGATGTGTGTGAGAACTGCCATTATCCCATAGTCCCACCTGGATGGCAAGG	777	gtggatggGTTACTACAACGGGCACACGAAGTGGCGGTGAAGAGCCTGA
51	CGATGTGTGTGAGAACTGCCATTATCCCATAGTCCCACCTGGATGGCAAGG	809	-----GGTACTACAACGGGCACACGAAGTGGCGGTGAAGAGCCTGA
101	GCACGCTGCTCATCCGAAATGGCTCTGAGGTGCGGGACCCACCTGGTTACC	827	AGCAGGGCAGCATGTCCCAGGACGCCTTCTGGCCGAGGGCAACCTCATG
101	GCACGCTGCTCATCCGAAATGGCTCTGAGGTGCGGGACCCACCTGGTTACC	848	AGCAGGGCAGCATGTCCCAGGACGCCTTCTGGCCGAGGGCAACCTCATG
151	TACGAAGGCTCCAATCCGCGGCTTCCCACCTGCAAG-----	877	AAGCAGCTGCAACACCGCGGCTGGTTCGGCTCTACGCTGTGGTCAACCA
151	TACGAAGGCTCCAATCCGCGGCTTCCCACCTGCAAG <u>ggtgac</u> ccccaggca	898	AAGCAGCTGCAACACCGCGGCTGGTTCGGCTCTACGCTGTGGTCAACCA
187	-----	927	GGAGCCCATCTACATCATCACTGAATACATGGAGAATGGGAGTCTAGTGG
201	gcaggcctgaaagacaaggctgsggatccctggctgtggcttccacc	948	GGAGCCCATCTACATCATCACTGAATACATGGAGAATGGGAGTCTAGTGG
187	-----	977	ATTTTCTCAAGACCCCTTCAGGCATCAAGTTGACCATCAACAACTCCTG
251	tctcccccaactaettctccccggcttctcctgtgcccccaacct	998	ATTTTCTCAAGACCCCTTCAGGCATCAAGTTGACCATCAACAACTCCTG
187	-----	1027	GACATGGCAGCCAAATTCGAGAAGGCATGGCATTCAATGAAGAGCGGAA
301	gtaactccaggctcctctgcccagatcccagctcggttctccctgatgccct	1048	GACATGGCAGCCAAATTCGAGAAGGCATGGCATTCAATGAAGAGCGGAA
187	-----	1077	TTATATTCATCGTGACCTTCGGGCTGCCAACATCTGGTGTCTGACACCC
351	tgctcttacagACAACCTGGTTATCGCTCTGCACAGCTATGAGCCCTCTC	1098	TTATATTCATCGTGACCTTCGGGCTGCCAACATCTGGTGTCTGACACCC
227	ACGACGGAGATCTGGCTTTGAGAAGGGGGAACAGCTCCGCATCCTGGAG	1127	TGAGCTGCAAGATTGCAGACTTTGGCCTAGCAGCCTCATTGAGGACAAC
401	ACGACGGAGATCTGGCTTTGAGAAGGGGGAACAGCTCCGCATCCTGGAG	1148	TGAGCTGCAAGATTGCAGACTTTGGCCTAGCAGCCTCATTGAGGACAAC
277	CAGAGCGGCGAGTGGTGAAGGCGAGTCCCTGACCAACCGGCGAGGAAGG	1177	GAGTACACAGCCAGGGAGGGGCAAGTTTCCCATTAAGTGGACAGCGCC
451	CAGAGCGGCGAGTGGTGAAGGCGAGTCCCTGACCAACCGGCGAGGAAGG	1198	GAGTACACAGCCAGGGAGGGGCAAGTTTCCCATTAAGTGGACAGCGCC
327	CTTCATCCCCTTCAATTTTGTGGCCAAAGCGAAGCAGCTGGAGCCCGAAC	1227	AGAAGCCATTAACACGGGACATTCACCATCAAGTCAGATGTGGTCTT
501	CTTCATCCCCTTCAATTTTGTGGCCAAAGCGAAGCAGCTGGAGCCCGAAC	1248	AGAAGCCATTAACACGGGACATTCACCATCAAGTCAGATGTGGTCTT
377	CCTGGTCTTCAAGAACCTGAGCCGCAAGGACGCGGAGCGGCAGCTCCTG	1277	TTGGATCCTGCTGACGGAAATTTGTCAACCGCCGCGCATCCCTTACCCA
551	CCTGGTCTTCAAGAACCTGAGCCGCAAGGACGCGGAGCGGCAGCTCCTG	1298	TTGGATCCTGCTGACGGAAATTTGTCAACCGCCGCGCATCCCTTACCCA
427	CGCCCGGGAACACTCACGGCTCCTTCTCATCCGGGAGAGCGAGAGCAC	1327	GGGATGACCAACCCGGAGGTGATTCAGAACTGGAGCGAGGCTACCCGAT
601	CGCCCGGGAACACTCACGGCTCCTTCTCATCCGGGAGAGCGAGAGCAC	1348	GGGATGACCAACCCGGAGGTGATTCAGAACTGGAGCGAGGCTACCCGAT
477	CGCGGGATCGTTTTCACTGTGCGTCCGGGACTTCGACCAAGAACAGGGAG	1377	GGTGGCCCTGACAACCTGCCAGAGGAGCTGTACCAACTCATGAGGCTGT
651	CGCGGGATCGTTTTCACTGTGCGTCCGGGACTTCGACCAAGAACAGGGAG	1398	GGTGGCCCTGACAACCTGCCAGAGGAGCTGTACCAACTCATGAGGCTGT
527	AGTGGTGAACAATTACAGATCCGTAATCTGGACAACCGTGGCTTCTAC	1427	GCTGGAAGGAGCGCCAGAGGACCGGCCACCTTTGACTACCTGCGCAGT
701	AGTGGTGAACAATTACAGATCCGTAATCTGGACAACCGTGGCTTCTAC	1448	GCTGGAAGGAGCGCCAGAGGACCGGCCACCTTTGACTACCTGCGCAGT
577	ATCTCCCCTCGAATCACTTTTCCCGGCTGCATGAACCTGGTCCGCAATTA	1477	GTGCTGGAGGACTTCTTACGGCCACAGAGGGCCAGTACCAGCCTACGC
751	ATCTCCCCTCGAATCACTTTTCCCGGCTGCATGAACCTGGTCCGCAATTA	1498	GTGCTGGAGGACTTCTTACGGCCACAGAGGGCCAGTACCAGCCTACGC
627	CACCAAtgcttccagatgggctgtgacacaggttgagccgccccctgccaga	1527	TTGA
801	CACCA-----	1548	TTGA
677	cccagaagccccagaagcgtggtgggaggacgagtgagggttcccagg		
809	-----		

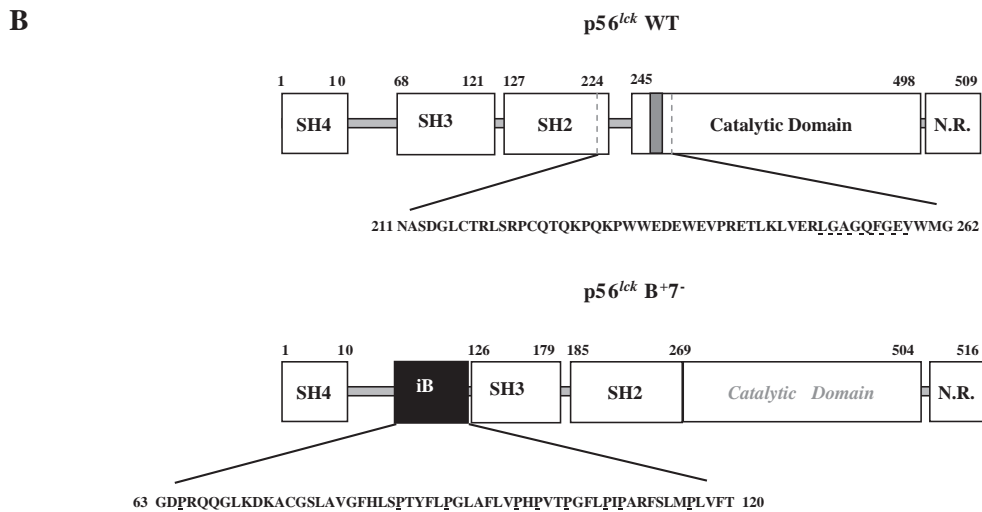


Fig. 1. Sequence and putative structure of the new p56lck protein isoform. (A) Alignment of the coding sequences of the full length 1530 bp (upper) and 1551 bp (lower) LCK cDNAs. Caps/small letter transitions delineate exon 2/intron B/exon 3 boundaries. Underlined residues correspond to the 5' and 3' splice sites, respectively. The possible branch point location for intron B splicing is shown in bold face. G204 and C221 present in the 1551 bp cDNA and missing in Genbank accession no. X14053 are shown in bold underlined letters. (B) Putative structure of the B<sup>+</sup>7<sup>-</sup> isoform versus p56lck full length. Numbering above the schematic representations refers to aminoacid residue positions delineating the p56lck functional domains. Sequence below full length isoform corresponds to the aminoacids encoded by exon 7. Underlined residues correspond to a consensus ATP binding site. Aminoacids below p56lck B<sup>+</sup>7<sup>-</sup> isoform correspond to the residues encoded by intron B 173 bp insert. Proline residues are underlined.

ing intron B was obtained independently using a cDNA template synthesized with the GeneRacer™ protocol (Invitrogen) that selects *bona fide* capped and polyadenylated

mRNAs (data not shown). Finally, database mining identified an EST cloned from brain (Genbank accession no. AL036698) corresponding to the 5'-end of type IIc LCK

mRNA up to exon 5 and containing intron B (data not shown).

### 3.2. Sequencing of the splicing junctions

Intron insertion or exon deletion due to mutations in donor and acceptor splice sites can produce alternative mRNAs with the preservation of the open reading frame (Carstens et al., 1991). Such a variation has already been reported for the LCK-deficient T cell line JCaM1 that contains a single base mutation in the 5' splice site of intron G responsible for the skipping of exon 7 in LCK mRNA encoding an inactive p56lck kinase (Rouer et al., 1999). To determine if the expression of B<sup>+</sup>7<sup>-</sup> LCK mRNA isoform was due to splice site mutations, we screened a panel of 187 LCK genomic DNA sequences determined in a previous study (see (Nervi et al., 2002) for details). No variation was detected neither in intron B nor in intron G sequences, and their boundaries (data not shown).

Comparison of the DNA sequences of many different genes revealed certain similarities at the intron–exon junction. The 5' splice site is usually MAG/GTRAGT whereas the 3' splice site corresponds to the sequence Y<sub>n</sub>NCAG/G (Mount, 1982). Sequences fitting these consensus are present at both exon 2/intron B and intron B/exon 3 junctions (AAG/GTGACC and TTACAG/ACA, respectively) in agreement with a strict GT-AG rule. Similarly, the branch point signal in intron B (TGTAAC, Fig. 1A) fits the consensus YRYRAC and lies 50 nucleotides upstream of the 3' splice site as expected (Harris and Senapathy, 1990). However, the G to C substitution at position 5 of the 5' splicing site might decrease splicing efficiency (Seraphin and Rosbash, 1990). We therefore tested the presence of the B<sup>+</sup>7<sup>-</sup> transcript isoform in cytoplasmic and nuclear fractions from resting and PHA+IL-2-activated peripheral blood T lymphocytes. This variant was systematically detected in the nuclear fraction but its presence was at detection limit by RT-PCR assays in the cytoplasmic fraction and did not allow to exclude minor contamination by nuclear RNAs (data not shown).

Most of these observations suggest that the B<sup>+</sup>7<sup>-</sup> LCK mRNA is not an abnormal transcript but rather results from an alternative splicing and exon skipping mechanism. However, the nuclear retention of this intron-containing mRNA suggests the existence of a quality control that limits its cytoplasmic export under physiological conditions (Sommer and Nehrbass, 2005).

### 3.3. Screening of the intron B variant frequency

To confirm the presence of this particular splice variant in different cell types, we designed a pair of primers located in exon 2 and exon 4 and flanking iB. RT-PCR analysis allowed the detection of two bands (262 and 412 bp), corresponding respectively to the full length and B<sup>+</sup>mRNA-isoforms while excluding a contamination with genomic

DNA which would have generated a larger PCR product containing not only intron B but also intron C. This pair of primers was then used to screen the presence of the intron B isoform in resting or CD3-activated PBMC from 15 individuals and in various cell lines (human NK cells, Jurkat T cells) and tissues (human thymus, liver). Each total RNA sample contained the LCK B<sup>+</sup> mRNA evidencing that all individuals and cell lines tested expressed at least these two isoforms of LCK mRNA as illustrated in a representative experiment (Fig. 2).

Similar RT-PCR analysis performed on established T cell clones or in vitro activated and polarized T cell subsets documented the presence of this isoform without obvious correlation with Th1/Th2 T-cell differentiation (data not shown).

### 3.4. The intron B<sup>+</sup>/exon 7<sup>-</sup> alternate cDNA encodes a new form of LCK protein

In silico translation of the sequence of this new LCK cDNA evidenced a putative open reading frame of 516 amino-acids encompassing the whole 1551 nucleotide cDNA. Interestingly, comparison with mouse LCK genomic DNA revealed that intron B open reading frame was not conserved (data not shown) suggesting that the B<sup>+</sup>7<sup>-</sup> LCK mRNA encoded a human-specific LCK protein isoform. The human B<sup>+</sup>7<sup>-</sup> cDNA was then inserted into the pcDNA3 expression vector and transiently expressed in COS-7 cells. Western blot analysis using the 3A5 mAb recognizing an epitope between aminoacids 54–222 evidenced that the pcDNA-LCK<sup>B<sup>+</sup>7<sup>-</sup></sup>-encoded protein is produced with an apparent molecular weight of 55 kDa in agreement with the prediction of the translation of its open reading frame (Figs. 1B and 3A). Nevertheless, expression level of this isoform was weak in comparison to the wild-type polypeptide encoded by pcDNA-LCK and synthesized in the same

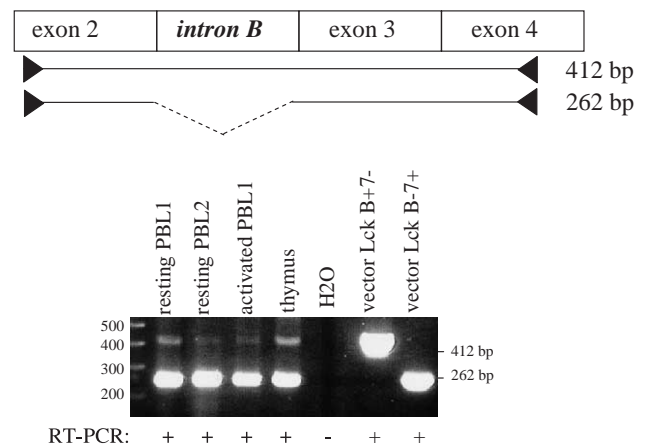


Fig. 2. Detection of intron B mRNA transcripts in normal lymphocytes. PCR analysis using primers in exons 2 and 4 allowed detection of two bands at 262 and 412 bps in total RNA extracted from resting/activated PBLs and thymus samples. pcDNA3 vectors containing LCK variant and full length isoforms were used as controls.

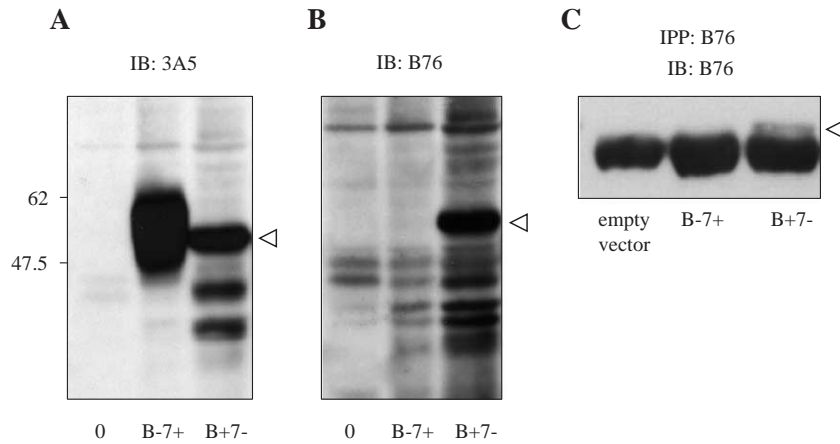


Fig. 3. Expression of the intron B isoform in transfected COS cells. Lysates of COS cells transfected with either full length or B<sup>+</sup>7<sup>-</sup> cDNA were directly immunoblotted with the pan anti-LCK 3A5 mAb (Fig. 3A) or the polyclonal antiserum B76 (Fig. 3B), or immunoprecipitated and then immunoblotted with the polyclonal antiserum B76 (Fig. 3C). Open arrow head: intron B isoform; IB: immunoblot; IP: immunoprecipitation.

conditions. This apparent difference in expression level might be linked to a conformational change precluding detection with this antibody. Alternatively, the B<sup>+</sup>7<sup>-</sup> isoform might be unstable since prolonged exposure of the film also revealed the presence of three LCK-specific degradation products. Such instability has already been suggested for the exon 7-less isoform (Goldman et al., 1998).

The expected size (516 aa) of the B<sup>+</sup>7<sup>-</sup> variant is too close to full length p56lck protein (509 aa) to allow a clear discrimination with the 3A5 antibody. We then raised a polyclonal serum directed against an intron peptide which specifically recognized the B<sup>+</sup>7<sup>-</sup> isoform by western blotting (Fig. 3B) and immunoprecipitation (Fig. 3C) in COS-7 cells. Nevertheless, this serum did not allow detection of endogenous B<sup>+</sup>7<sup>-</sup> LCK protein in any T lymphocyte samples tested (data not shown). This could be explained by the poor quality of the intron-specific antiserum, the low abundance of this variant or the nuclear retention of this alternate form in physiological conditions.

### 3.5. Intron B insertion changes its SH3 interactions

SH3 domains play two major roles in Src-family kinase functions: the recruitment of proteins via the interaction with proline-rich regions, and the regulation of kinase activity through the intramolecular interactions with the SH3/SH2-catalytic domain linker (Palacios and Weiss, 2004). Tec family kinases also use intramolecular interactions between the SH3 and the adjacent proline-rich domains which prevent interaction with their respective ligands Sam68 and Grb-2 (Andreotti et al., 1997). Interestingly, iB insertion adds a proline-rich region next to the SH3 domain, similarly to the Tec family (Fig. 1B). We therefore addressed whether this sequence, like in the case of Tec, affected SH3 binding to previously described molecular partners such as the RNA-binding adaptor Sam68 (Feuillet et al., 2002; Fusaki et al., 1997), the ubiquitine ligase c-Cbl (Fukazawa et al., 1995) and the

cytoskeleton regulator WASp (Banin et al., 1996). Pull-down experiments using GST-SH3 and GST-iB-SH3 were performed on quiescent or TCR-stimulated Jurkat cells or its Lck-deficient mutant line JCam1.6. GST-only constructs failed to precipitate any material (data not shown). Far Western blotting of the precipitated material using a biotinylated Lck-SH3 domain showed partial or complete disappearance of several bands, suggesting that the iB

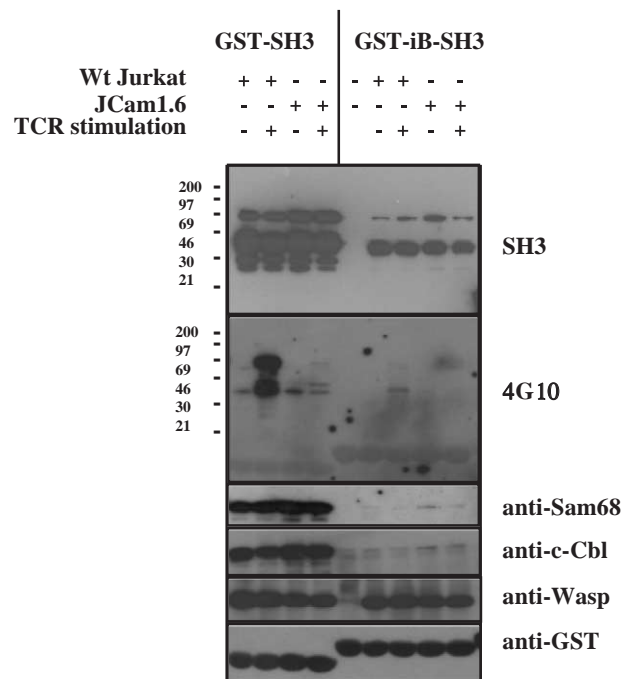


Fig. 4. Intron B sequences alter SH3-mediated molecular interactions. Pull-down experiments using GST-Lck-SH3 and GST-iB-SH3 were performed on quiescent or TCR-stimulated Jurkat T cells or Lck-deficient JCam1.6 cell line. After gel separation and blotting, precipitated polypeptides were sequentially revealed with a biotinylated Lck SH3 domain, anti-phosphotyrosine 4G10, Sam68 (68 kDa), c-Cbl (120 kDa), WASp (53 kDa) and GST specific antibodies, as indicated on panel right side. Molecular weights are indicated (kDa).

modifies but does not abolish SH3 binding capability (Fig. 4, top panel). Some of these SH3 binding proteins are tyrosine phosphorylated upon Jurkat T cell activation, as shown by Western blot using 4G10 antibody. Further, their tyrosine phosphorylation was dependent on p56lck expression as they were absent in Lck-deficient JCaml.6 cell extracts. Interestingly, GST-iB-SH3 protein did not bind to any tyrosine phosphorylated bands in TCR stimulated Jurkat cell lysates. These results show that iB insertion might prevent binding of proteins to SH3 domain, including proteins phosphorylated during TCR stimulation. Several downstream effector of the src kinases bind via a combined interaction with SH3 and SH2 domains (Sam68, *c-Cbl*) whereas others preferentially interact mostly via the SH3 domain (WASp). Some of these proteins and phosphoproteins have apparent molecular weights of 70 and 120 Kd; this is compatible with the size and CD3 induced tyrosine phosphorylation characteristic of Sam68 and *c-Cbl*. Interestingly, using specific antibodies we could show that the interactions of SH3 domain with Sam68 and *c-Cbl* were abrogated in the presence of iB. The SH3 seems to retain functionality since iB does not prevent its association with WASp. These data suggest that iB impairs some but not all SH3-dependent interactions. Since the full length p56lck is negatively regulated after SH3 dependent recruitment of *c-Cbl* (Hawash et al., 2002; Rao et al., 2002), hence, iB containing Lck could potentially escape *c-Cbl*-mediated downregulation of kinase recruitment to the immune synapse whereas WASP-mediated effects on cytoskeleton reorganization would remain unaffected.

### 3.6. Conclusions

Altogether our results indicate that the B<sup>+</sup>7<sup>-</sup> LCK mRNA corresponds to a rare transcript that results from a physiological alternate splicing mechanism. Its preferential retention in the nucleus is most probably due to a mRNA quality control that limits nuclear export (Sommer and Nehrbass, 2005). Alternative transcripts of the *LCK* gene are present in diverse pathological conditions in human, including several malignancies (Sartor et al., 1989) and a case of an infant with Severe Combined Immunodeficiency (SCID) expressing the 7-less variant (Goldman et al., 1998). A large screening of various pathologies and more specifically those involving T cell dysfunctions using our B<sup>+</sup>7<sup>-</sup> LCK-specific immune serum might allow the identification of a pathological situation where the B<sup>+</sup>7<sup>-</sup> isoform is functionally expressed.

### Acknowledgement

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Ministère de la Santé (Projet Hospitalier de Recherche

Clinique) and European Union (Euro-Thymaïde, LSHB-CT-2003-503410).

### References

- Andreotti, A.H., Bunnell, S.C., Feng, S., Berg, L.J., Schreiber, S.L., 1997. Regulatory intramolecular association in a tyrosine kinase of the Tec family. *Nature* 385, 93–97.
- Banin, S., Truong, O., Katz, D.R., Waterfield, M.D., Brickell, P.M., Gout, I., 1996. Wiskott–Aldrich syndrome protein (WASp) is a binding partner for *c-Src* family protein-tyrosine kinases. *Curr. Biol.* 6, 981–988.
- Carstens, R.P., Fenton, W.A., Rosenberg, L.R., 1991. Identification of RNA splicing errors resulting in human ornithine transcarbamylase deficiency. *Am. J. Hum. Genet.* 48, 1105–1114.
- Feuillet, V., et al., 2002. The distinct capacity of Fyn and Lck to phosphorylate Sam68 in T cells is essentially governed by SH3/SH2-catalytic domain linker interactions. *Oncogene* 21, 7205–7213.
- Fukazawa, T., et al., 1995. The SH3 domain-binding T cell tyrosyl phosphoprotein p120. Demonstration of its identity with the *c-cbl* protooncogene product and in vivo complexes with Fyn, Grb2, and phosphatidylinositol 3-kinase. *J. Biol. Chem.* 270, 19141–19150.
- Fusaki, N., Iwamatsu, A., Iwashima, M., Fujisawa, J., 1997. Interaction between Sam68 and Src family tyrosine kinases, Fyn and Lck, in T cell receptor signaling. *J. Biol. Chem.* 272, 6214–6219.
- Germani, A., Malherbe, S., Rouer, E., 2003. The exon 7-spliced Lck isoform in T lymphocytes: a potential regulator of p56lck signaling pathways. *Biochem. Biophys. Res. Commun.* 301, 680–685.
- Goldman, F.D., et al., 1998. Defective expression of p56lck in an infant with severe combined immunodeficiency. *J. Clin. Invest.* 102, 421–429.
- Harris, N.L., Senapathy, P., 1990. Distribution and consensus of branch point signals in eukaryotic genes: a computerized statistical analysis. *Nucleic Acids Res.* 18, 3015–3019.
- Hawash, I.Y., Kesavan, K.P., Magee, A.I., Geahlen, R.L., Harrison, M.L., 2002. The Lck SH3 domain negatively regulates localization to lipid rafts through an interaction with *c-Cbl*. *J. Biol. Chem.* 277, 5683–5691.
- Huse, M., Eck, M.J., Harrison, S.C., 1998. A Zn<sup>2+</sup> ion links the cytoplasmic tail of CD4 and the N-terminal region of Lck. *J. Biol. Chem.* 273, 18729–18733.
- Majolini, M.B., Boncristiano, M., Baldari, C.T., 1999. Dysregulation of the protein tyrosine kinase LCK in lymphoproliferative disorders and in other neoplasias. *Leuk. Lymphoma* 35, 245–254.
- Modrek, B., Resch, A., Grasso, C., Lee, C., 2001. Genome-wide detection of alternative splicing in expressed sequences of human genes. *Nucleic Acids Res.* 29, 2850–2859.
- Mount, S.M., 1982. A catalogue of splice junction sequences. *Nucleic Acids Res.* 10, 459–472.
- Nervi, S., et al., 2000. Specific deficiency of p56lck expression in T lymphocytes from type 1 diabetic patients. *J. Immunol.* 165, 5874–5883.
- Nervi, S., et al., 2002. No association between *lck* gene polymorphisms and protein level in type 1 diabetes. *Diabetes* 51, 3326–3330.
- Nunes, J., et al., 1993. Signalling through CD28 T-cell activation pathway involves an inositolphospholipid-specific phospholipase C activity. *Biochem. J.* 293, 835–842.
- Palacios, E.H., Weiss, A., 2004. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene* 23, 7990–8000.
- Patel, V.P., Moran, M., Low, T.A., Miceli, M.C., 2001. A molecular framework for two-step T cell signaling: Lck Src homology 3 mutations discriminate distinctly regulated lipid raft reorganization events. *J. Immunol.* 166, 754–764.
- Penninger, J.M., Wallace, V.A., Kishihara, K., Mak, T.W., 1993. The role of p56lck and p59fyn tyrosine kinases and CD45 protein tyrosine

- phosphatase in T-cell development and clonal selection. *Immunol. Rev.* 135, 183–214.
- Rao, N., et al., 2002. Negative regulation of Lck by Cbl ubiquitin ligase. *Proc. Natl. Acad. Sci. U. S. A.* 99, 3794–3799.
- Rouer, E., Benarous, R., 1992. Alternative splicing in the human *lck* gene leads to the deletion of exon 1' and results in a new type II lck transcript. *Oncogene* 7, 2535–2538.
- Rouer, E., Brule, F., Benarous, R., 1999. A single base mutation in the 5' splice site of intron 7 of the *lck* gene is responsible for the deletion of exon 7 in lck mRNA of the JCaM1 cell line. *Oncogene* 18, 4262–4268.
- Sartor, O., Gregory, F.S., Templeton, N.S., Pawar, S., Perlmutter, R.M., Rosen, N., 1989. Selective expression of alternative lck mRNAs in human malignant cell lines. *Mol. Cell. Biol.* 9, 2983–2988.
- Seraphin, B., Rosbash, M., 1990. Exon mutations uncouple 5' splice site selection from U1 snRNA pairing. *Cell* 63, 619–629.
- Sommer, P., Nehrbass, U., 2005. Quality control of messenger ribonucleo-protein particles in the nucleus and at the pore. *Curr. Opin. Cell Biol.* 17, 294–301.
- Straus, D.B., Weiss, A., 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell* 70, 585–593.
- Takadera, T., et al., 1989. Structure of the two promoters of the human *lck* gene: differential accumulation of two classes of lck transcripts in T cells. *Mol. Cell. Biol.* 9, 2173–2180.
- Togni, M., et al., 2004. The role of adaptor proteins in lymphocyte activation. *Mol. Immunol.* 41, 615–630.
- Yamashita, M., Hashimoto, K., Kimura, M., Kubo, M., Tada, T., Nakayama, T., 1998. Requirement for p56(lck) tyrosine kinase activation in Th subset differentiation. *Int. Immunol.* 10, 577–591.