The Journal of Immunology

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J Immunol 2000; 164:5199-5206; ;
doi: 10.4049/jimmunol.164.10.5199
http://www.jimmunol.org/content/164/10/5199

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cloning and Characterization of Human Lnk, an Adaptor Protein with Pleckstrin Homology and Src Homology 2 Domains that Can Inhibit T Cell Activation[1]

Yijin Li,* Xiaoqing He,* Josephine Schembri-King,† Scott Jakes,† and Jun Hayashi[2]*

Lnk was originally cloned from a rat lymph node cDNA library and shown to participate in T cell signaling. Human Lnk (hLnk) was cloned by screening a Jurkat cell cDNA library. hLnk has a calculated molecular mass of 63 kDa, and its deduced amino acid sequence indicates the presence of an N-terminal proline-rich region, a pleckstrin homology domain, and a Src homology 2 domain. When expressed in COS cells, hLnk migrates with an apparent molecular mass of 75 kDa. Confocal fluorescence microscope analysis indicates that in COS cells transfected with an expression vector encoding a chimeric Lnk-green fluorescent protein, hLnk is found at the juxtanuclear compartment and also appears to be localized at the plasma membrane. Lnk is tyrosine-phosphorylated by p56^{lck}. Following phosphorylation, p56^{lck} binds to tyrosine-phosphorylated hLnk through its Src homology 2 domain. In COS cells cotransfected with hLnk, p56^{lck}, and CD8-ζ, hLnk associated with tyrosine-phosphorylated TCR ζ-chain through its Src homology 2 domain. The overexpression of Lnk in Jurkat cells led to an inhibition of anti-CD3 mediated NF-AT-Luc activation. Our study reveals a potentially new mechanism of T cell-negative regulation. ***The Journal of Immunology,*** **2000, 164: 5199–5206.**

The engagement of TCR with peptide Ag-bound MHC molecule initiates multiple intracellular cascades of biochemical events leading to enhanced gene transcription, cell proliferation, and differentiation (reviewed in Refs. 1–3). TCR is a multisubunit complex consisting of a variable $\alpha\beta$ heterodimer, which recognizes the Ag-MHC complex, and six invariant chains (CD3γ, CD3δ, two copies of CD3ϵ, and a ζ homodimer) that play an important role in mediating the TCR signal transduction (4). The cytoplasmic domains of CD3 and ζ contain the immunoreceptor tyrosine-based activation motif (ITAM)[3] (5), consisting of two tandem YXXL/I motifs separated by 7–8 aa. The src family protein tyrosine kinases (PTK) p56^{lck} and p59^{fyn}, activated by TCR ligation, can phosphorylate tyrosine residues within ITAMs. The doubly tyrosine-phosphorylated ITAMs serve as docking sites for the Syk family PTK ζ-associated protein-70 (ZAP-70) to help its phosphorylation and activation by p56^{lck} or p59^{fyn} (6). These PTKs phosphorylate additional substrates, leading to downstream events including activation of phospholipase Cγ-1 (PLCγ-1), Ras, and phosphatidylinositol 3-kinase (PI3-kinase). All of these events contribute to the activation and binding of transcription factors, such as NF-AT and AP-1, to the promoter regions of specific genes such as IL-2, the production of which is a prelude for clonal expansion and lymphokine secretion.

Extensive investigation has been focused on resolving how the early tyrosine-phosphorylation events are coupled to downstream biochemical cascades. It has been suggested that the transduction mechanism involves assembly of multimolecular signaling complexes at and near the TCR (2). A group of molecules called adaptor proteins, which possess no intrinsic enzymatic function but can mediate protein-protein interactions, have been characterized and shown to play a crucial role in this process (reviewed in Refs. 7 and 8). For example, the transmembrane protein linker for activation of T cells (LAT) can serve as a substrate of ZAP-70 upon TCR stimulation and act as a central adaptor by direct association with Grb-2 (through Grb2's Src homology 2 (SH2) domain) and PLCγ-1 (9, 10). Other proteins also found in the complex include Vav, Cbl, the p85 subunit of PI3-kinase, and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), and their interaction with LAT may occur in an indirect manner. The interaction of LAT with other signaling molecules has been demonstrated to be necessary for T cell activation (11). Another ZAP-70 substrate SLP-76, which contains a proline-rich region, an SH2 domain and multiple tyrosine-phosphorylation sites, can also help to form a multimolecular complex by binding to Grb-2 and Vav (12, 13). A mutant T cell line lacking SLP-76 expression shows impaired coupling of the TCR to the Ras pathway and reduced PLCγ-1 tyrosine phosphorylation, which can be restored by the reexpression of SLP-76 (14).

Adaptor proteins can also play a role as negative regulators of signaling. Cbl, a widely expressed adaptor molecule with a phosphotyrosine-binding domain, a proline-rich region, and multiple tyrosine-phosphorylation sites, can interact with Vav, Crk, Grb2, PI3-kinase, p59^{fyn}, ZAP-70, and Syk (15–20). Recent evidence suggests that Cbl acts as a negative regulator in lymphocytes (21). Overexpression of Cbl in Jurkat T cells inhibits AP-1 activity after TCR ligation (20). Cbl has also been found to negatively regulate Syk tyrosine kinase in mast cells (22).

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Received for publication June 10, 1999. Accepted for publication March 7, 2000.

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[1] This work was supported by Council for Tobacco Research Grant 4332 and by Boehringer Ingelheim.

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[3] Abbreviations used in this paper: ITAM, immunoreceptor tyrosine-based activation motif; PTK, protein tyrosine kinase; GFP, green fluorescent protein; ZAP-70, ζ-associated protein of 70 kDa; PLCγ-1, phospholipase Cγ-1; PI3-kinase, phosphatidylinositol 3-kinase; LAT, linker for activation of T cells; SH2, Src homology 2; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; hLnk, human Lnk; PH, pleckstrin homology; APS, adaptor molecule containing PH and SH2 domains.

0022-1767/00/$02.00

Here we report that another adaptor protein, Lnk, can also play a role as a negative regulator in T cells. Lnk was originally cloned from a rat lymph node cDNA library (23). The deduced amino acid sequence of Lnk reveals the presence of an SH2 domain and a putative tyrosine-phosphorylation site. Lnk mRNA is preferentially expressed in lymph node and spleen lymphocytes. Mouse Lnk cDNA has also been cloned, and recombinant mouse Lnk protein was able to serve as a substrate of $p56^{lck}$ and ZAP-70 (24), although it was only weakly tyrosine-phosphorylated upon TCR stimulation. We recently cloned human Lnk (hLnk) by screening a Jurkat cell cDNA library and found that compared with the reported rat and mouse sequences it has an extended coding region at the 5′ end resulting in an additional 267 amino-terminal amino acids. The additional coding sequence reveals a pleckstrin homology (PH) domain and a proline-rich region. We show here that hLnk can bind to tyrosine-phosphorylated ζ-chain ITAM residues via its SH2 domain. $p56^{lck}$ phosphorylates hLnk and binds to the tyrosine-phosphorylated hLnk through its SH2 domain. In addition, we have found that when overexpressed in Jurkat cells, hLnk inhibits anti-CD3-mediated activation of NF-AT transcription activity.

Materials and Methods

Abs

Rabbit Ab generated against His-tagged rat Lnk, which also recognizes human Lnk, has been previously described (23). Mouse anti-$p56^{lck}$ and rabbit anti-CD8 were obtained from Santa Cruz Laboratories (Santa Cruz, CA). Anti-phosphotyrosine mAb (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). OKT3 ascites used for stimulation of Jurkat cells was generated from hybridoma acquired from the American Type Culture Collection (Manassas, VA).

Constructs

The cDNA of constitutively active $p56^{lck}$ (LckY505F) (25), a gift from Dr. Kristin M. Abraham (University of Maryland School of Medicine, Baltimore, MD), was cloned into pCDNA3 (Invitrogen, Carlsbad, CA). The kinase inactive form of $p56^{lck}$ (LckR273) and another variant carrying complete deletion of the SH2 domain (LckΔSH2) (25) were kind gifts from Dr. André Veillette (McGill University, Montréal, Canada). The Syk expression vector was kindly provided by Dr. Edward Clark (University of Washington, Seattle, WA). The chimeric receptor CD8-ζ, originally described by Dr. Arthur Weiss (University of California, San Fransisco, CA) (26), was a gift form Dr. Cox Terhorst (Beth Israel Hospital, Harvard Medical School, Cambridge, MA) and inserted into pCDNA3.1$^-$ (Invitrogen). The reporter construct NF-AT-Luc was kindly provided by Dr. Gerald Crabtree (Stanford University Medical School, Stanford, CA).

Conjugation of phospho-peptides and binding assay

Five tyrosine-phosphorylated peptides representing sequences of half ITAMs within TCR ζ were obtained. The five peptides were: NQLpYNELNL (ζ ITAM1 amino), RREEpYDVLDK (ζ ITAM1 carboxyl), EGLpYNELQK (ζ ITAM2 amino), (Nle)AEApYSEIG(Nle) (ζ ITAM2 carboxyl), and HDGLpYQGLST (ζ ITAM3 amino). The residue norleucine (Nle) was substituted for methionine in the appropriate peptides to prevent oxidation. Next, 100 μg of each peptide was dissolved in 100 μl of coupling buffer (0.1 M $NaHCO_3$ plus 0.5 M NaCl, pH 8.3) and added to 750 μl of normal human serum-activated Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Piscataway, NJ). Coupling was done at room temperature for 3 h. Nonreacted groups on the gel were blocked with 1 M Tris, pH 7.4. The gel was washed alternatively with 0.1 M $NaHCO_3$ plus 0.5 M NaCl, pH 8.0, and 0.1 M Na acetate plus 0.5 M NaCl, pH 4.0. Then, 30 μl of peptide conjugated beads were added to the lysate of COS cells transfected with Lnk or Lnk(R392K) and incubated at 4°C for 3 h. Unconjugated beads with their reaction groups blocked were used as negative control. After washing three times with PBS containing 0.1% Nonidet P-40, beads were boiled together with 30 μl of 2× SDS sample buffer. The supernatant was used for Western blot analysis with anti-Lnk Ab.

cDNA cloning of hLnk

Primers corresponding to various regions of rat Lnk cDNA sequence were synthesized. PCR was conducted using Jurkat cDNA prepared with random primer. Partial hLnk sequence (~600 bp) was obtained with the forward primer (5′-CATGCTCGAGATCACTTCCTGTCCTGCTAC-3′) and the reverse primer (5′-GATGGATCCGGTGTACTGGTTGTCAATGG-3′) and was cloned into SK vector at sites of *Bam*HI and *Xho*I. The insert was regenerated, labeled with [α-^{32}P]dATP (ICN Pharmaceuticals, Costa Mesa, CA) using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN), and used as a probe to screen a λgt11 Jurkat library (Clontech, Palo Alto, CA). Phage DNA from positive plaques was purified with Nucleobond AX (The Nest Group, Southboro, MA). Sequence analysis was done by dideoxy chain terminator and automated fluorescent DNA sequencer.

Northern blot

Total RNA from Jurkat cells was isolated using TRIZOL reagent (Life Technologies, Gaithersburg, MD). Poly(A)$^+$ RNA was isolated using Dynabeads Oligo$(dT)_{25}$ (Dynal, Lake Success, NY). About 1.5 μg of Poly(A)$^+$ RNA was electrophoretically separated on a 1% agarose gel and transferred onto Zeta-Probe Blotting Membrane (Bio-Rad, Hercules, CA). The membrane was hybridized with the same ^{32}P-labeled cDNA fragment that was used in the library screening. The size of the mRNA was measured using an RNA ladder as standard (Life Technologies).

Construction of hLnk expression vector and mutagenesis

The full coding region plus 17 bp of noncoding sequence at the 5′ end of hLnk cDNA was generated by PCR and inserted into pCDNA3. The same sequence without the stop codon was inserted into pEGFP-N1 (Clontech) to express a Lnk-green fluorescent protein (GFP) chimeric protein. The point mutation in the SH2 domain of Lnk(R392K) was generated using a method previously described (27). Briefly, two mutagenic primers were designed to encode the R→K mutation as well as a silent mutation to introduce a flanking *Sac*II restriction site. Two separated PCR were conducted. An upstream primer and the antisense mutagenic primer (5′-TTTTTTCCGCG GCGCGTCTCGCTCTGCTTCACCAGGAACAC-3′) were used in the PCR to generate the upstream fragment, while the sense mutagenic primer (5′-TTTTTTCCGCGGGGAATACGTGCTCACTTT-3′) and a downstream primer were used in another PCR to generate the downstream fragment. The two fragments were purified and ligated after *Sac*II digestion and then ligated into the pCDNA3 expression vector. The mutation was confirmed by sequencing.

Confocal microscopy

COS-7 cells transfected with the Lnk-GFP construct were grown on sterile glass coverslips. Then, 48 h after transfection, cells were viewed using a Zeiss laser scanning 410 confocal microscope (Zeiss, Oberkochen, Germany). The GFP molecules were excited using a 488-nm laser and imaged using a 515- to 540-nm bandpass filter.

COS cell transfection, immunoprecipitation, and Western blotting

COS-7 cells were cultured in DME-F12 medium containing 10% iron supplemented calf serum. Cells were transfected with 10 μg each of expression vector containing either Lnk or Lnk(R392K) with or without Lck(Y505F), Lck(R273), Lck(ΔSH2), or Syk using the DEAE-dextran method (28). Some of these cells were cotransfected with 10 μg of CD8-ζ together with or without 10 μg of Lck(Y505F). Total amount of DNA was equalized with pCDNA3 empty vector. Then, 48 h after the transfection, cells were lysed in RIPA buffer containing 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 1 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride hydrochloride. Immunoprecipitation was conducted using 2 μg of rabbit anti-CD8 Ab followed by protein A-Sepharose 4 Fast Flow (Pharmacia). Immunoprecipitates were resolved on a SDS/10% PAGE under reducing conditions and transferred to Immobilon-P membranes (Millipore, Bedford, MA). After blocking with 5% skim milk in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20), blots were probed with primary Abs (anti-Lnk, anti-$p56^{lck}$, anti-phosphotyrosine Abs) followed by incubation with HRP-conjugated secondary Abs (Cappel, Durham, NC) and developed using enhanced chemiluminescent substrate (Pierce, Rockford, IL). Western blot analyses were also conducted using aliquots of cell lysates from each transfection with anti-Lnk, anti-CD8, and anti-$p56^{lck}$ to detect the expression of each construct.

Jurkat cell transfection, stimulation, and luciferase assay

Jurkat cells were grown in RPMI 1640 supplemented with 10% FBS. Transient transfection was performed by electroporating (at 250V and 960 μF)

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MNGPALQPSSPSSAPSASPAAAPRGWSEFCELHAVAAARELARQYWLFAREHPQHAPLRA  60
ELVSLQFTDLFQRYFCREVRDGRAPGRDYRDTGRGPPAKAEASPEPGPGPAAPGLPKARS 120
SEELAPPRPPGPCSFQHFRRSLRHIFRRRSAGELPAAHTAAAPGTPGEAAETPARPGLAK 180
KFLPWSLAREPPPEALKEAVLRYSLADEASMDSGARWQRGRLALRRAPGPDGPDRVLELF 240
DPPKSSRPKLQAACSSIQEVRWCTRLEMPDNLYTFVLKVKDRTDIIFEVGDEQQLNSWMA 300
ELSECTGRGLESTEAEMHIPSALEPSTSSSPRGSTDSLNQGASPGGLLDPACQKTDHFLS 360
CYPWFHGPISRVKAAQLVQLQGPDAHGVFLVRQSETRRGEYVLTFNFQGIAKHLRLSLTE 420
RGQCRVQHLHFPSVVDMLHHFQRSPIPLECGAACDVRLSSYVVVVSQPPGSCNTVLFPFS 480
LPHWDSESLPHWGSELGLPHLSSSGCPRGLSPEGLPGRSSPPEQIFHLVPSPEELANSLQ 540
HLEHEPVNRARDSDYEMDSSSRSHLRAIDNQYTPL*
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FIGURE 1. Amino acid sequence of hLnk. The three proline-rich sequences within the N-terminal region are underlined. The PH domain is double-underlined, and the SH2 domain is dash-underlined.

10^7 cells in 0.4 ml RPMI 1640 in a Gene Pulse cuvette (Bio-Rad) with 10 μg of Lnk or the Lnk(R392K) mutant together with 10 μg of NF-AT-Luc. Transfected cells were cultured in 10 ml of growth medium for 24 h and then seeded at 10^6 cells/ml into 24-well plates. Cells were either left unstimulated or stimulated with OKT3 ascites at 1:500 dilution or with PMA (10ng/ml) plus ionomycin (1 μg/ml) for 8 h. Luciferase assays were performed according to the manufacturer's protocol (Promega, Madison, WI). Luciferase activities, determined using a Microplate Luminometer (Packard, Meriden, CT), were shown as fold increases compared with levels from control unstimulated cells. In some experiments, the relative luciferase activities were normalized against the expressed protein levels of hLnk and hLnk(R392K). The levels of these proteins expressed in the transfected cells were determined by Western blot analyses using anti-hLnk Ab and [^{125}I]-goat anti-rabbit Ab. The relative radiolabels associated with hLnk and hLnk(R392K) were measured using phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Results

Cloning of hLnk cDNA

A partial hLnk sequence (~600 bp) was obtained by PCR using cDNA from Jurkat cells and primers designed from the rat Lnk sequence. The partial hLnk sequence was used as a probe to screen a λgt11 Jurkat cDNA library. Five positive plaques were isolated from 360,000 plaques screened. After combining all the sequence information, a signal open reading frame encoding a protein with a predicted molecular mass of 63 kDa was obtained. The hLnk cDNA (GenBank accession no. NM005475) shows high homology to both rat and mouse Lnk with 73% amino acid sequence identity. However, we found that hLnk has a much larger 5′-coding region, indicating that the previously reported rat and mouse sequences were not complete. To confirm the new 5′-coding region sequence, PCR was conducted using cDNA from other human cell lines including Daudi, Raji, IM-9, and HepG2. All of the sequences obtained from the various cell lines were 100% identical with the one from the Jurkat cDNA library. The full coding region of Lnk contains an N-terminal proline-rich region, a PH domain, and a SH2 domain (Fig. 1). It is also consistent with the sequence recently submitted to the GenBank by Bartholomew et al. (GenBank accession no. AJ012793).

hLnk is structurally related to two other adaptor molecules, APS and SH2-B

The PH and SH2 domains of hLnk show similarity to those of APS (adaptor molecule containing PH and SH2 domains) and SH2-B (Fig. 2, *A* and *B*). APS was cloned from a human B cell cDNA library (29), and SH2-B was cloned from a rat mast cell cDNA library (30). The PH domain of hLnk is 35% identical with that of APS and 29% to SH2-B, and the SH2 domain shares 65% and 68%

A

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rSH2B   AAGLTSGGGGQPQWQKCRLLLRSEGEGGGGSR-LEFFVPPKASRPRLSIPCS
hLnk    ADEASMDSG--ARWQRGRLALRRAPGPDGPDRVLELFDPPKSSRPKLQAACS
hAPS    ADDAAAGSGGSAQWQKCRLLLRRAV-AEERFR-LEFFVPPKASRPKVSIPLS

rSH2B   TITDVRTATALEMPDRENTFVVKVEGPSEYILETTDALHVKAWVSDIQECLS
hLnk    SIQEVRWCTRLEMPDNLYTFVLKVKDRTDIIFEVGDEQQLNSWMAELSECTG
hAPS    AIIEVRTTMPLEMPEKDNTFVLKVENGAEYILETTDSLQKHSWVADIQGCVD
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B

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rSH2B   WFHGMLSRLKAAQLVLEGGTSSHGVFLVRQSETRRGEYVLTFNFQGKAKH
hLnk    WFHGPISRVKAAQLVQLQGPDAHGVFLVRQSETRRGEYVLTFNFQGIAKH
hAPS    WFHGTLSRVKAAQLVLAGGPRNHGLFVIRQSETRPGEYVLTFNFQGKAKH

rSH2B   LRLSLNEEGQCRVQHLWFQSIFDMLEHFRVHPIPLESGGSSDVVLVSYV
hLnk    LRLSLTERGQCRVQHLHFPSVVDMLHHFQRSPIPLECGAACDVRLSSYV
hAPS    LRLSLNGHGQCHVQHLWFQSVLDMLRHFHTHPIPLESGGSADITLRSYV
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FIGURE 2. Aliments of the PH domain (*A*) and SH2 domain (*B*) of hLnk with those of rat SH2B and human APS. The identical amino acid residues are indicated with vertical bars.

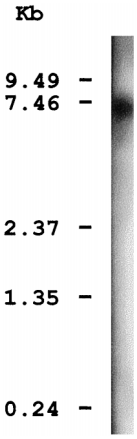

FIGURE 3. Northern blot of hLnk. About 1.5 μg of poly(A)$^+$ RNA from Jurkat cells was hybridized with a [^{32}P]-labeled hLnk cDNA fragment as a probe. hLnk cDNA probe hybridized to mRNA with an apparent size of 6.8 kb.

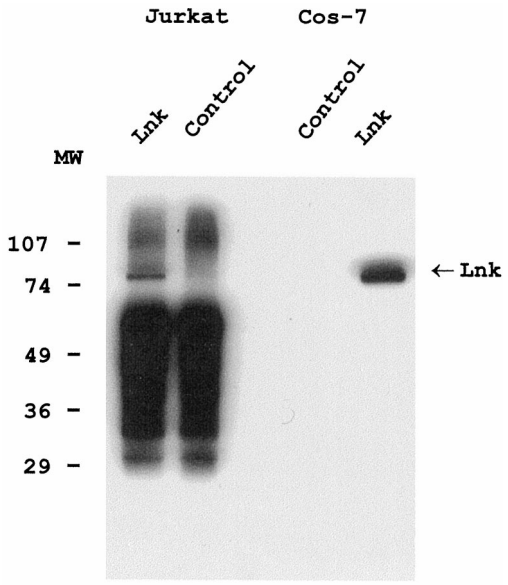

FIGURE 4. Expression of hLnk in Jurkat and COS cells. Jurkat and COS cells were transiently transfected either with expression vector containing hLnk or with control empty vector. hLnk was immunoprecipitated from Jurkat lysates. Western blot analyses were conducted using immunoprecipitates from Jurkat and cell lysates from COS cells using anti-Lnk Ab.

identity to APS and SH2-B, respectively. Each of these three proteins has a proline-rich region at the N terminus. It has been suggested that these three molecules form a new family of adaptor protein (29).

Expression of hLnk

The hLnk cDNA probe hybridized to a 6.8-kb mRNA in Northern blot analysis using poly(A) RNA from Jurkat cells. No signal was observed even after long exposure when 20 μg of total RNA was used, indicating the message level of mRNA was very low. The message was detected when 1.5 μg of poly(A)$^+$ RNA was used in the assay followed by overnight exposure (Fig. 3). Similar results were also obtained from other human cell lines such as Daudi, Raji, IM-9, and HepG2 (data not shown). The endogenous hLnk protein could not be detected in Jurkat cells, presumably due to low level of expression. When COS-7 and Jurkat cells were transfected with an expression vector containing the hLnk cDNA, anti-Lnk Ab detected a band with an apparent molecular mass of 75 kDa, which was absent from the empty vector control cell extract (Fig. 4).

Cellular distribution of hLnk

To visualize the cellular localization of hLnk, COS-7 cells were transfected with expression vector containing a chimeric protein of Lnk-GFP. In the transfected cells, the majority of Lnk-GFP was found around juxtanuclear region while some appeared to be localized to the plasma membrane. In the control cells transfected with GFP only, GFP was diffusely expressed throughout the cytosol (Fig. 5).

hLnk can be tyrosine-phosphorylated by p56^{lck} but not by Syk

COS-7 cells were cotransfected with expression vectors containing hLnk, constitutively active p56^{lck} kinase, Lck(Y505F), or with Syk, which belongs to the same family of PTK as ZAP-70. As shown in Fig. 6, hLnk can be strongly phosphorylated by Lck(Y505F), while Syk had no effect on its phosphorylation. The expressed Syk was active in the assay as judged by its ability of autophosphorylation (data not shown). hLnk can also be coimmunoprecipitated with Lck(Y505F) (Fig. 7*A*). A point mutation (R392K) within the highly conserved FLVERS motif (31) in the SH2 domain of hLnk did not affect its association with Lck(Y505F). However, when Lnk was cotransfected with a kinase inactive form of p56^{lck} (LckR273) or with p56^{lck} completely lacking the SH2 domain (LckΔSH2), neither of the two mutated Lck proteins could be coimmunoprecipitated with Lnk (Fig. 7*B*). These results indicate that the interaction of hLnk and p56^{lck} requires hLnk to be tyrosine-phosphorylated by p56^{lck} and that the SH2 domain of p56^{lck} interacts with the phosphotyrosine residue of hLnk.

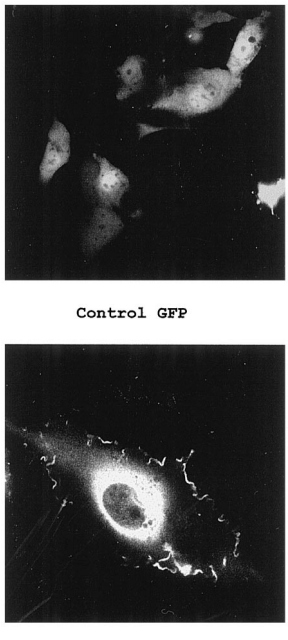

FIGURE 5. Cellular distribution of hLnk. COS cells were transiently transfected with expression vector containing hLnk-GFP chimeric protein or with GFP only as a control. Then, 48 h after transfection, cells were viewed using a confocal microscope.

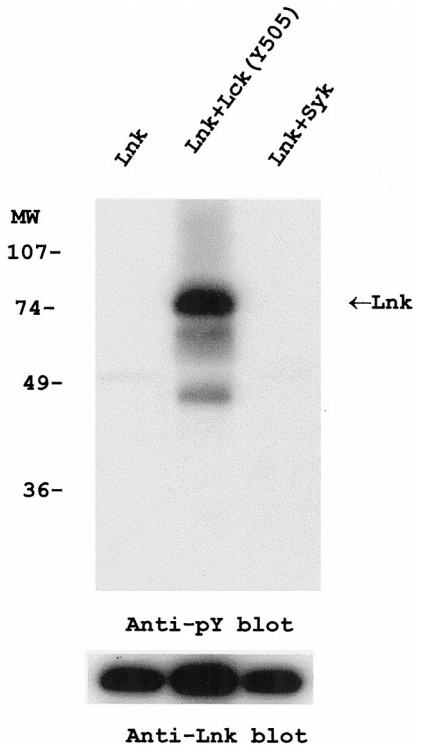

FIGURE 6. Tyrosine-phosphorylation of hLnk. COS cells were cotransfected with expression vectors containing hLnk, Lck(Y505F), and Syk. Cell lysates were immunoprecipitated with anti-Lnk Ab and blotted with anti-phosphotyrosine Ab. An aliquot of cell lysate from each transfected cells was blotted with anti-Lnk Ab to show the equal expression of Lnk.

hLnk can bind to the tyrosine-phosphorylated TCR ζ-chain

Because SH2-B, the protein that is structurally related to Lnk, can bind to the ITAM sequences of IgE receptor FcεRIγ (30), we investigated the possibility that hLnk may be able to associate with the TCR ζ-chain. COS cells were cotransfected with expression vectors containing hLnk and a chimeric receptor CD8-ζ with or without Lck(Y505F). The chimeric receptor CD8-ζ has a cytoplasmic domain of TCR ζ-chain fused to the transmembrane and extracellular domains of CD8. Lck(Y505F) can tyrosine-phosphorylate the ITAMs in the ζ-chain. As shown in Fig. 8, hLnk was able to bind to the ζ-chain in a p56^{lck}-dependent manner. This association was not detected with hLnk containing a R392K point mutation within the SH2 domain. The cytoplasmic domain of ζ-chain contains three ITAM sequences, numbered ITAM1 through 3 in the order of its relative position from N to C terminal. Within each ITAM, there are two tyrosine residues that become phosphorylated by p56^{lck}. To determine whether hLnk can actually bind to the phosphotyrosine residues of the ζ-chain ITAM, nona- or decapeptides containing phosphotyrosine corresponding to each ITAMs were constructed having phosphotyrosine at the center, except for the ITAM3 carboxyl end tyrosine residue. These peptides designated as ITAM1-N, ITAM1-C, ITAM2-N, ITAM2-C, and ITAM3-N were conjugated to agarose beads and incubated with lysate of COS cells transfected with hLnk. As shown in Fig. 9, *top panel*, hLnk preferentially bound to the ITAM1-N and ITAM2-N residues. The association of hLnk with ITAM1-C, ITAM2-C, and ITAM3-N was minimal if any. When agarose beads conjugated with ITAM1-N and ITAM2-N were incubated with lysate of COS cells transfected with hLnk (R392K), its association was significantly diminished, indicating that the association of hLnk with ITAM1-N and ITAM2-N was SH2 domain dependent (Fig. 9, *mid-*

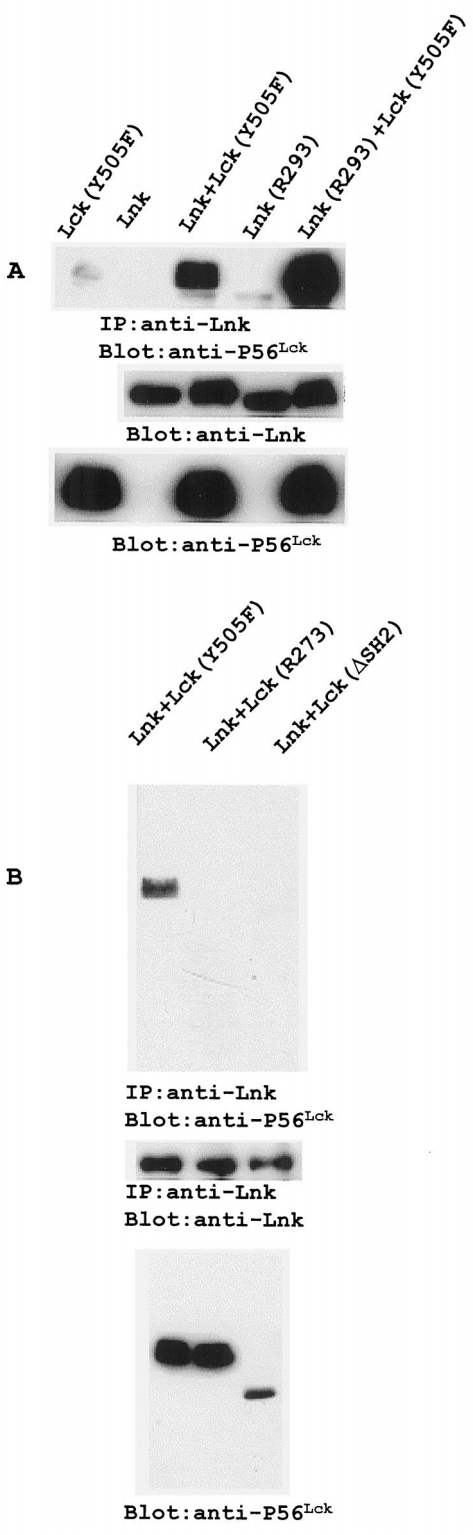

FIGURE 7. The association of hLnk and p56^{lck}. *A*, COS cells were transfected with expression vectors containing hLnk or hLnk(R392K) with or without Lck(Y505F). The cell lysates were immunoprecipitated with anti-Lnk Ab and blotted with anti-p56^{lck}. An aliquot of cell lysate from each transfected cells was blotted with anti-Lnk or anti-p56^{lck} to show their levels of expression. *B*, COS cells were cotransfected with expression vectors containing hLnk and either Lck(Y505F), Lck(R273), or Lck(ΔSH2). The cell lysates were immunoprecipitated with anti-Lnk Ab and blotted with anti-p56^{lck}. The same membrane was stripped and blotted with anti-Lnk to show the levels of hLnk expression. An aliquot of cell lysate from each transfection was also blotted with anti-p56^{lck} to show the levels of expression of each Lck variants.

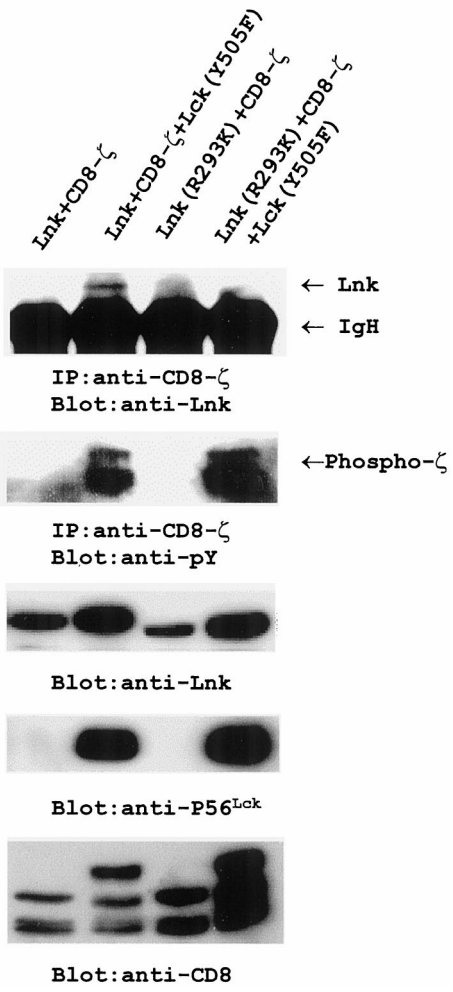

FIGURE 8. hLnk binds to the tyrosine-phosphorylated ζ-chain. COS cells were cotransfected with expression vector containing hLnk or hLnk(R392K) and the chimeric receptor CD8-ζ with or without Lck(Y505F). The cell lysate were immunoprecipitated using anti-CD8 and blotted with anti-Lnk. The membrane was later stripped and blotted with anti-phosphotyrosine Ab to show the phosphorylation of the ζ-chain. An aliquot of cell lysate from each transfection was blotted with anti-Lnk, anti-p56^{lck}, or anti-CD8 to show the levels of expression of each protein.

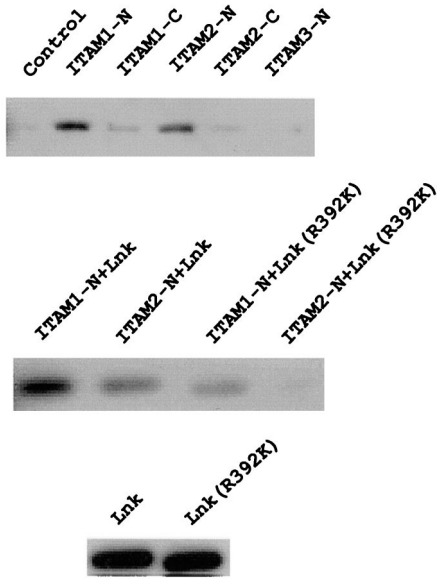

FIGURE 9. Association of Lnk with ζ-chain ITAM peptides. Western blots of Lnk associated with nona- or decapeptides containing phosphotyrosine of ζ-chain ITAMs are shown. A preferential association of hLnk with ITAM1-N and ITAM2-N is shown (*top*). This association was significantly diminished with hLnk(R392K), indicating that the association is mediated by the SH2 domain of hLnk (*middle*). COS cell lysates used in these experiments contained equivalent levels of hLnk and hLnk(R392K) (*bottom*).

dle panel). COS cells transfected with hLnk and hLnk(R392K) expressed and contained equivalent amounts of hLnk proteins in its lysates (Fig. 9, *bottom panel*).

Inhibitory role of hLnk in TCR signal transduction pathway

The endogenous expression of hLnk protein in Jurkat cell is very low and could not be detected. Because hLnk can bind to the TCR ζ-chain via its SH2 domain, we examined the effect of hLnk on TCR signaling in Jurkat cells overexpressing hLnk. The assay was conducted by measuring transcriptional activation of NF-AT using a reporter construct of IL-2 driven by the NF-AT response element (NF-AT-Luc). An expression vector containing hLnk or a control empty vector was transiently cotransfected into Jurkat cells with NF-AT-Luc. Upon stimulation by OKT3-mediated CD3 cross-linking, cells transfected with the control vector showed over a 5-fold increase in the luciferase activity. In contrast, cells transfected with Lnk showed only about a 2-fold increase (***, $p < 0.0003$, two-tailed Student *t* test, $n = 3$), indicating that the TCR-mediated activation of NF-AT activity was inhibited by hLnk expressed in Jurkat cells (Fig. 10*A*). The 60% inhibition is comparable to the inhibition of AP-1 activity (measured by the AP-1-seap reporter construct) by Cbl overexpression (20). Interestingly, when cells were stimulated with PMA and ionomycin, which can bypass the proximal TCR events and activate NF-AT-Luc activity, Lnk had little or no inhibitory effect ($p > 0.35$, not significant, two-tailed Student *t* test, $n = 3$). The values between controls of vector- and Lnk-transfected cells were also not statistically significant (two-tailed Student *t* test, $n = 3$). The transfection efficiencies between experiments were comparable. To test whether the hLnk-mediated inhibition of OKT3-mediated activation of NF-AT-Luc activation is due to the binding of hLnk to the ζ-chain, NF-AT-Luc activities were also examined in Jurkat cells transfected with hLnk(R392K). hLnk with the SH2 point mutation (R392K) cannot bind to the ζ-chain ITAM (see above, Fig. 9). The expressed protein levels were quantitated using Western blot analyses of hLnk developed with [^{125}I]-goat anti-rabbit Ab on a phosphoimager (Molecular Dynamics Storm System). The relative luciferase activities were normalized to the expressed protein levels of hLnk and hLnk(R392K). As shown in Fig. 10*B*, both hLnk ($p < 0.005$, two-tailed Student *t* test, $n = 3$) and hLnk(R392K) ($p < 0.001$, two-tailed Student *t* test, $n = 3$) significantly inhibited OKT3-mediated luciferase activity compared with the vector control-transfected cells. However, the levels of inhibition between hLnk and hLnk(R392K) were not significant ($p > 0.09$, two-tailed Student *t* test, $n = 3$). As in Fig. 10*A*, there were no significant differences in the luciferase activities among control-, hLnk-, and hLnk(R392K)-transfected cells when these cells were treated with PMA plus ionomycin ($p > 0.1$, ANOVA test). These results indicate that hLnk-mediated inhibition of the OKT3-mediated stimulation of NF-AT-Luc activity is mediated by other functional domain(s) besides the SH2 domain of hLnk. Furthermore, these results indicate that the binding of hLnk to the tyrosine-phosphorylated ITAM by itself have little effect on the inhibition of OKT3-mediated NF-AT-Luc activation.

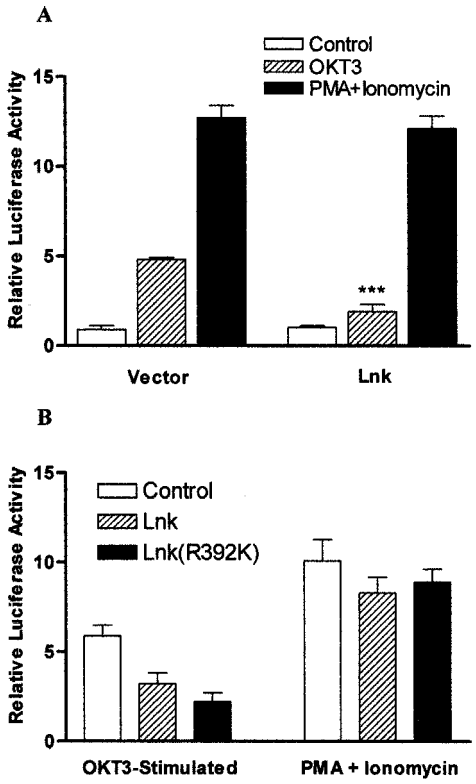

FIGURE 10. Inhibition of NF-AT transcription by hLnk. *A*, Jurkat cells were transiently transfected with hLnk or empty expression vector together with NF-AT-Luc. Then, 24 h after transfection, cells were collected and stimulated or left unstimulated with OKT3 or with PMA and ionomycin. The luciferase activities were assayed in triplicate and shown as fold increase compared with levels from control unstimulated cells. *B*, Jurkat cells were transiently transfected with hLnk or Lnk(R392K) together with NF-AT-Luc. The same procedure was used for stimulation with OKT3 and PMA plus ionomycin. Luciferase assay was performed as in *A*. The relative luciferase activities from hLnk- and hLnk(R392K)-transfected cell lysates were normalized against the levels of hLnk and hLnk(R392K) expressed in the transfected cells. The levels of hLnk and hLnk(R392K) expressed in the transfected cells were obtained by Western blot analyses of hLnk using cell lysates from an equal number of cells developed by [^{125}I]-goat anti-rabbit IgG analyzed on a phosphoimager (Molecular Dynamics Storm System).

Discussion

In this report, we describe the cloning and characterization of hLnk, an adaptor molecule with multifunctional domains (PH and SH2), an N-terminal proline-rich region, and possible tyrosine-phosphorylation site(s) by p56^{lck}. The PH domains, which display remarkable conservation of three-dimensional organization while lacking primary sequence similarity, are suggested to function as membrane adaptors, linking their host proteins to the membrane surface (32). Thus, it is possible that the PH domain of Lnk is responsible for its plasma membrane localization, visualized by Lnk-GFP. We have found that hLnk can bind to the tyrosine-phosphorylated ζ-chain through its SH2 domain. Results from the experiments using synthetic peptides with phosphotyrosines corresponding to each of the tyrosine residues within the ζ-chain ITAMs indicated that hLnk binds preferentially to the ITAM1-N and ITAM2-N phosphotyrosines in a SH2 domain-dependent manner. Provided that the amounts of peptides conjugated to the agarose beads were equal, more hLnk appears to have bound to the ITAM1-N than to the ITAM2-N. However, additional studies are needed to elucidate this point. Our preliminary results indicated that both wild-type hLnk and hLnk(R392K) with dysfunctional SH2 domain inhibited OKT-3-mediated NF-AT-Luc activation to a comparable levels. Thus, it is possible that other functional domains of hLnk other than the SH2 domain may contribute to the inhibition of NF-AT-Luc activation.

The tyrosine-phosphorylation sites of hLnk is not presently clear. The half ITAM motif (**D**NQ**Y**TP**L**, consensus sequence in bold) found at the C terminus is a possible phosphorylation site for p56^{lck}. Previous studies of in vitro kinase assays using recombinant hLnk indicated that both p56^{lck} and ZAP-70 kinases could phosphorylate hLnk (24). In our experiments using COS cells overexpressing p56^{lck} and hLnk with Y→F mutation at the half ITAM site (hLnk Y572F), tyrosine phosphorylation by p56^{lck} was observed indicating that hLnk can be tyrosine phosphorylated at sites other than Y_{572}. The role of ZAP-70 kinase on hLnk phosphorylation is difficult to assess in cells because the activation of ZAP-70 requires active p56^{lck}, which not only can activate ZAP-70, but also can phosphorylate hLnk. In contrast to ZAP-70, Syk kinase that belongs to the same family of kinase as ZAP-70 does not require the presence of p56^{lck} for its activation. In our experiments using COS cells overexpressing Syk and hLnk, phosphorylation of hLnk did not take place while Syk activity was clearly demonstrated by its autophosphorylation. It is not clear whether this is due to the differences in substrate specificity of these enzymes or to the experimental conditions (lack of TCR/CD3 receptor complex expression in COS cells) under which hLnk will not be tyrosine-phosphorylated by Syk family kinases. It is also possible that hLnk phosphorylation observed by in vitro kinase assay may not necessarily reflect the role these enzymes play in the cells.

At present we do not know what other protein(s) hLnk can bind to with its functional domains. Collectively, hLnk has the potential to interact with multiple molecules through its functional domains.

The most interesting finding about hLnk is its ability to inhibit T cell activation upon TCR stimulation. Considering the fact that hLnk can bind to the ITAM of TCR ζ-chain via its SH2 domain led to our initial speculation that Lnk binding to the tyrosine-phosphorylated ITAM within the ζ-chain may prevent the association of other molecules. ITAMs serve as docking sites to several key signal transduction molecules such as ZAP-70, p56^{lck}, and Shc (33). However, due to the low level of binding between Lnk and ζ-chain observed in our experiments, it is not clear whether this mechanism is solely responsible for the inhibitory role. When hLnk- and hLnk(R392K)-mediated inhibition of NF-AT-Luc activation was normalized to the respective levels of hLnk and hLnk(R392K) protein expression, the inhibitory effects were comparable. These results indicate that the binding of hLnk SH2 domain to the ITAM may not be the mechanism for hLnk-mediated inhibition of NF-AT-Luc activation. We also showed that Lnk could associate with p56^{lck} with the association being mediated by the SH2 domian of p56^{lck} and the kinase activity of p56^{lck} a requirement. It is possible that Lnk may sequester p56^{lck} from other molecules through this interaction. It has also been reported that a peptide corresponding to the C-terminal putative phosphorylation site of Lnk inhibited association between two signaling molecules in T cells, Shb and p36/LAT (34). Thus Lnk may act as an inhibitory molecule by interaction with multiple proteins via its different domains.

We have been puzzled by the fact that the expression level of hLnk is extremely low in all the cell lines we have examined so far. The expression of Lnk in human tissues has not been tested yet. Pervious studies on rat tissues showed that although it was preferentially expressed in lymphoid tissues such as lymph node and spleen, the expression level in these tissues was lower compared with other adaptor proteins such as Shc or Grb-2. In cell lines, it is

difficult to detect the levels of endogenous Lnk even using 2×10^7 cells for immunoprecipitation. Thus it is not clear whether hLnk can exert an inhibitory role at such low levels. It is possible that Lnk is kept at a low expression level so the resting T cells can be activated upon stimulation. During certain states, when the response of T cells need to be inhibited, Lnk may be induced and play its inhibitory role. Future studies on the regulation of Lnk expression may tell us its physiological function.

The formation of multimolecular complexes has been a major focus in the study of signal transduction. Many adaptor proteins have been characterized and shown to mediate protein-protein association and thus facilitate transduction of signals. The study of adaptor proteins that act as negative regulators is still at an early stage, and not many molecules with such a function have been found. By forming or preventing the formation of a particular multimolecular complex, an adaptor protein may exert its inhibitory action. Further characterization of hLnk may reveal new mechanisms of negative regulation of T cell signal transduction.

Acknowledgments

We thank Dr. Kristin M. Abraham (University of Maryland School of Medicine, Baltimore, MD), Dr. André Veillette (McGill University, Montréal, Canada), Dr. Edward Clark (University of Washington, Seattle, WA), Dr. Cox Terhorst (Beth Israel Hospital, Harvard Medical School, Cambridge, MA), and Dr. Gerald Crabtree (Department of Pathology, Stanford University Medical School, Stanford, CA) for providing the cDNA and constructs used in our experiments. We also thank Dr. Daniel J. Sussman (University of Maryland School of Medicine) for critical reading of the manuscript.

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