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Immunology

Cloning and characterization of Lnk, a signal transduction protein that links T-cell receptor activation signal to phospholipase $C\gamma_1$, Grb2, and phosphatidylinositol 3-kinase

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ABSTRACT **A cDNA encoding a signal transduction protein with a Src homology 2 (SH2) domain and a tyrosine phosphorylation site was cloned from a rat lymph node cDNA library. This protein, which we designate Lnk, has a calculated molecular weight of 33,988. When T lymphocytes were activated by antibody-mediated crosslinking of the T-cell receptor and CD4, Lnk became tyrosine phosphorylated. In activated T lymphocytes, phospholipase $C\gamma_1$, phosphatidylinositol 3-kinase, and Grb-2 coimmunoprecipitated with Lnk. Our results suggest that Lnk becomes tyrosine phosphorylated and links the immediate tyrosine phosphorylation signals of the TCR to the distal phosphatidylinositol 3-kinase, phospholipase $C\gamma_1$ and Ras signaling pathways through its multifunctional tyrosine phosphorylation site.**

Resting T lymphocytes are activated when the T-cell receptor (TCR) recognizes antigen bound to major histocompatibility complex (MHC) molecules. The TCR is a multisubunit complex of the TCR α and β chains, which recognize the antigen–MHC complex, and CD3 γ, δ, and ε chains as well as ζ and η chains, which function as signal transducers (1). The immediate biochemical event following TCR activation is tyrosine phosphorylation at the immunoreceptor tyrosine-based activation motif (ITAM) of the CD3 and $\zeta(\eta)$ chain complex (2). The ITAM sequence is a tandem repeat of the YXXL motif separated by 6–8 aa. It has been suggested that tyrosine phosphorylation at the ITAM sequence plays an essential role in TCR signaling (3, 4). Cytoplasmic protein tyrosine kinases such as Lck, Fyn, and ZAP-70 are involved in TCR-mediated phosphotyrosine signaling (2). It has been suggested that Lck and Fyn are involved in tyrosine phosphorylation of the CD3 ITAM (5, 6). Tyrosine phosphorylation at the ITAMs of CD3 subunits and ζ chain is thought to provide a key molecular switch leading to the recruitment of ZAP-70 to the TCR through its Src homology 2 (SH2) domains (7, 8). ZAP-70 has been shown to play an essential role in the development of T cells and in TCR activation signal transduction (9, 10).

In T cells, antigen recognition by the TCR leads to activation of several signaling pathways initiated by phospholipase $C\gamma_1$ ($PLC\gamma_1$), phosphatidylinositol 3-kinase (PI3K), and Grb-2. These proteins bind to the upstream activation molecules through their SH2 or SH3 domains (11–13). Adaptor proteins such as Vav and Shc have been shown to play a role in mediating TCR activation signaling (14, 15). In addition to these adaptor proteins, 36- and 38-kDa phosphotyrosine (pY)-containing proteins have been described as candidate molecules playing a role in the transduction of the TCR activation signals to the distal $PLC\gamma_1$, PI3K, and Grb-2 signaling pathways (7, 16, 17).

Here we report the cloning of cDNA encoding a protein with a calculated molecular weight of 33,988 that contains a SH2 domain and a multifunctional tyrosine phosphorylation site that associates with the downstream signaling molecules $PLC\gamma_1$, Grb-2, and PI3K upon TCR activation.||

EXPERIMENTAL PROCEDURES

mRNA Preparations. TEA3A1 rat thymic epithelial cells were cultured as described (18). Either Fisher or Lewis rats were used as tissue sources. Lymph node lymphocytes were prepared and suspended in RPMI 1640 medium. Total RNA was prepared from TEA3A1 cells and rat tissues by use of RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX). mRNA was purified with oligo(dT) cellulose (Becton Dickinson). The cDNA template was synthesized by SuperScript reverse transcriptase (GIBCO/BRL) with either oligo(dT) primer or random primer.

cDNA Cloning of Lnk. The original 1.0-kb cDNA fragment was cloned from TEA3A1 cells by 3′RACE PCR (19) with the forward primer (5′-GAAGCTAAGAGTCAGGGCGGCT-CTAAT-3′) and reverse adaptor primer (5′-GACTCGAG-TCGACATCG-3′). The size-selected ($\geq$2 kb) and oligo(dT)-primed rat lymph node cDNA library was constructed with a λZAP cDNA synthesis kit (Stratagene). The whole cDNA library was screened by plaque hybridization at high stringency with the 1.0-kb fragment as probe. Twelve positive plaques were isolated and phage DNAs were excised *in vivo* to form phagemid (ExAssist/SOLR system; Stratagene). To obtain the 5′ ends of the gene, 5′RACE PCR (19) was carried out. All of the PCR products were subcloned into the plasmid vector pBluescript KS (Stratagene). DNA sequences were analyzed by dideoxy chain termination with the Sequenase 2.0 kit (United States Biochemical) and *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems).

Generation of His-Tagged Fusion Protein of Lnk. DNA corresponding to the open reading frame of Lnk was synthesized by PCR and was ligated into pET-19b (Novagen) and used to transform *Escherichia coli* BL21(DE3)/pLysS (Novagen). Production of the recombinant protein was induced with 1 mM isopropyl β-D-thiogalactopyranoside. *E. coli* cell lysate was prepared by sonication in SDS sample buffer and the recombinant protein was purified by preparative electrophoresis (Bio-Rad model 491 prep cell). Purified recombinant

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Abbreviations: CTP, C-terminal peptide; GST, glutathione *S*-transferase; ITAM, immunoreceptor tyrosine-based activation motif; PI3K, phosphatidylinositol 3-kinase; $PLC\gamma_1$, phospholipase $C\gamma_1$; pY, phosphotyrosine; SH2, Src homology 2; TCR, T-cell receptor.

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||The sequence reported in this paper has been deposited in the GenBank database (accession no. U24653).

protein was dialyzed against 0.1 M $NaHCO_3$/0.5 M NaCl and conjugated to CNBr-activated Sepharose 4B.

Preparation of Anti-Lnk Antibodies. Rabbit polyclonal antisera were generated against the His-tagged Lnk fusion protein and the synthetic peptide (ESVSSARDSDYEMDSSS-RSH) corresponding to the C-terminal region of Lnk made on a multiple antigen peptide resin (20). The antibodies were affinity purified by chromatography on Sepharose 4B (Sigma) conjugated to either synthetic peptide or His-tagged Lnk fusion protein, respectively. Antibodies to His-tagged Lnk (anti-Lnk) were conjugated to agarose (ImmunoPure antigen/antibody immobilization kit; Pierce) and used for immunoprecipitation studies. The antibody against the synthetic C-terminal peptide (anti-CTP) was used for Western immunoblot analyses.

COS Cell Transfection. COS-7 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. DNA corresponding to the sequence from 64 bp upstream of the Lnk translational initiation site to the 3′ end of the Lnk open reading frame was synthesized by PCR. The amplified DNA was subcloned into the expression vector pSVL SV40 (Pharmacia) and used to transfect COS-7 cells by the DEAE-dextran method (21). Control COS-7 cells were transfected with an empty pSVL SV40 vector. The transfected COS-7 cells were incubated for 48 hr and lysed in SDS sample buffer. Western immunoblot analyses of the cell lysates were carried out with anti-CTP.

Immunoprecipitation and Western Blot Studies. Activation of TCR signaling was carried out (15) with either Con A (30 μg/ml; Miles) for 5 min at 37°C or monoclonal anti-rat TCRα/β and anti-rat CD4 (2 μg/ml each; Harlan Bioscience, Indianapolis). Cells were pelleted and lysed with lysis buffer (15). Lysates were centrifuged (13,000 × *g*) and the supernatants were incubated at 4°C for 2 hr (or as indicated) with 2 μg of affinity-purified anti-Lnk conjugated to agarose beads. The beads were washed with lysis buffer plus 0.1% Nonidet P-40. Immunoprecipitates were resolved by SDS/12.5% PAGE under reducing conditions and electrotransferred to a poly(vinylidene difluoride) membrane (Millipore). Blots were blocked with 5% skim milk/10 mM Tris, pH 7.6/150 mM NaCl/0.05% Tween 20, probed with antibodies against the Lnk CTP, pY, PLCγ_1, PI3K, Grb-2, or Sos-1 (Upstate Biotechnology, Lake Placid, NY). Filters were incubated with horseradish peroxidase-conjugated secondary antibodies (Cappel) and developed by enhanced chemiluminescence (ECL; Amersham).

***In Vitro* Binding Studies.** *E. coli* cells containing plasmids encoding glutathione *S*-transferase (GST) fusion proteins of the SH2 domains of Grb-2 and PI3K (N- and C-terminal SH2) were obtained from Tony Pawson (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto). GST and GST fusion proteins were purified on glutathione-agarose beads (Pharmacia) (22). Agarose beads bound GST fusion proteins of PLCγ_1 N- and C-terminal SH2 domains were obtained from Upstate Biotechnology. Rat lymph node lymphocytes (10^8 per ml) were stimulated and lysed as described above. Clarified lysates were incubated with 3 μg of immobilized GST or GST-SH2 fusion proteins at 4°C for 2 hr. The protein complexes were washed with 0.5 M NaCl containing 1% Nonidet P-40, phosphatase inhibitors and proteinase inhibitors, resolved by SDS/12.5% PAGE, and transferred to a poly(vinylidene difluoride) membrane. Blots were probed with anti-CTP and developed by chemiluminescence.

Peptides CH13L (CHLRAIDNQYTPLSQL) and CV14R (CVSSARDSDYEMDSSSR) were synthesized by Quality Controlled Biochemicals (Hopkinton, MA) with the tyrosine residues either phosphorylated or nonphosphorylated. A cysteine was added to the N terminus of each peptide for conjugation to SulfoLinK coupling gel (Pierce). Six micrograms of peptide in 30 μl of 50% (vol/vol) agarose beads was incubated with the lysates from unstimulated rat lymph node lymphocytes (2×10^7 per ml) for 2 hr at 4°C. The beads were washed with washing buffer and the precipitated proteins were resolved by SDS/12.5% PAGE. Western immunoblot analyses used monoclonal antibodies to Grb-2, PI3K, and PLCγ_1. For PLCγ_1 immunoblot, the beads were washed with 0.5 M NaCl containing 1% Nonidet P-40, phosphatase inhibitors, and proteinase inhibitors.

RESULTS

Isolation of Lnk cDNA. We isolated a cDNA fragment with a novel sequence from TEA3A1 rat thymic epithelial cells during our effort to clone the cDNA encoding a thymic hormone, thymulin. This cDNA fragment was 1.0 kb in size and hybridized to a 4.3-kb message in Northern blot analysis. The protein encoded by this message was named Lnk on the basis of its functional properties, described later. Lnk mRNA was preferentially expressed in lymph node and spleen (Fig. 1). Additional analyses by RNase protection assays showed that Lnk mRNA was preferentially expressed by lymphocytes in these organs (data not shown).

A rat lymph node cDNA library was screened with the 1.0-kb cDNA as probe. A 3-kb cDNA was subsequently isolated from this library. To obtain additional 5′ sequence information, 5′RACE PCR was carried out. After combining all of the sequence information, we obtained a single open reading frame (Fig. 2*A*).

Lnk Contains a SH2 Domain and a Possible Tyrosine Phosphorylation Site. A homology search of the deduced amino acid sequences in GenBank revealed a span of 99-aa SH2 domain in the central region of Lnk (Fig. 2*B*). The SH2 domain of Lnk contains all of the conserved basic amino acid residues that are responsible for pY binding (24). Among the SH2 domains found in other proteins, the SH2 domain of Lnk has the highest sequence homology (42%) with Shc (25). The SH2 domain of Lnk has a leucine residue at the βD5 position, as is the case with Shc, indicating that the SH2 domain of Lnk belongs to the same group III SH2 domains as the SH2 domain of Shc (26). Proteins with SH2 domain(s) play an important role in protein tyrosine kinase-mediated signal transduction (24). Thus, it is possible that Lnk plays a role in the signal transduction of lymphocytes. Computer analysis of the putative Lnk protein sequence also suggested a possible tyrosine phosphorylation site (DNQYTPL) in the C-terminal region.

Identification of Lnk Protein in Rat Lymph Node Lymphocytes. Anti-Lnk and anti-Lnk CTP antibodies were developed. When Western blots of anti-Lnk immunoprecipitates from

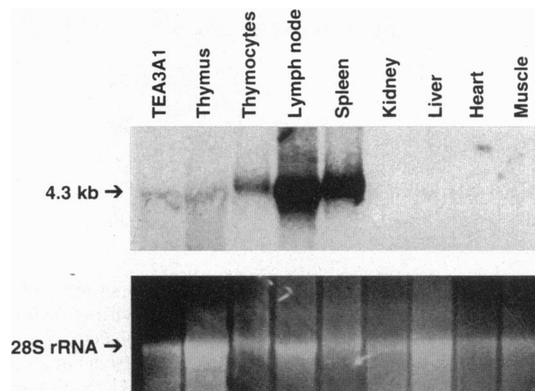

FIG. 1. Preferential expression of Lnk mRNA in rat spleen and lymph node. Northern blot analysis of poly(A)$^+$ RNA (5 μg per lane) from the indicated tissues was carried out with the 1.0-kb Lnk cDNA fragment as probe. Ethidium bromide staining of the 28S rRNA is shown below for estimating the relative amount of RNA loaded for each sample.

A

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MPDNLYTFVLKVQGQTDIIFEVGDEQQLNSWLAELRASTGLGLEHLDTELPLSLVAEPGPAISPRGSTDSLDQGASPGVMLDPACQKTDHFLSCYPWFHGPIS  103
RVRAAQLVQLQGPDAHGVFLVRQSESRRGEYVLTFNLQGRAKHLRLVLTERGQCRVQHLHFPSVVDMLRHFQRSPIPLECGAACDVRLSGYVVVVSQAPGSSN  206
TVLFPFSLPHWDSELGHPHLSSAGCPPGHGAEALRGQVTPPEQIFHLVPSPEELANSLRQLELESVSSARDSDYEMDSSSRSHLRAIDNQYTPLSQLCREANL  309
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B

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Lnk   WFHGPISRVRAAQLVQLQ-GPDAHGVFLVRQSESRRGEYVLTFN-LQ---GR-AKHLRL-VL
Shc   WFHGKLSRREAEALLQLN------GDFLVRESTTTPGQYVLTG--LQS--GQ-PKHLLL-V-
Matk  WFHGKISGQEAVQ--QLQ-PPE-DGLFLVRESARHPGDYVLCVS-F----GRDVIHYR--VL
Abl   WYHGPVSRN-AAEYL-LSSG--INGSFLVRESESSPSQRSISLR-YE---GR-VYHYRIN--
Src   WYFGKITRRESERLL-LN-PENPRGTFLVRESETTKGAYCLSVSDFDNAKGLNVKHYKIRKL

Lnk   TER-GQCRV-QHLHF-PSVVDMLRHFQRS---PIPLECG-AACDVRL-SGYV
Shc   -DPEGVVRTKDHR-F-ESV-SHLISYHMDNHLPII-SAGSELC---L-QQPV
Matk  -HRDGHL-TIDEAVFFCNLMDMVEHY--SK------DKG-AICT-KLVRPKR
Abl   TASDGKLYVSSESRF-NTLAE-LVHHH-ST-V----ADG-LITT--L-HYPA
Src   -DSGG-FYITSRTQF-SSL-QQLVAYY-SKH-----ADG--LCH-RL--TNV
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FIG. 2. (*A*) Amino acid sequence of Lnk. cDNA sequence including the 3′ and 5′ noncoding regions is available through GenBank (accession no. U24653). (*B*) Alignment of the SH2 domain of Lnk with the SH2 domains. The SH2 domain of Lnk is aligned with the SH2 domains of Shc, Matk (23), Abl, and Src. Amino acid residues with identity to Lnk are highlighted.

lymph node lymphocyte lysates were probed with anti-CTP, a protein band at 40 kDa was detected (Fig. 3). This immunoreactive band was not detected when anti-CTP was preincubated with excess recombinant Lnk (data not shown), indicating that the 40-kDa protein band was indeed Lnk. The calculated molecular weight of Lnk is 33,988. Lnk from COS cells transfected with an expression vector containing the Lnk cDNA was also identified as a 40-kDa protein by Western blot analysis (Fig. 3). This protein was not detected in lysates of COS cells transfected with an empty vector. These results indicate that Lnk can be immunoprecipitated by anti-Lnk and detected by anti-CTP antibodies as 40-kDa protein.

Tyrosine Phosphorylation of Lnk in Activated Lymph Node T Cells. Both SH2 domains of Lnk and Shc belong to group III, indicating that Lnk may have similar tyrosine phosphoprotein binding specificity as Shc. In T cells, Shc has been shown to associate directly with the TCR complex (15). In addition, Lnk contains a putative tyrosine phosphorylation site, DNQYTPL, resembling the first half of the ITAM consensus sequence (DXXYXXL). Thus, it seemed reasonable that Lnk may be involved in TCR signaling. To test this hypothesis, we examined whether Lnk is tyrosine phosphorylated upon TCR activation. Freshly isolated rat lymph node lymphocytes were stimulated by antibody-mediated crosslinking of the TCR and CD4, a method commonly used to activate TCR-mediated signal transduction (15). Tyrosine phosphorylation of Lnk was detected as soon as 30 sec and up to 10 min after the activation of rat lymphocytes, whereas it was not detected in unstimulated lymphocytes (Fig. 4). Tyrosine phosphorylation of Lnk was not detected after antibody-mediated crosslinking of TCR alone or CD4 alone or after antibody-mediated crosslinking of CD3 with CD4 (data not shown). These results indicate that Lnk is immediately tyrosine phosphorylated upon activation of the TCR of $CD4^+$.

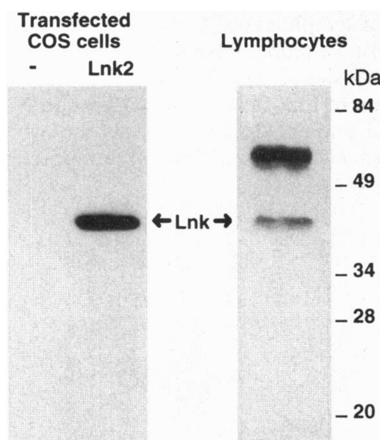

FIG. 3. Western blot analysis of Lnk. Lysates from rat lymph node lymphocytes (10^8 cells) and COS cells transfected with an expression vector containing either Lnk cDNA or an empty vector (−) were prepared as described. Lysates from lymphocytes were incubated with anti-Lnk-agarose as indicated. The cell lysates from COS cells and the immunoprecipitate from lymph node lymphocytes were resolved by SDS/12.5% PAGE and blotted onto a poly(vinylidene difluoride) membrane for Western blot analysis with anti-CTP. Crossreacting proteins (40-kDa Lnk and immunoglobulin heavy chain from lymphocyte immunoprecipitates) were visualized by chemiluminescence after incubation of the filter with horseradish peroxidase-conjugated goat anti-rabbit antibodies.

Coimmunoprecipitation of PLCγ_1, Grb-2, and PI3K with Lnk. TCR activation leads to the activation of signal transduction pathways initiated by PLCγ_1 (11), Grb-2 (17), and PI3K (12). These proteins associate with yet unidentified tyrosine phosphoproteins of 36–38 kDa in activated T cells (7, 16, 17). Thus, we examined whether Lnk is involved in the activation of these signal transduction proteins. Lymph node lymphocytes were activated by crosslinking of TCR and CD4 or by Con A treatment. Lysates from these cells were incubated with anti-Lnk-conjugated agarose beads. Immunoprecipitates were analyzed by Western blot with monoclonal antibodies to PLCγ_1, Grb-2, and PI3K. Increased amounts of Grb-2, PLCγ_1, and PI3K were coimmunoprecipitated with Lnk in lysates of activated T cells (Fig. 5). Western blot analysis of Lnk immunoprecipitates with anti-Grb-2 was also carried out with cell lysates from Con A-treated lymphocytes. This procedure ensured that what was detected with anti-Grb-2 was not the immunoglobulin light chain, which runs at a similar position in SDS/PAGE. Sos also coimmunoprecipitated with Lnk together with Grb-2 from TCR-activated cell lysates (Fig. 5). These results strongly indicate that upon TCR activation, PLCγ_1, Grb-2, and PI3K associate with Lnk and that these

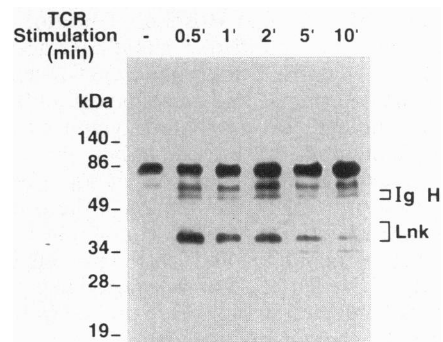

FIG. 4. Tyrosine phosphorylation of Lnk. Lymph node lymphocytes (10^8 cells per condition) were activated by antibody-mediated crosslinking of TCR and CD4 at 37°C for 0.5–10 min. Immunoprecipitation was carried out with anti-Lnk-agarose, and Western blot analysis of the immunoprecipitates was carried out with monoclonal anti-pY. Blot was visualized by chemiluminescence. Lane −, nonactivated control cells. Ig H, immunoglobulin heavy chain.

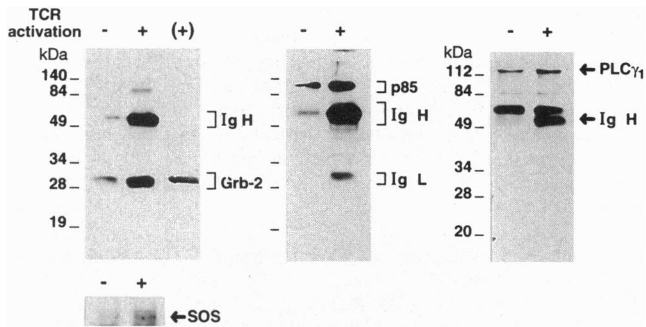

FIG. 5. Coimmunoprecipitation of Grb-2, PI3K, and $PLC\gamma_1$ with Lnk. Immunoprecipitation was carried out with lysates from control (−) and TCR-activated (+) lymphocytes (10^8 cells per condition) and anti-Lnk agarose. Western blot analyses of the immunoprecipitates were carried out with monoclonal antibodies to Grb-2 (*Left*), PI3K (*Center*), PI and $PLC\gamma_1$ (*Right*) and polyclonal antibody to Sos (*Lower Left*). Immunoreactive bands were visualized by chemiluminescence. For the Grb-2 Western blot, anti-Lnk agarose immunoprecipitate from Con A-activated lymphocytes [lane (+)] was also used. Ig H and L, immunoglobulin heavy and light chains.

associations are possibly mediated by the SH2 domains of these proteins. These proteins were also detectable in preparation of unstimulated cells. Since activation of some T cells must be continually occurring in rats kept under non-aseptic conditions, this may be due to such residual activated T cells.

SH2 Domains of $PLC\gamma_1$, Grb-2, and PI3K Bind to Lnk *in Vitro*. $PLC\gamma_1$, Grb-2, and PI3K are known to associate with upstream signal transduction proteins through their SH2 domains. There are two SH2 domains in $PLC\gamma_1$ and PI3K and one in Grb-2. To examine whether SH2 domains of $PLC\gamma_1$, Grb-2, and PI3K associate with tyrosine-phosphorylated Lnk, GST fusion proteins of SH2 domains of $PLC\gamma_1$ (N- and C-terminal SH2), Grb-2, and PI 3-kinase (N- and C-terminal SH2) bound to glutathione-agarose beads were incubated with lysates from activated T cells. GST-SH2 fusion proteins of Grb-2, N-terminal $PLC\gamma_1$, and N-terminal PI3K precipitated Lnk from TCR-activated cell lysates (Fig. 6). The C-terminal SH2 domains of both $PLC\gamma_1$ and PI3K, as well as the control GST, did not interact with Lnk. These results indicate that $PLC\gamma_1$, Grb-2, and PI3K associate with Lnk in a specific manner. Moreover, our results showed a difference in the binding specificity of N-terminal and C-terminal SH2 domains of $PLC\gamma_1$ and PI3K with Lnk.

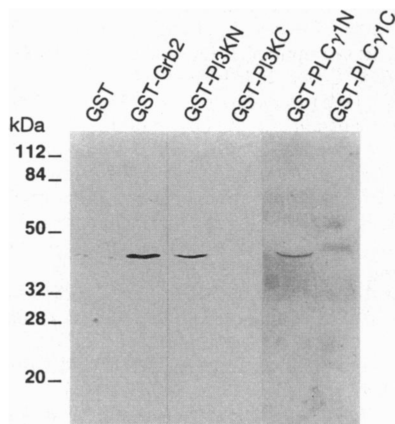

FIG. 6. Precipitation of Lnk with GST fusion proteins of SH2 domains of Grb-2, PI3K, and $PLC\gamma_1$. Lysates were prepared from TCR activated lymphocytes (10^8 cells per condition). The lysates were incubated with either GST alone as a negative control or with GST fusion proteins with the Grb-2-SH2 domain, PI3K N- and C-terminal SH2 domains, and $PLC\gamma_1$ N- and C-terminal SH2 domains. All fusion proteins were bound to glutathione-agarose beads as described. Western blot analysis of the precipitated proteins was carried out with anti-CTP; visualization was achieved with chemiluminescence.

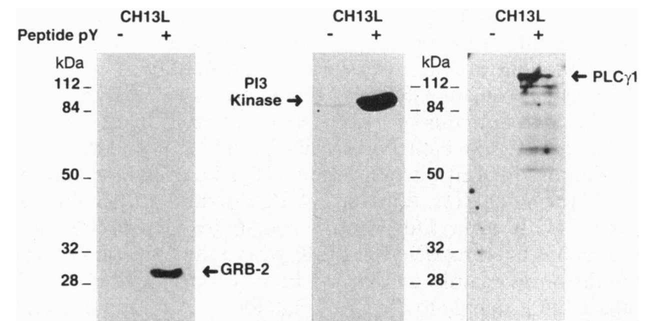

FIG. 7. Precipitation of Grb-2, PI3K, and $PLC\gamma_1$ by tyrosine phosphopeptide of Lnk. Unphosphorylated tyrosine (−) and phosphotyrosine (+)-containing synthetic peptides conjugated to agarose beads were incubated with lymphocyte lysates (2×10^7 cells per condition) at 4°C for 2 hr. The agarose beads were washed three times with washing buffer. For the $PLC\gamma_1$ immunoblot, agarose beads were washed three times with 0.5 M NaCl containing 1% Nonidet P-40, phosphatase inhibitors, and proteinase inhibitors. Western blot analyses of the precipitated proteins were done with monoclonal antibodies to Grb-2 (*Left*), PI3K (*Center*), and $PLC\gamma_1$ (*Right*) with visualization by chemiluminescence.

Association of $PLC\gamma_1$, Grb-2, and PI3K with Lnk pY-Containing Peptides. Experiments were carried out to map the possible pY residue(s) that interact with the SH2 domains of $PLC\gamma_1$, Grb-2, and PI3K. Tyrosine residues in the hydrophilic region of Lnk were identified by computer-assisted analysis. Out of a total of six tyrosine residues, two were in the hydrophilic region and were found in the C-terminal region of the molecule. Thus, two synthetic peptides corresponding to the sequences surrounding these two tyrosine residues were made with either pY or nonphosphorylated tyrosine (see *Experimental Procedures*). Both of these peptides were conjugated to agarose beads through a cysteine placed at the N terminus. Cell lysates from lymph node lymphocytes were incubated with these peptide-conjugated agarose beads. The proteins bound to the beads were immunoblotted with the antibodies to $PLC\gamma_1$, Grb-2, and PI3K. $PLC\gamma_1$, Grb-2, and PI3K associated with only the pY-CH13L peptide, the computer-predicted tyrosine phosphorylation site of Lnk, but not with the nonphosphorylated CH13L peptide (Fig. 7). We observed no association of $PLC\gamma_1$, Grb-2, and PI3K with other peptides whether they contained pY or not (data not shown). Our results indicate that the association of $PLC\gamma_1$, Grb-2, and PI3K with Lnk is dependent upon the tyrosine phosphorylation of Lnk and that the tyrosine phosphorylation site of Lnk is multifunctional and may associate with the SH2 domains of $PLC\gamma_1$, Grb-2, and PI3K upon TCR activation.

DISCUSSION

In this paper we report the molecular cloning and characterization of a protein we call Lnk from rat lymph node lymphocytes. Lnk is preferentially expressed in mature lymphocytes and contains a putative SH2 domain and a possible tyrosine phosphorylation site. Using specific antibodies, we found that Lnk became tyrosine phosphorylated following TCR activation by antibody-mediated crosslinking of TCR and CD4.

The association of downstream proteins with Lnk was not strictly activation dependent. Detectable amounts of $PLC\gamma_1$, Grb-2, and PI3K coimmunoprecipitated with Lnk in lysates of nonactivated lymphocytes. This may be due in part to the use of lymph node lymphocytes freshly isolated from the rat, some of which may have been in an activated state. Alternatively, it is possible that Lnk coimmunoprecipitates as a large functional complex of loosely associated signal transduction proteins that

becomes activated by TCR. Upon activation of TCR, their phosphorylation increases the affinity of protein association for an efficient execution of signal transduction.

Experiments using GST-SH2 fusion proteins clearly showed that the SH2 domains of PLCγ_1, Grb-2, and PI3K are associated with Lnk from activated T cells. Both PLCγ_1 and PI3K are associated with Lnk through their N-terminal SH2 domains. The C-terminal SH2 domains of PLCγ_1 and PI3K did not interact with Lnk. Differential specificity of SH2-domain binding has been reported in PI3K, where the C-terminal SH2 domain binds mainly to Tyr^{740} whereas the N-terminal SH2 domain binds mainly to Tyr^{751} of platelet-derived growth factor receptor (27). The results from experiments using synthetic tyrosine phosphopeptide corresponding to the possible tyrosine phosphorylation site complemented the results from experiments with GST-SH2 fusion proteins. The results clearly showed that Lnk's tyrosine phosphorylation site is multifunctional and interacts with the SH2 domains of PLCγ_1, Grb-2, and PI3K. The control synthetic peptide with nonphosphorylated tyrosine did not bind to these proteins. Therefore, at least in activated T cells, tyrosine phosphorylation of Lnk contributes to the increased association of PLCγ_1, Grb-2, and PI3K. pY mapping of Lnk from activated T cells must be carried out to determine the tyrosine phosphorylation site of Lnk. Results from studies in the literature indicate that the interaction of tyrosine-phosphorylated protein and SH2 domain is sequence specific. The amino acid sequence that follows the putative tyrosine phosphorylation site of Lnk is TPL and is different from the sequences known to interact with PLCγ_1, Grb-2, and PI3K SH2 domains. However, the association of SH2 domains of PLCγ_1, Grb-2, and PI3K with tyrosine phosphopeptide of Lnk appears specific. Perhaps the SH2-domain binding site of Lnk is multifunctional because it does not contain a specific consensus sequence that prefers binding of a particular SH2 domain. This may be the way that Lnk facilitates an equal-opportunity binding of downstream signal transduction proteins. A flexible and compensatory nature of autophosphorylation sites in their association with multiple SH2-containing proteins has also been reported for epidermal growth factor receptor (28).

The upstream protein that Lnk associates with via its SH2 domain is not known. Preliminary studies indicate that Lnk associates with one of the CD3 chains (data not shown).

In summary, our results indicate that Lnk plays a role in the transduction of the TCR activation signal in CD4$^+$ T cells. We have shown that Lnk becomes tyrosine phosphorylated upon TCR activation and recruits PLCγ_1, Grb-2, and PI3K. These associations may facilitate the activation of multiple signal transduction pathways leading to the transcriptional activation of the interleukin 2 gene. As the transcriptional activation of that gene requires nearly simultaneous binding of multiple transcription factors (29), a well-coordinated concurrent activation of multiple distal signal transduction pathways leading to the generation of multiple transcription factors must take place. The presence of signal transduction proteins of 36–38 kDa that become tyrosine phosphorylated upon TCR activation has been postulated to play an important role in recruiting PLCγ_1 and Grb-2 (16, 17). Lnk appears to be a prime candidate that fits this criterion in the TCR activation signaling in CD4$^+$ T cells.

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