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The T-Cell Antigen CD5 Acts as a Receptor and Substrate for the Protein-Tyrosine Kinase p56^{lck}

MONIKA RAAB, MASAHIRO YAMAMOTO, AND CHRISTOPHER E. RUDD*

Division of Tumor Immunology, Dana-Farber Cancer Institute, and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

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CD5 is a T-cell-specific antigen which binds to the B-cell antigen CD72 and acts as a coreceptor in the stimulation of T-cell growth. CD5 associates with the T-cell receptor ζ chain (TcRζ)/CD3 complex and is rapidly phosphosphorylated on tyrosine residues as a result of TcRζ/CD3 ligation. However, despite this, the mechanism by which CD5 generates intracellular signals is unclear. In this study, we demonstrate that CD5 is coupled to the protein-tyrosine kinase p56^{lck} and can act as a substrate for p56^{lck}. Coexpression of CD5 with p56^{lck} in the baculovirus expression system resulted in the phosphorylation of CD5 on tyrosine residues. Further, anti-CD5 and anti-p56^{lck} coprecipitated each other in a variety of detergents, including Nonidet P-40 and Triton X-100. Anti-CD5 also precipitated the kinase from various T cells irrespective of the expression of TcRζ/CD3 or CD4. No binding between p59$^{fyn(T)}$ and CD5 was detected in T cells. The binding of p56^{lck} to CD5 induced a 10- to 15-fold increase in p56^{lck} catalytic activity, as measured by in vitro kinase analysis. In vivo labelling with $^{32}P_i$ also showed a four- to fivefold increase in Y-394 occupancy in p56^{lck} when associated with CD5. The use of glutathione *S*-transferase–Lck fusion proteins in precipitation analysis showed that the SH2 domain of p56^{lck} could recognize CD5 as expressed in the baculovirus expression system. CD5 interaction with p56^{lck} represents a novel variant of a receptor-kinase complex in which receptor can also serve as substrate. The CD5-p56^{lck} interaction is likely to play roles in T-cell signalling and T-B collaboration.

The pan-T-cell marker CD5/Ly-1 antigen is a 69-kDa monomeric antigen that belongs to a family of receptors typified by the scavenger receptor cysteine-rich family of extracellular domain-like structures (19, 22, 38). This family includes the type I macrophage scavenger receptor, the human complement factor 1, the sea urchin speract receptor, and the lymphoid antigen CD6 (3, 25). Although highly conserved, members of this receptor family have been reported to bind ligands as diverse as speract peptides and the B-cell antigen CD72 (25). As in the case of CD2, CD4, CD6, and CD28, CD5 has been hypothesized to act as a second signal in the activation pathway of T cells. In this sense, CD5 can provide costimulatory signals in the proliferation of T cells (1, 10, 28, 51). Costimulation increases intracellular Ca^{2+} and cyclic GMP levels (27), interleukin 2 (IL-2) secretion, and interleukin 2 receptor (IL-2R) expression (10, 23). Other monoclonal antibodies (MAbs) to CD5 have been reported to stimulate T-cell growth directly or in conjunction with CD28 (10, 28, 63). CD5 has been reported to bind to the B-cell antigen CD72 (31, 59), a finding consistent with the observation that CD5 augments T-cell help for B-cell immunoglobulin production (55).

CD5 can physically associate with the T-cell receptor ζ chain (TcRζ)/CD3 complex on the surface of T cells, an interaction may account for the functional dependency of CD5 on TcRζ/CD3 expression (5, 9, 36). In this sense, CD5 shares a number of properties with TcRζ. Both can be coprecipitated with TcRζ/CD3 in gentle detergents and are rapidly phosphorylated on tyrosine residues in response to TcRζ/CD3 ligation (9, 14, 45). Both TcRζ and CD5 possess part of a potential tyrosine kinase phosphorylation motif (Y-X_{11}-Y-XXL) found in the other CD3 chains, B29 and MB1 (40). The Y-XX-L submotif found in the TcRζ and CD3 chains has been replaced by a Y-XX-P submotif in CD5. Intriguingly, the first tyrosine residue is surrounded by residues with homology to the autophosphorylation site of Src family members (DNEY) (22). The receptor therefore appears to be well constructed to act as a substrate for Src-related kinases. CD5 binding to the B-cell antigen CD72 may facilitate collaboration between T and B lymphocytes.

Over the past few years, a variety of T-cell antigens involved in T-cell signalling have been found to be complexed to members of the Src family of protein-tyrosine kinases. Initially, CD4 and CD8 were found to associate with protein-tyrosine kinase p56^{lck} (4, 44; for a review, see reference 42). Other regulatory receptors such as TcRζ/CD3, IL-2R β chain, Thy-1, and Ly-6 have also been shown to associate with Src kinases (18, 52). Binding is mediated by C-X-C-P motif in the CD4/CD8 α-chain cytoplasmic tail and by N-region sequences of p56^{lck} in the case of CD4-p56^{lck} and CD8-p56^{lck} (47, 48, 57). By contrast, the N-terminal region of the kinase domain of p56^{lck} mediates binding to the IL2-R β chain (18). CD4-p56^{lck} activity is regulated by ligand-induced receptor cross-linking (30, 62). The CD4-p56^{lck} interaction is required for coreceptor function (17), while p56^{lck} expression is required for T-cell stimulation and thymic differentiation (33, 53).

Given our previous findings showing the CD5 associates with the TcRζ/CD3 complex and is rapidly phosphorylated with receptor ligation, we were interested in whether CD5 could interface with Src-related kinases such as p56^{lck}. In this report, we demonstrate that CD5 has the unusual property of acting as both a substrate and a receptor for p56^{lck}. The binding of p56^{lck} with CD5 also resulted in the stimulation of p56^{lck} activity. CD5-p56^{lck} is likely to account for the costimulatory function of CD5 and may play a role in the regulation of T-B collaboration in the immune response.

* Corresponding author. Mailing address: Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115.

MATERIALS AND METHODS

MAbs, antisera, and cells. MAbs used included antibodies to CD5 (ImmunoGen, Inc., Cambridge, Mass.) (24T6G12; immunoglobulin G2) (9) and to CD4 (19Thy5D7; immunoglobulin G2) (39). Anti-p56^{lck} MAbs were generated against a synthetic peptide corresponding to the N-terminal residues 39 to 64 (gift of P. Kanteti). Antiphosphotyrosine MAb 4G10 was kindly provided by B. Druker (Dana-Farber Cancer Institute, Boston, Mass.). Rabbit anti-mouse immunoglobulin was obtained from Dako Corporation (Carpinteria, Calif.). Anti-CD27 and anti-CD26 antibodies have been described before (34, 43). T cells (Jurkat, Peer IV) were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 1% (wt/vol) penicillin-streptomycin at 37°C in an atmosphere containing 5% CO_2. TcRζ/CD3-negative Jurkat T cells were obtained from the American Type Culture Collection. *Spodoptera frugiperda* cell line IPLB-Sf21 (Sf21) (60) was propagated as a monolayer culture in Sf 900 insect medium (Gibco/BRL) supplemented with 10% fetal calf serum and 50 μg of gentamicin per ml as described by Brown and Faulkner (8). Viral infections were performed at a multiplicity of infection of 5 for protein production and for labelling experiments or of 0.1 for virus production.

cDNA cloning. Isolation of a cDNA clone encoding CD5 was performed by cDNA synthesis as described previously (21). Briefly, total cellular RNA was extracted from Jurkat cells by guanidinium-cesium chloride, and cDNA synthesis was performed with a random primer. After PCR amplification using CD5-specific oligonucleotides with restriction sites for *Eco*RI, the resultant PCR product was purified and cloned in the *Eco*RI site of pVl1393 to generate pVl1393-CD5. For cDNA encoding CD4 (kindly provided by Richard Axel, Columbia University, New York, N.Y.), recombinant transfer vectors were prepared by CD4 DNA amplification by PCR using specific oligonucleotides and cloning into the *Eco*RI site of the transfer vector pVl1393. The *S. frugiperda* cell line was then transfected with a mixture of linear wild-type baculoviral DNA (Invitrogen) and pVl1393-DNA constructs. Two days after infection, cell supernatants containing both wild-type and recombinant viruses were removed and plaqued on a monolayer of Sf21 cells. Recombinant virus plaques were identified visually by their occlusion-negative phenotype (54). Recombinant virus was purified from contaminating wild-type virus by two rounds of plaque purification, and high-titered lysates (10^7 to 10^8 PFU/ml) of recombinant baculovirus were obtained. Lck-baculovirus recombinants were kindly provided by Gerhard Raab.

DNA sequences encoding the SH2 (residues 127 to 234), SH3 (62 to 126), and SH2/SH3 (62 to 234) domains of Lck tyrosine kinase were amplified by PCR from plasmid containing full-length murine *lck* cDNA, using specific 3′ and 5′ primers that included restriction sites for *Bam*HI and *Eco*RI, and subcloned into the pGEX-2T vector (Pharmacia, Uppsala, Sweden) cut with *Bam*HI and *Eco*RI. Competent *Escherichia coli* DH5α was used for transformation and expression of the fusion proteins (49).

Protein production and Western blotting (immunoblotting). Sf21 cells (1.5×10^6) were seeded into 35-mm-diameter cell culture dishes. Plates were infected with either baculovirus encoding p56^{lck} or coinfected with baculovirus encoding p56^{lck} and baculovirus encoding CD5. At different times postinfection, the confluent monolayers of Sf21 cells were rinsed with phosphate-buffered saline (8 mM K_2HPO_4, 2 mM KH_2PO_4, 145 mM NaCl) and subsequently lysed with 200 μl lysis buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% [vol/vol] Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin). The cellular debris was removed by centrifugation (12,000 × *g*, 10 min, 4°C). The proteins were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE) (26), and transferred to nitrocellulose (56). Nitrocellulose was blocked with 2% gelatin in Tris-buffered saline (TBS), washed thrice in TBS, and incubated with anti-Lck (1:2,000) in TBS with Tween 20 (TBST). Samples were then washed thrice with TBST prior to incubation with mouse anti-rabbit alkaline phosphatase. Bands were visualized by alkaline phosphatase reaction (Promega, Madison, Wis.).

Immunoprecipitation and kinase assay. Cells (1×10^6 to 2×10^6) were lysed in 200 μl of lysis buffer for 30 min on ice. To remove the cellular debris, lysates were centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was precleared with 100 μl of SAC (10% suspension in lysis buffer). Immunoprecipitation was then carried out by incubation of the lysate with the antibody for 1 h at 4°C. The mixture was further incubated with 50 μl of protein A-Sepharose beads (10% [wt/vol]) for 1 h. Immunoprecipitates were washed three times with ice-cold lysis buffer and suspended in 30 μl of kinase buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.2], 3.5 mM $MgCl_2$, 3.5 mM $MnCl_2$) containing 10 to 20 μCi of [γ-^{32}P]ATP and incubated at room temperature for 10 min. The sample were boiled for 5 min in SDS sample buffer then subjected to SDS-PAGE analysis, and the phosphorylated proteins were visualized by autoradiography.

For reprecipitations, SDS (1% [wt/vol]) and β-mercaptoethanol (0.1% [vol/vol]) were added to the samples, which were then denatured by boiling for 5 min, diluted to 0.1% SDS with lysis buffer, and subjected to immunoprecipitation as described previously (44). For the reprecipitation analysis of Jurkat cells, cells were solubilized in 1% Brij 96 lysis buffer with 1 mM phenylmethylsulfonyl fluoride and subjected to the first immunoprecipitation and in vitro kinase assay. The precipitated and ^{32}P-labelled proteins were solubilized again radioimmunoprecipitation assay lysis buffer, subjected to immunoprecipitation with the second antibody, and then analyzed by SDS-PAGE. Two-dimensional nonequilibrium pH gradient gel electrophoresis was conducted with ampholines of a pH range between 3 and 11 as described previously (35, 41).

In vivo labelling studies. Sf21 cells were infected with recombinant virus at a multiplicity of infection of 5. At 48 h postinfection, cells were washed and resuspended in phosphate-free medium. Labelling of 1×10^6 to 2×10^6 cells was performed with 1 mCi of $^{32}P_i$ (NEN) for 3 h in phosphate-free medium. The cells were subsequently harvested, and protein analysis was performed by SDS-PAGE and autoradiography or subjected to immunoprecipitations.

Phosphoamino acid analysis and phosphotryptic peptide mapping. Protein phosphorylated in vivo or in vitro was purified from SDS–10% polyacrylamide gels visualized by autoradiography. The extracted protein was subjected to acid hydrolysis for 3 h at 110°C with 200 μl of 6 M HCl. H_2O (200 μl) was added, and the samples were lyophilized and resuspended in 10 μl of H_2O. Aliquots (0.5 to 2 μl) were separated on a thin-layer cellulose plate (E. Merck, Darmstadt, Germany), using 3 μl of a mixture containing phosphoserine, phosphothreonine, and phosphotyrosine at a concentration of 1 mg/ml as reference. Separation of the amino acids was performed at 1,000 V (12). The phosphoamino acid markers were visualized by using ninhydrin spray, and the radioactivity was located by autoradiography.

For two-dimensional tryptic peptide mapping, proteins were eluted from gel slices and digested with 10 μg of trypsin, and

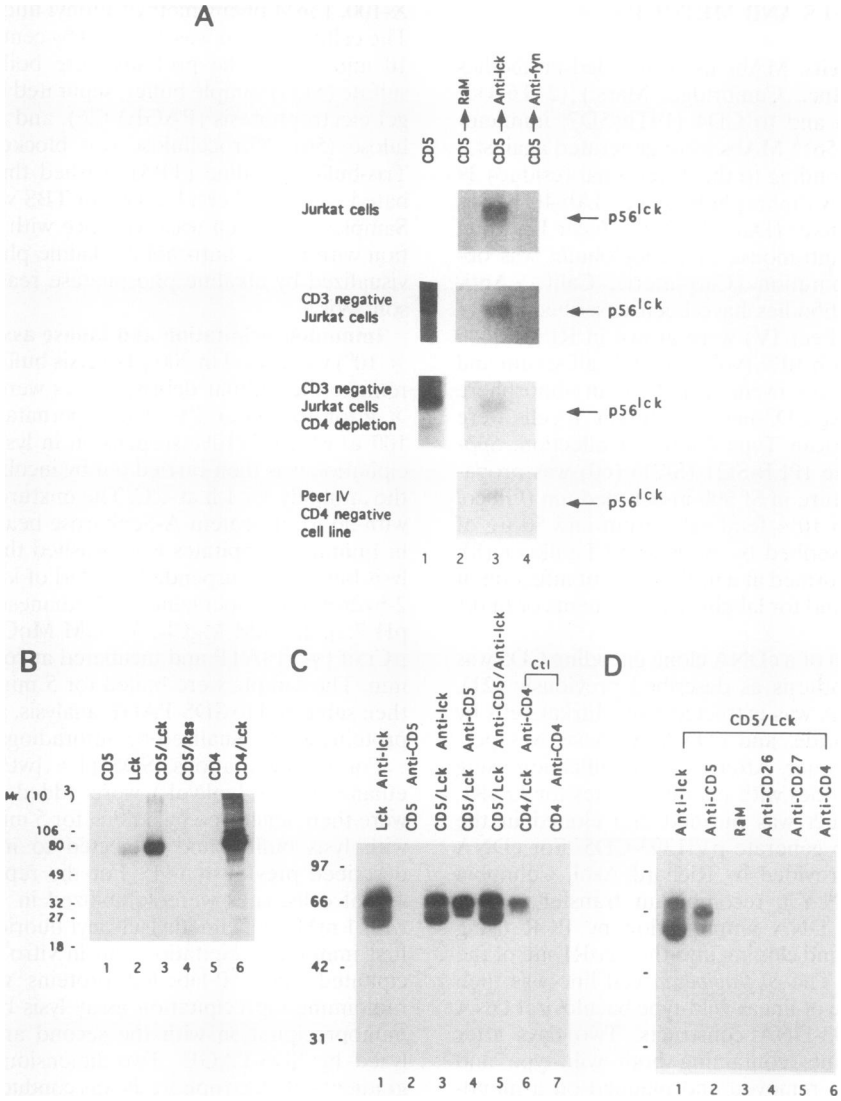

FIG. 1. p56^{lck} associates with CD5. (A) Reprecipitation analysis of T cells. ^{32}P-labelled anti-CD5 immunoprecipitates (lane 1) in 50 × 10^6 cell lysates in Brij 96 lysis buffer were solubilized again 1% radioimmunoprecipitation assay lysis buffer and subjected to immunoprecipitations by second antibodies followed by SDS-PAGE. Lane 2, rabbit anti-mouse; lane 3, anti-Lck; lane 4, anti-Fyn. CD4 depletion was performed with 100 × 10^6 CD3-negative Jurkat cell lysates in 1% Brij 96 lysis buffer by repeated anti-CD4 immunoprecipitations. (B) In vivo-labelled whole-cell lysates prepared from 1.5 × 10^5 cells from various combinations of infection were separated on an SDS–10% polyacrylamide gel and subjected to antiphosphotyrosine blotting. Lane 1, CD5 infection; lane 2, Lck infection; lane 3, CD5/Lck coinfection; lane 4, CD5/Ras coinfection; Lane 5, CD4 infection; lane 6, CD4/Lck coinfection. (C) Lysates of virus-infected Sf21 cells were prepared by using 1% Triton X-100 lysis buffer, immunoprecipitated with the indicated antibodies, labelled in a phosphotransferase assay, and separated on an SDS–10% polyacrylamide gel. Lane 1, Lck infection, anti-Lck; lane 2, CD5 infection, anti-CD5; lanes 3 to 5, CD5/Lck coinfection, anti-Lck (lane 3), anti-CD5 (lane 4), and combination of anti-Lck and anti-CD5 (lane 5); lane 6, CD4/Lck coinfection, anti-CD4; lane 7, CD4 infection, anti-CD4. (D) Immunoprecipitation of cell lysates from CD5/Lck coinfection. Lane 1, anti-Lck; lane 2, anti-CD5; lane 3, rabbit anti-mouse; lane 4, anti-CD26; lane 5, anti-CD27; lane 6, anti-CD4.

peptides were separated by thin-layer chromatography. The first dimension was conducted on 0.1-mm-thick cellulose plates (E. Merck) by electrophoresis in 50 mM ammonium bicarbonate (pH 8.9; 1,000 V for 30 min) followed by ascending chromatography in the second dimension [butanol-pyridine-acetic acid-water [75:50:15:60) (20, 37).

RESULTS

In an effort to establish whether CD5 was capable of direct binding to Src family members, the receptor was analyzed from various T cells and as expressed in the baculovirus expression system. With use of nondisruptive detergents, anti-CD5 has been found to coprecipitate p56^{lck}, p59^{fyn}, and the TcRζ/CD3 complex (5, 9). However, these studies did not distinguish between the direct binding of p56^{lck} to CD5 and the coprecipitation of other receptor complexes such as CD4-p56^{lck} or TcRζ-p59^{fyn}. To circumvent the possible coprecipitation of TcRζ/CD3 or CD4-p56^{lck} complexes, CD5 was precipitated from different TcRζ/CD3 and CD4-negative T-cell lines and assayed for coprecipitated kinase activity. [^{32}P]ATP-labelled immune complexes were then denatured by boiling in SDS and subjected to reprecipitation with anti-Lck and anti-Fyn antisera. As seen in Fig. 1A, p56^{lck}, but not p59^{fyn}, was readily coprecipitated with CD5 from lysates from Jurkat cells lacking

TcRζ/CD3 expression and from the same lysates that had been subjected to depletion with anti-CD4 antibody (Fig. 1A, lanes 3 and 4). Similarly, CD5 precipitations from CD4-negative Peer IV T cells were found to coprecipitate p56^{lck} (Fig. 1A, lower panel, lane 3). Control antibodies such as rabbit anti-mouse (lane 2) failed to reprecipitate material. These data showed that CD5 and p56^{lck} can interact in a cellular environment where CD4, CD8, and TcRζ/CD3 are not present.

To directly assess this possibility, CD5 and p56^{lck} were coexpressed in Sf21 insect cells and assayed for complex formation. Cell lysates from Sf21 cells that had been infected with various proteins (CD5, Lck, Ras, and CD4) were separated by SDS-PAGE and subjected to immunoblotting with an antiphosphotyrosine antibody (Fig. 1B). As expected, expression of p56^{lck} resulted in the detection of a band at 55 to 60 kDa corresponding to the kinase (lane 2), while expression of CD5 alone (lane 1), CD5 and p21^{ras} (lane 4), and CD4 (lane 5) failed to be detected with the antiphosphotyrosine antibody. By contrast, the coexpression of CD5 with p56^{lck} showed a greatly enhanced in vivo labelling of material in the molecular mass range of p56^{lck} (i.e., 55 to 60 kDa) (lane 3). A similar effect was observed in Sf21 cells which coexpressed CD4 and p56^{lck} (lane 6). This change in phosphotyrosine labelling upon coexpression of CD5 and p56^{lck} suggested a possible interaction.

In an effort to directly establish whether CD5 and p56^{lck} could physically interact, anti-CD5 was used to immunoprecipitate antigen from a Triton X-100-based detergent cell lysates, followed by labelling in an in vitro kinase assay with [^{32}P]ATP. Significantly, under these conditions, anti-CD5 precipitated high levels of p56^{lck} activity, resulting in the labelling of bands at 62 kDa and an intermediate band at 59 kDa (Fig. 1C, lane 4). By contrast, CD5 alone failed to show coprecipitated in vitro kinase activity, indicating that CD5 did not bind to detectable levels of an endogenous kinase in the Sf21 cells and that CD5 is itself not a kinase (Fig. 1C, lane 2). The level of activity in anti-CD5 from CD5/p56^{lck}-coinfected cells was similar to that precipitated by anti-p56^{lck} from cells infected with p56^{lck} alone (lane 1) or from cells infected with CD5 and p56^{lck} (lane 3). The major difference in the patterns was that anti-p56^{lck} coprecipitated bands primarily at 56 and 62 kDa, while anti-CD5 precipitated bands at 62 and 59 kDa (lanes 1 and 3 versus lane 4). As a positive control, anti-CD4 precipitates from cells coexpressing CD4 and p56^{lck} showed a band at 62 kDa (lane 6), while no material was detected from cells expressing CD4 alone (lane 7). As a negative control for the specificity of precipitation, rabbit anti-mouse antibodies (Fig. 1D, lane 3) and antibodies to CD26 (Fig. 1D, lane 4), CD27 (Fig. 1D, lane 5), or CD4 (Fig. 1D, lane 6) failed to precipitated kinase activity.

Glycosylated CD5 has a molecular mass of 69 kDa; however, incomplete processing of the protein expressed in Sf21 cells resulted in the synthesis of CD5 as a 55-kDa polypeptide. Two-dimensional isoelectric focusing of the labelled CD5-p56^{lck} complex showed two sets of spots at opposite ends of the pH gradient (Fig. 2A). A doublet corresponding to p56^{lck} was noted at the acidic end of the gradient at pH of 4.9 to 5.2, as previously described (4, 44). This corresponded to p56^{lck}, as shown in the pattern of anti-p56^{lck} precipitates from cells expressing *lck* alone (Fig. 2C). Coprecipitation of CD5-p56^{lck} was reciprocal, as shown by the ability of anti-p56^{lck} to coprecipitate CD5 (Fig. 2B) and vice versa (Fig. 2A). Peptide map analysis further showed the basic 55-kDa spot to have a pattern distinct from the upper and lower molecular mass p56^{lck} bands (Fig. 2D, lane 1 versus lanes 2 and 3). Reprecipitation analysis further showed that labelled CD5 could be precipitated from labelled immune complexes (Fig. 2E, lane 6).

The CD5-p56^{lck} complex could also be extracted in various detergents, as detected by immunoblotting with an anti-Lck antibody (Fig. 3). Anti-CD5 precipitates from Brij 96 (lane 4)-, Nonidet P-40 (NP-40) (lane 5)-, and Triton X-100 (lane 6)-based lysates coprecipitated kinase activity (upper panel) as well as p56^{lck}, as detected by immunoblotting (lower panel). The activity and amount of precipitable p56^{lck} did not vary significantly with different detergents. For the purpose of comparison, anti-CD4 also precipitated high levels of kinase activity from Brij 96-, NP-40-, and Triton X-100-based detergents (upper panel; lanes 1, 2, and 3, respectively). The amount of p56^{lck} protein precipitated by CD4 was significantly higher than that detected in association with CD5. Excluding the possibility that an additional component from insect cells might facilitate an interaction between receptor and kinase, these data strongly suggest that p56^{lck} associates with CD5 and that the interaction is stable in variety of detergents.

Given that CD5 and p56^{lck} associate in a complex, an important issue concerned whether CD5 binding could regulate p56^{lck} activity. Previous studies have shown that the interaction of middle T antigen of polyomavirus activates pp60^{src} activity (6, 13). In vitro kinase analysis demonstrated that coexpression of CD5 and p56^{lck} resulted in a marked increase in the autophosphorylation of p56^{lck} (Fig. 2A and B versus C). This was particularly the case when anti-CD5 was used to precipitate the kinase (Fig. 2A versus C). Further, under these conditions, the amount of p56^{lck} coprecipitated by CD5 (Fig. 3, lanes 4 to 6) was considerably less than that precipitated by the anti-p56^{lck} sera (data not shown). Importantly, similar observations were obtained from cells labelled in vivo with $^{32}P_i$. As seen in Fig. 4, anti-p56^{lck} precipitated a well-labelled doublet at 56 and 62 kDa from cells infected with the kinase alone (lane 1). No material was detected from noninfected cells (data not shown). Anti-CD5 precipitated labelled material in the 59- to 62-kDa range from cells coinfected with CD5 and p56^{lck} (lane 3). The bands comigrated with bands precipitated by anti-p56^{lck} (lane 4). As a negative control, rabbit anti-mouse antibodies failed to precipitate material (lane 2). Similarly, anti-CD5 failed to precipitated material from cells infected with CD5 alone (lane 5). As a positive control, coinfection of CD4 and kinase allowed the detection of the same doublet by anti-Lck (lane 8) or anti-CD4 antibodies (lane 7).

Two-dimensional tryptic peptide mapping of p56^{lck} labelled with $^{32}P_i$ showed two peptides, one possessing Y-394 and another corresponding to a peptide carrying Y-505 (Fig. 5A to C). The expression of p56^{lck} alone showed the labelling of both peptides, with more heavily labelling of Y-505 carrying peptide than Y-394 peptide (Fig. 5A). However, in contrast, the coexpression of p56^{lck} with CD5 caused a marked shift in the level of labelling of Y-394 relative to Y-505 (Fig. 5B and C). The labelling of Y-394 was further enriched when anti-CD5 was used to copurify p56^{lck}, indicating that altered phosphorylation pattern occurred on p56^{lck} associated with CD5 (Fig. 5B). However, a significant increase in Y-394 phosphorylation was also observed in anti-Lck precipitates, which should precipitate both CD5-bound and unbound enzyme (Fig. 5C). Quantitation of eluted peptides further demonstrated that p56^{lck} binding to CD5 induced a four- to fivefold increase in Y-394 phosphorylation (Fig. 5D). The increase in Y-394 phosphorylation was usually accompanied by a coordinate decrease in Y-505 phosphorylation (Fig. 5D). Taken together, in vitro and in vivo analyses indicate that kinase binding to CD5 was sufficient to stimulate p56^{lck} catalytic activity.

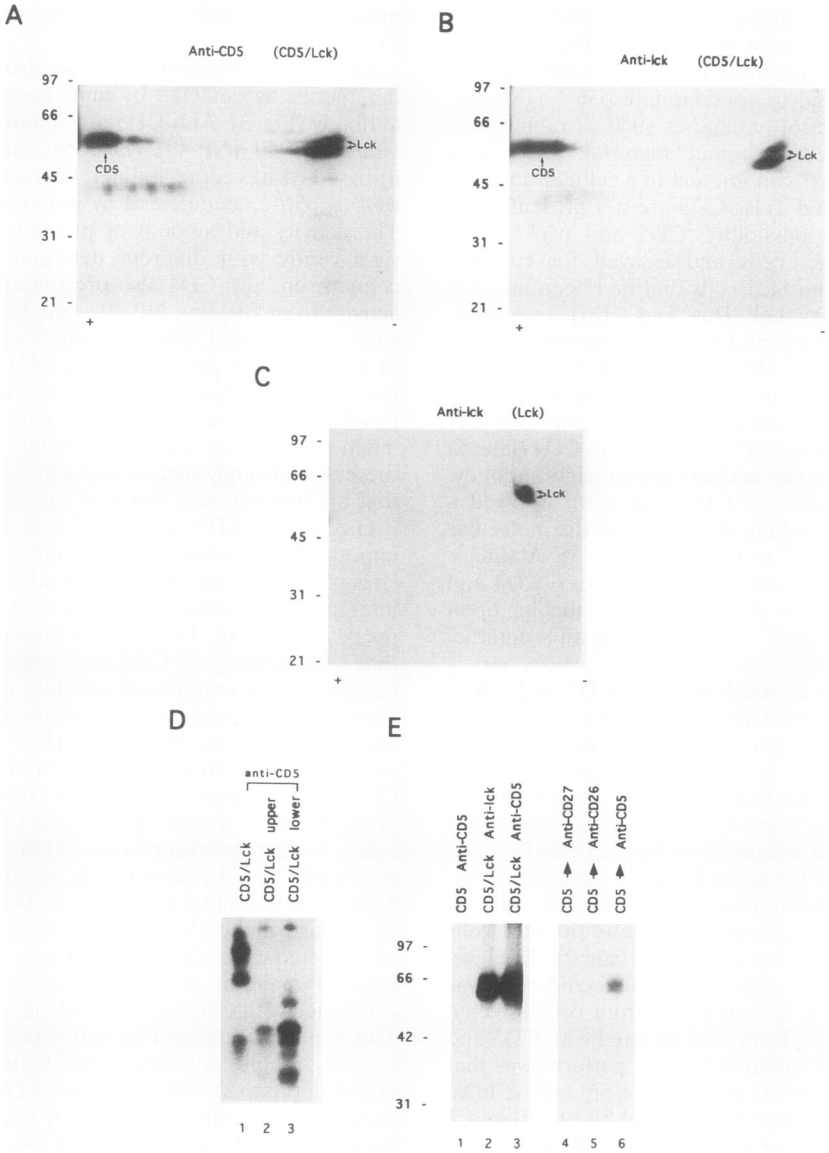

FIG. 2. Detection of the CD5-p56^{lck} complex. (A to C) Two-dimensional gel electrophoresis demonstrates the presence of p56^{lck} in complex formation with CD5 antigen. Immunoprecipitates from Triton X-100-based detergent lysates from Sf21 cells infected with CD5/Lck (A and B) or Lck alone (C) were conducted by using the designated antibody and were subjected to in vitro kinase labelling and two-dimensional isoelectric focusing. (D) Peptide map analysis from the two-dimensional gel electrophoresis in panel A. Phospholabelled CD5 and Lck proteins generated from anti-CD5 immunoprecipitates were eluted from the two-dimensional gel, digested with V8 protease, and analyzed by SDS-PAGE and autoradiography. Lane 1, basic 55-kDa spot; lane 2, upper Lck spot; lane 3, lower Lck spot. (E) Reprecipitation analysis of CD5/Lck complex. Anti-CD5 immunoprecipitates from CD5/Lck-coinfected Sf21 cells were labelled in a phophotransferase assay, denatured by boiling in SDS, and subjected to reprecipitation with the indicated antibodies. Lane 4, anti-CD27; lane 5, anti-CD26; lane 7, anti-CD5. Lane 1 to 3 represent the initial pattern of bands after immunoprecipitation with the first antibody. Sizes are indicated in kilodaltons.

It was next of interest to assess whether CD5 was itself phosphorylated in vivo by p56^{lck}. Previous work by ourselves (9) and others (14) has shown that CD5 is rapidly phosphorylated on tyrosine residues as a result of TcRζ/CD3 ligation. Furthermore, CD5 was readily labelled by p56^{lck} in vitro (Fig. 2). Two-dimensional gel analysis of anti-CD5 precipitates from Sf21 cells coinfected with CD5 and p56^{lck} and labelled in vivo with $^{32}P_i$ clearly showed the labelling of CD5 (Fig. 6A). Reprecipitation with anti-CD5 also precipitated CD5 (Fig. 6B, lane 4). Two bands were precipitated by anti-Lck (Fig. 6B, lane 3). Specificity was demonstrated by the inability of an irrelevant antibody against CD27 to precipitate antigen (Fig. 6B, lane 2). Phosphoamino acid analysis of in vivo-labelled CD5 revealed phosphorylation on tyrosine (Fig. 6A, right panel; Fig. 6B, right panel). As a positive control, p56^{lck} was also found labelled on tyrosine (Fig. 6A, right panel). CD5 was not labelled in the absence of coexpressed p56^{lck} (Fig. 4, lane 5). These data indicate that in addition to associating with the kinase, CD5 serves as a substrate for p56^{lck}.

Two approaches were used to begin to map the region within p56^{lck} responsible for binding to CD5. Initially, glutathione *S*-transferase (GST) fusion proteins consisting of the Lck SH2, Lck SH3, or Lck SH2/SH3 domains were used to precipitate CD5 from Sf21 cell lysates infected with CD5. These precipi-

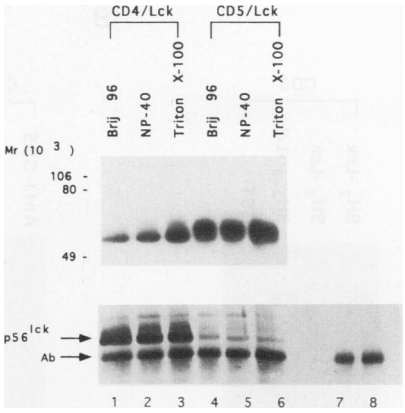

FIG. 3. CD5-p56^{lck} interaction is stable in different detergents. Sf21 cells (1.5×10^6) were coinfected with CD4/Lck (lane 1 to 3) or CD5/Lck (lane 4 to 6). The cells were lysed with either 1% Brij 96 (lanes 1 and 4), 1% NP-40 (lanes 2 and 5), or 1% Triton X-100 (lanes 3 and 6) lysis buffer, and immunoprecipitated with anti-CD4 (lanes 1 to 3) or anti-CD5 (lanes 4 to 6). The immunoprecipitates were labelled in a phosphotransferase assay (upper panel) or blotted with anti-Lck (lower panel). Lane 7, anti-CD4 MAb; lane 8, anti-CD5 MAb.

tates were then subjected to immunoblotting with a cocktail of anti-CD5 antibodies (Fig. 7A). Under these conditions, the GST-Lck SH2 domain precipitated significant amounts of CD5 (lane 1), while no material was precipitated with use of the Lck SH3 domain or the GST control (lanes 2 and 4). In another approach, the GST-SH2 protein was also found to precipitate a band corresponding to CD5 from insect cells that were infected with CD5 and labelled with [^{35}S]methionine (Fig. 7B). The GST-SH2 protein precipitated a heavily labelled band at 55 to 60 kDa (lane 3) that comigrated with a band precipitated by anti-CD5 (lane 1). The relatively faint amount of CD5 precipitated by the anti-CD5 antibody is reflected by the inability of antibody to function efficiently in immunoprecipitation (data not shown). The GST fusion protein failed to precipitate material (lane 2). Coexpression of Lck and CD5 followed by in vitro labelling showed an apparent increase in the level of material in the 55- to 60-kDa range precipitated by

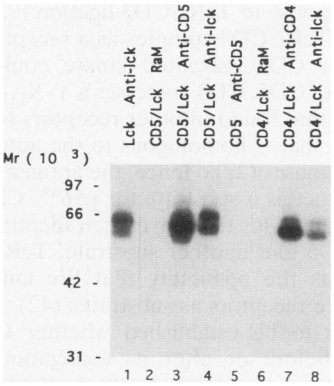

FIG. 4. In vivo ^{32}P labelling. Sf21 cells were either infected with Lck (lane 1) or CD5 (lane 5) alone or coinfected with CD5/Lck (lane 2 to 4) or CD4/Lck (lane 6 to 8) for 48 h and labelled with $^{32}P_i$ for a further 3 h. Equivalent portions of each lysate were immunoprecipitated with anti-Lck (lanes 1, 4, and 8), rabbit anti-mouse (lanes 2 and 6), anti-CD5 (lanes 3 and 5), and anti-CD4 (lane 7) antibodies. The labelled immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

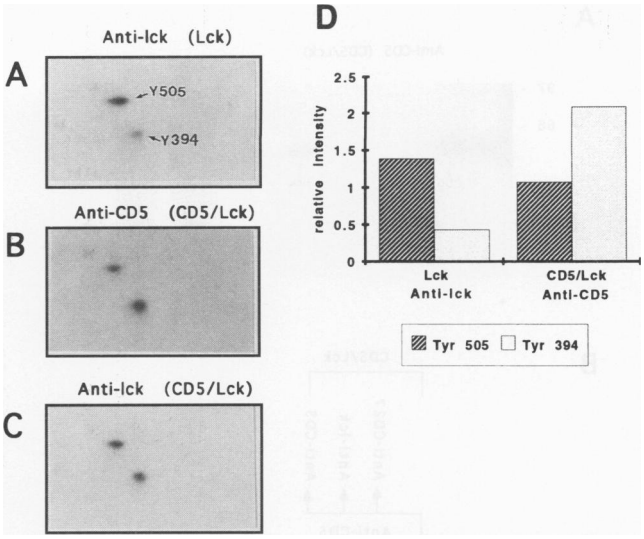

FIG. 5. Regulation of p56^{lck} activity by CD5. (A to C) Two-dimensional tryptic peptide maps of in vivo-phosphorylated p56^{lck}. Lck-infected Sf21 cells (A) and CD5/Lck-infected Sf21 cells (B and C) were labelled with $^{32}P_i$ in vivo, solubilized, and immunoprecipitated with either anti-Lck (A and C) or anti-CD5 (B) antibodies. The samples were separated by SDS-PAGE, and radiolabelled Lck proteins were subjected to digestion with trypsin. Peptides were then separated by electrophoresis and chromatography in two dimensions and analyzed by autoradiography. (D) Densitometric analysis of Tyr-505 and Tyr-394 from either anti-Lck or anti-CD5 immunoprecipitates. The autoradiography were scanned by a Scantjet laser scanner (Hewlet Packard Co.).

the SH2 domain (data not shown). This observation indicates that the p56^{lck} SH2 domain is capable of recognizing CD5 as expressed in insect cells.

DISCUSSION

Stimulation of T cells via the TcRζ/CD3 complex involves the combined action of coreceptors, protein-tyrosine kinases, and phosphatases. CD4 and CD8 binding to p56^{lck} is likely to contribute the kinase to a multimeric complex composed of the CD3 γ, δ, and ε chains and the TcR ζ and η chains. CD3 subunits and TcRζ in turn bind to other protein-tyrosine kinases, p59$^{fyn(T)}$ and ZAP-70 (11, 46). In this study, we have described another type of receptor-kinase interaction in which antigen can serve as both receptor and substrate for p56^{lck}. CD5-associated p56^{lck} was detected in T cells, either in the absence or in the presence of the TcRζ/CD3 or CD4-p56^{lck} complex (Fig. 1), and was expressed in the baculovirus expression system (Fig. 2 to 6). Coprecipitation of CD5 and p56^{lck} was reciprocal, being detected with either anti-CD5 or anti-Lck antibodies (Fig. 2). Complexes were detected at various stages (24 to 70 h) of the infection cycle and were not dependent on levels of protein expression (data not shown). The CD5-p56^{lck} complex is relatively stable, since it was also readily detected in different detergents (including Triton X-100 and NP-40), as detected by in vitro kinase labelling and immunoblotting (Fig. 3).

The CD5-p56^{lck} interaction exhibits several novel characteristics. First, coexpression of CD5 with p56^{lck} induced an increase in kinase activity, as measured by increased in vitro activity (Fig. 2). Increased in vitro phosphorylation of enolase was also detected (data not shown). When standardized for amounts of protein, the in vitro activity of p56^{lck} showed a 10-

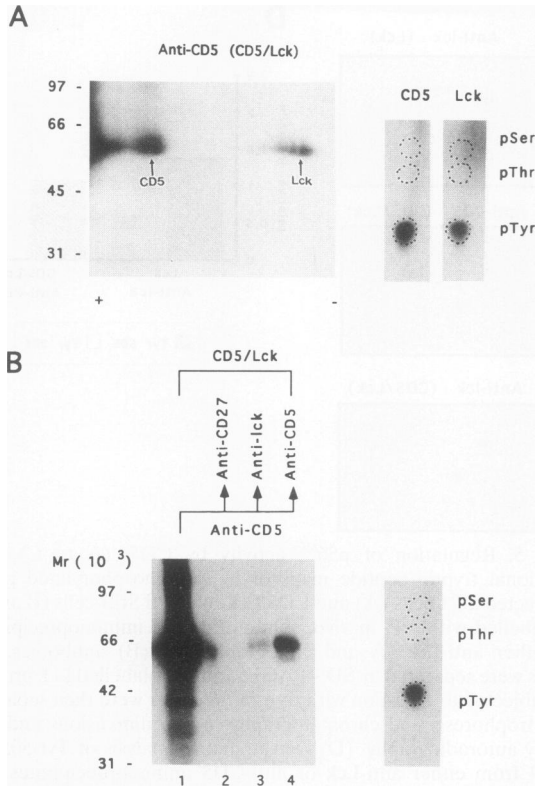

FIG. 6. CD5 is phosphorylate by associated p56^{lck} in vivo. Sf21 cells (1.5×10^6) were coinfected with CD5/Lck for 48 h and labelled with $^{32}P_i$ for a further 3 h. (A) Two-dimensional gel electrophoresis after immunoprecipitation with anti-CD5 (left) and phosphoamino acid analysis of the CD5 and Lck spots (right). The bands representing CD5 and Lck were eluted from the gel and processed as described previously (33, 39). The samples were spotted onto a thin-layer chromatography plate and separated by electrophoresis in one dimension. Positions of ninhydrin-stained phosphoamino acid standards phosphoserine, phosphotyrosine, and phosphothreonine (pSer, pTyr, and pThr, respectively) are circled. Sizes are indicated in kilodaltons. (B) Reprecipitation analysis. The in vivo-labelled CD5/Lck complexes were initially precipitated with anti-CD5 (lane 1) and subjected to reprecipitation with anti-CD27 (lane 2), anti-Lck (lane 3), and anti-CD5 (lane 4). Phosphoamino acid analysis of the phospholabelled CD5 antigen (lane 4) was conducted as described in Materials and Methods.

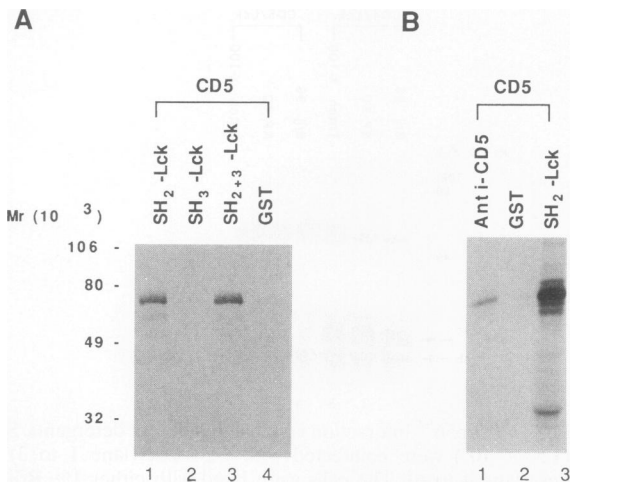

FIG. 7. GST-SH2 domain of Lck precipitates CD5 expressed in insect cells. (A) Sf21 cells were infected with CD5, lysed in 1% Triton X-100, and subjected to precipitation with GST-Lck SH2 (lane 1), GST-Lck SH3 (lane 2), GST-Lck SH2/SH3 (lane 3), and GST alone (lane 4). The precipitates were separated on an SDS–10% polyacrylamide gel and subjected to anti-CD5 blotting. (B) Sf21 cells expressing CD5 were labelled in vivo with [^{35}S]methionine, lysed, and precipitated with anti-CD5 antibody (lane 1), GST (lane 2), and GST-Lck SH2 (lane 3).

to 15-fold increase in kinase activity relative to free kinase. Importantly, in vivo phosphorylation studies also showed a four- to fivefold increase in phosphorylation at Y-394 (Fig. 5). Different antibodies to the N-terminal and C-terminal regions of Lck precipitated the same levels of activity, arguing that antibody recognition did not itself alter the activity of the kinase (data not shown). Whether the activation of p56^{lck} is induced by intermolecular binding to cytoplasmic sequences or by the formation of CD5 dimers remains to be established. Positively charged peptides have been reported to activate p56^{lck} (7). Consistent with this, the CD5 cytoplasmic sequence possesses a region enriched in positively charged residues (KKLVKKFRQKK) that is conserved in humans and mice (19). CD5-p56^{lck} therefore appears to differ from other receptor systems in that some activation occurs in the absence of an extracellular ligand. The functional rationale for this mode of regulation is unknown but may involve the need for primed receptor-kinase complexes in the case of low-affinity cell-to-cell interactions. CD5 on T cells has been reported to bind to CD72 on B cells and as such to play a role in T-B collaboration (31, 59). Interchain regulation of Src kinase has previously been noted for middle T antigen of polyomavirus or p47$^{gag\text{-}crk}$ binding to pp60^{src} (13, 32). Whether other Src-related kinases can interact with CD5 is unclear. While we have previously shown that p59$^{fyn(T)}$ can be coprecipitated with CD5 from peripheral T cells that had been solubilized in the detergent Brij 96 (9), the association was generally not seen in anti-CD5 precipitates from Jurkat cells (Fig. 1A). Unlike CD5 and p56^{lck}, the coexpression of CD5 with p59$^{fyn(T)}$ yielded inconsistent results (data not shown).

Unlike CD4 and CD8-p56^{lck} (44), CD5 serves as a substrate for the kinase to which it binds. Coexpression of CD5 and p56^{lck} resulted in the tyrosine phosphorylation of CD5, as detected by in vivo labelling (Fig. 6). This observation is consistent with the previous finding that CD5 is rapidly phosphorylated in response to TcRζ/CD3 ligation (9, 14). p56^{lck} in proximity to the TcRζ/CD3 complex as a receptor-free kinase or CD4/CD8- or CD5-associated kinase could induce the phosphorylation of CD5. CD5 possesses a Y-X_{11}-Y-XXL motif that is similar to that found in other receptors (40), as well as a motif (DNEY) that is homologous to the autophosphorylation site for Src kinases (22). Hence, the antigen appears to be well designed to act as a substrate for p56^{lck}. CD5 represents one of the few substrates that have been identified for p56^{lck}. The fact that CD5 and another substrate, TcRζ, are surface receptors suggests the possibility that Src kinases have an affinity for surface receptors as substrates (42). In the case of CD5, it remains to be established whether CD5 becomes phosphorylated before or after its association with p56^{lck}. p56^{lck} could function in a kind of autoregulatory loop, phosphorylating the site to which it eventually binds by means of its SH2 domain.

Binding and phosphorylation of CD5 by p56^{lck} is likely to contribute to the functional properties of this antigen. Not surprisingly, anti-CD5 antibody can provide costimulatory signals in the proliferation of T cells, similar to CD4 and CD8 (1, 2, 16, 51). Similarly, certain MAbs to CD5 have been

reported to directly stimulate T-cell growth or to stimulate in conjunction with CD28 (10, 28, 63). Anti-CD5 cross-linking can also induce the tyrosine phosphorylation of numerous substrates (1, 9). Therefore, CD5-p56^{lck} may provide signals analogous to those of the CD4- and CD8-p56^{lck} complexes. CD5 association with the TcRζ/CD3 complex would facilitate this coreceptor function (5, 9). The ability of CD5 to act as a substrate for p56^{lck} is also reminiscent of the TcR/CD3-associated ζ chain. Both can be coprecipitated with the TcR/CD3 complex in mild detergents and are rapidly phosphorylated upon TcR/CD3 ligation. TcRζ phosphorylation allows for the recruitment of ZAP-70 by means of SH2 binding (11). CD5-CD72 binding in T-B collaboration may regulate p56^{lck} binding and activity. Phosphorylation of CD5 may in turn allow for the subsequent association of intracellular proteins.

The use of GST fusion proteins provided evidence in support of the notion that the SH2 domain of p56^{lck} may bind to CD5. GST-p56^{lck} SH2 fusion proteins precipitated CD5 from insect cell lysates, while GST alone failed to bind to the antigen (Fig. 7). SH2 domains are composed of a β-pleated sheet surrounded by two α helices that bind to phosphotyrosine and adjacent hydrophobic residues (15, 64). Src kinase SH2 domains bind a certain defined motifs, with optimal binding to pYEEI (50). CD5 carries several potential binding sites that include Y-407 (YKKL), Y-458 (YSQP), Y-470 (YPAL), and Y-492 (YDLH) (19, 22). Although none are optimal, the Y-407 (YKKL) and Y-470 (YPAL) motifs share residues at the first and third positions with the alternate Src SH2 domain binding motif pYXXL (50). GST-SH2 recognition of CD5 was made against lysates from insect cells expressing CD5. Insect cells carry endogenous tyrosine kinases that may have provided sufficient phosphorylation to allow for SH2 domain recognition of CD5. The phosphorylation may have been too low to be detected by antiphosphotyrosine immunoblotting (Fig. 1B, lane 1) or by in vivo labelling (Fig. 4, lane 5). Further work is required to determine whether Lck SH2 domain recognition of CD5 occurs in T cells and whether other Src-related kinases are capable of associating with CD5.

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