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### Permalink

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### Journal

Journal of Experimental Medicine, 183(1)

### ISSN

0022-1007

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### Publication Date

1996

### DOI

10.1084/jem.183.1.169

Peer reviewed

## The SH3 Domain of p56<sup>lck</sup> Binds to Proline-rich Sequences in the Cytoplasmic Domain of CD2

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### Summary

CD2, a cell surface glycoprotein expressed on T cells and natural killer cells, can couple to signaling pathways that result in T cell proliferation. An Src-like protein tyrosine kinase, p56<sup>lck</sup>, coprecipitates with CD2, and perturbation of CD2 by monoclonal antibodies results in an increase in the activity of p56<sup>lck</sup>, suggesting that an interaction with p56<sup>lck</sup> contributes to CD2-mediated signaling. Herein, we investigate the mechanism by which CD2 associates with p56<sup>lck</sup>. We demonstrate that CD2 and p56<sup>lck</sup> associate when coexpressed in nonlymphoid cells, that this association requires the cytoplasmic domain of CD2, and that the SH3 domain of p56<sup>lck</sup> mediates its interactions with CD2. Using truncation mutants of CD2, we identify two regions in the cytoplasmic domain of CD2 involved in binding p56<sup>lck</sup>. Each region contains a proline-rich sequence that, in the form of a synthetic peptide, directly binds p56<sup>lck</sup>. Thus, proline-rich sequences in the cytoplasmic domain of CD2 allow this transmembrane receptor to bind to the SH3 domain of p56<sup>lck</sup>.

A variety of receptor–ligand interactions occur when T lymphocytes recognize and respond to antigenic peptides bound to MHC molecules on the surfaces of other cells. Although this response depends on the TCR, other cell surface molecules contribute to T cell activation by promoting adhesion to the APC and by providing biochemical signals. CD2, a 55-kD cell surface glycoprotein expressed by T lymphocytes and NK cells, subserves adhesion functions by binding CD48, CD58, and CD59 (1–3). It is likely that CD2 also has a role in generating transmembrane signals during the activation of T cells and NK cells (4, 5). Perturbation of CD2 can trigger the cytolytic activity of NK cells and can provide a potent stimulus for T cell activation, inducing proliferative responses comparable to those elicited by either mitogenic lectins or mAbs to the TCR (4, 5). In several experimental systems, CD2-mediated signaling augments antigen-induced activation of T cells (6, 7). Moreover, administration of nondepleting CD2 mAbs to mice induces T cell unresponsiveness, indicating that CD2 either has a critical role in T cell activation in vivo or can deliver signals leading to unresponsiveness (8). CD2-deficient mice, however, do not manifest an obvious phenotype, suggesting that, if CD2 does have a critical role in physiological T cell activation, other molecules can assume that role when T lymphocytes develop in the absence of CD2 (9). Alternatively, the functional deficit resulting from the absence of CD2 may be subtle. Of interest in this

regard, recent data suggest that CD2 plays a unique role in the reversal of T cell anergy in vitro (10).

The mechanism by which CD2 couples to signal transduction pathways is uncertain. When T cells are lysed with digitonin or Brij, CD2 coimmunoprecipitates with the TCR (11–14). Therefore, like CD4 and CD8, CD2 appears to be in close physical proximity with the TCR (11–14). Indeed, CD2-mediated activation requires coexpression of TCR or of receptor complexes, such as CD16, that contain the TCR- $\zeta$  chain or the closely related Fc $\epsilon$  receptor  $\gamma$  chain (15, 16). Thus, agonist mAbs to CD2 fail to activate T cell lines that express abundant CD2 but that lack TCR (15). As is the case with the TCR, appropriate stimulation of CD2 induces tyrosine phosphorylations and polyphosphoinositide turnover (17–20). These CD2-induced signaling events depend on the cytoplasmic domain of CD2, which is relatively large (116 amino acids), is highly conserved between human, rat, and mouse, and lacks intrinsic kinase activity (21–24).

The remarkable capacity of CD2 to activate T cells and NK cells raises the possibility that, like the TCR, CD4, and CD8, CD2 interacts with cytoplasmic protein tyrosine kinases (PTK).<sup>1</sup> Several observations support this possibility. Perturbation of CD2 induces protein tyrosine phosphoryla-

<sup>1</sup>Abbreviations used in this paper: F.L., full-length; GST, glutathione-S-transferase; PTK, protein tyrosine kinases; Sf9, *Spodoptera frugiperda*.

tions, and CD2 signaling requires expression of CD45, a transmembrane tyrosine phosphatase that regulates the activities of Src-like PTK (20, 25). Stimulation of CD2 on T cells leads to an increase in the specific activity of p56<sup>lck</sup>, a lymphoid-specific Src-like PTK (26). We and others have found that p56<sup>lck</sup> as well as p59<sup>fyn</sup> coimmunoprecipitate with CD2 from detergent lysates of rat T lymphocytes and NK cells, raising the possibility that CD2 associates directly with these Src-like PTK (12, 13, 27). Herein we examine the mechanism by which CD2 associates with p56<sup>lck</sup>.

## Materials and Methods

**Cell Lines, Cell Culture, mAbs, and Other Reagents.** COS 6M cells were grown in DME with 10% FCS. *Spodoptera frugiperda* (Sf9) cells were grown in complete Grace's medium. Anti-rat CD2 mAbs OX-34, OX-54, and OX-55 were the kind gift of A. Williams (Oxford University, Oxford, UK).

**Generation of Fusion Proteins and Anti-GST Antiserum.** The cloning of Wild Type p56<sup>lck</sup> into the baculovirus shuttle vector pBMS-1 has been previously reported (28). Domains encompassing the unique regions SH2 and SH3 were cloned in frame with GST into the pGEX-2T vector (Pharmacia, Uppsala, Sweden) by PCR. The unique sequence fusion encompassed amino acids 8–65, whereas the SH3 and SH2 fusions encompassed amino acids 66–113 and 114–235, respectively, of the Wild Type gene. The unique sequence and GST alone were produced with isopropyl- $\beta$ -D-thiogalactopyranoside according to the manufacturer's instructions (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and used for injections in New Zealand white rabbits for polyclonal antisera production. HIS-T7 epitope-tagged fusion proteins were made by cloning the appropriate portions of the rat CD2 cytoplasmic domain in frame into the pet-28 vector (Novagen, Inc., Madison, WI) by PCR. Protein production was induced with IPTG and purified according to the manufacturer's instructions.

**Generation of CD2 Mutants.** The CD2 truncation mutants were generated by PCR using the rat cDNA provided by A. Williams. The 5' oligonucleotide located upstream of the CD2 start site contained an EcoRI site and was used for all PCR. Its sequence is 5'-TATATATATAGAATTCCACTGGGA AAAGAA-TGTAAAGATGAGATG-3'. To generate the truncation mutants, we synthesized antisense oligonucleotides containing a stop codon after the appropriate amino acid followed by restriction sites. The sequences for CY6, CY40, CY78, CY85, and CY105 are 5'-TATTTAAGCTTGCGGCCGCTAGTTCGGTTTTTTT-CCTCTTCA-3', 5'-TATATATATGAATTCGTGCGACCTA-TGATGCTGGTGAGGCCTGGGTTGAGTGTGGCTTGGG-3', 5'-TATATAATAGTCGACGCGGCCGCTATCTCTTC-TTCGGCTGGTGCTCACG-3', 5'-TATAATATAGTCGAC-GCGGCCGCTACTGTGTGCCTGATGGAGGAGGTCTC-3', and 5'-TATATATATGAATTCGTGCGACCTAACAGGAGG-CCTTGGCTGAACTCGGGGCTGGGCAAAGG-3', respectively. The PCR products were cloned into pSM for expression in COS cells and pVL1393 for recombination with BaculoGold DNA and expression in Sf9 cells (PharMingen, San Diego, CA). The internal deletion designated CY49.69 (lacking amino acids 49–69 of the cytoplasmic tail of rat CD2) was generated by overlap PCR as described (29). 5' and 3' overlapping PCR fragments were generated for the internal deletion. Oligonucleotides used for the PCR reactions were as follows: 5' fragment of CY49.69, 5'-TATATATATAGAATTCCACTGGGAAAAGAATGTAAAGATGAGATG-3' and 5'-CTTCGGCTGGTGCTCACGGTT-

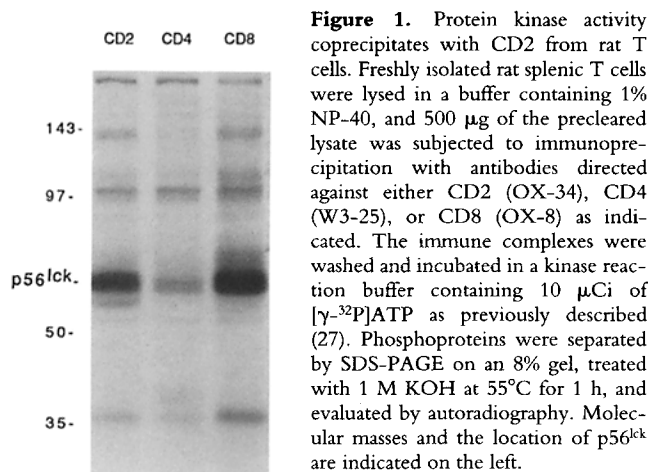
AGCTTGGGAAGCCACTGGATT-3'; and the 3' fragment of CY49.69, 5'-AATCCAGTGGCTTCCCAAGCTAACCGTGAG-CACCAGCCGAAG-3' and 5'-TATAAAGTCGACGCGGCC-GCTTAATTAGGGGGTGGCAACGAAAC-3'. The internal deletion designated CY7.41 (lacking amino acids 7–41 of the cytoplasmic tail of rat CD2) was also generated by overlap PCR using the following primers: 5' fragment of CY7.41, 5'-TATATAGAA-TTCCACTGGGAAAAGAATGTAAAGATGAGATG-3' and 5'-GTGCTCACGGTTGCCAGGTGTCTGGAGATG-3'; and the 3' fragment of CY7.41, 5'-CAGACACCTGGCAACCGTGAG-CACCAGCCG-3' and 5'-TATAAAGTCGACGCGGCCGCT-TAATTAGGGGGTGGCAACGAAAC-3'. The DNA sequence of the cytoplasmic domain of each mutant was verified.

**Expression of Full-Length and Mutant CD2 and p56<sup>lck</sup> in COS Cells and Sf9 Cells.** The various CD2 constructs and p56<sup>lck</sup> were expressed in COS cells by transient transfection using DEAE dextran (Sigma Chemical Co., St. Louis, MO) according to published methods (30). To express the constructs in Sf9 cells, we transfected the various constructs into Sf9 cells in the presence of BaculoGold DNA (PharMingen) according to the manufacturer's instructions, harvested, and amplified the recombinant baculovirus. The virus was then used for large-scale preparations of recombinant protein.

**Immunoprecipitations, In Vitro Kinase Assays, and Immunoblotting.** Anti-CD2 immunoprecipitations were performed using either mAb OX-34 (mouse IgG2b) coupled to Pansorbin cells (Calbiochem Corp., La Jolla, CA), or mAb OX-55 (mouse IgG1) coupled either to Pansorbin cells coated with rabbit anti-mouse Ig antiserum (Cappel Laboratories, Malverne, PA) or to Sepharose-coupled goat anti-mouse Ig (Cappel Laboratories). In vitro kinase assays were performed as described (27). Anti-GST immunoblots were performed by blocking the gel transferred to Immobilon-P (Millipore Corp., Bedford, MA) in 20 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20 (TBST) with 3% nonfat dried milk for 30 min followed by incubation with anti-GST antiserum (1:500 in TBST with 3% milk) for 2 h at room temperature. The blots were washed three times for 5 min in TBST and incubated with <sup>125</sup>I-labeled protein A (Amersham Corp., Arlington Heights, IL) at 0.5 mCi/ml in TBST with 3% milk for 1 h at room temperature. The blots were washed three times for 5 min in TBST, dried, and evaluated by autoradiography. Anti-rat CD2 immunoblots were performed as above using mAb OX-34 at 0.1  $\mu$ g/ml in TBST with 3% milk, and anti-T7 immunoblots were performed using anti-T7 mAb (Novagen, Inc.) according to the manufacturer's instructions.

**Peptide Generation.** The peptides used in these studies were purchased from either Chiron Mimotopes Peptide Systems (San Diego, CA) or Research Genetics (Huntsville, AL), analyzed by mass spectrometry or HPLC, and were  $\geq$ 70% pure. The sequences of each peptide are as follows: peptide 1, ASQAPPPGHH; peptide 2, PGHRPLPPSHR; peptide 3, PKKRPPPSGTQ; peptide 4, GQKGPLPRPRV; and peptide 5, RVQPKPPCGSG. (Peptide 4 is a 12-mer; a glycine was added to the NH<sub>2</sub> terminus to prevent cyclization of the NH<sub>2</sub>-terminal glutamine.) Equivalent molar quantities of the peptides were conjugated to activated CH Sepharose 4B (Pharmacia LKB Biotechnology Inc.) per the manufacturer's instructions and were used to precipitate purified GST/p56<sup>lck</sup>.

**Determination of Ca<sup>2+</sup> Fluorescence and IL-2 Production.** Jurkat cells were loaded with the Ca<sup>2+</sup>-sensitive fluor and Indo-1, and calcium fluorescence was monitored as previously described (31). To determine IL-2 production, we cultured Jurkat cells at 10<sup>6</sup> cells/ml in 96-well plates and stimulated the cells for 18 h with



**Figure 1.** Protein kinase activity coprecipitates with CD2 from rat T cells. Freshly isolated rat splenic T cells were lysed in a buffer containing 1% NP-40, and 500  $\mu$ g of the precleared lysate was subjected to immunoprecipitation with antibodies directed against either CD2 (OX-34), CD4 (W3-25), or CD8 (OX-8) as indicated. The immune complexes were washed and incubated in a kinase reaction buffer containing 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP as previously described (27). Phosphoproteins were separated by SDS-PAGE on an 8% gel, treated with 1 M KOH at 55°C for 1 h, and evaluated by autoradiography. Molecular masses and the location of p56<sup>lck</sup> are indicated on the left.

media, PMA alone (10 ng/ml), the combination of OX-54 (20  $\mu$ g/ml), OX-55 (20  $\mu$ g/ml) and PMA, or the combination of C305 (1:500 dilution from ascites) and PMA. Culture supernatants were assayed for IL-2 using a human IL-2 cytoscreen immunoassay kit (Biosource International, Camarillo, CA) according to the manufacturer's instructions.

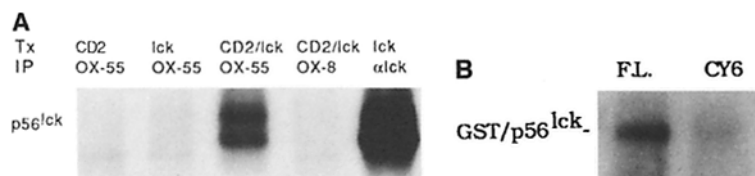
## Results

**CD2 and p56<sup>lck</sup> Associate in Rat T Cells, and This Association Can Be Reproduced in COS Cells.** As is the case with CD4 and CD8, immunoprecipitates of CD2 from 1% NP-40 lysates of splenic T cells contain a protein kinase activity detected by in vitro kinase assays (Fig. 1). We and others have identified p56<sup>lck</sup> as one of the PTKs present in these CD2 immunoprecipitates (12, 13, 27). To determine whether CD2 and p56<sup>lck</sup> associate in a nonlymphoid cell line, we transfected COS cells (a fibroblast line that does not express p56<sup>lck</sup>) with cDNAs encoding p56<sup>lck</sup> and rat CD2. After 72 h, the cells were solubilized in a buffer containing 1% NP-40, and the resulting lysate was subjected to immunoprecipitation with antibodies directed against CD2 and p56<sup>lck</sup> (Fig. 2 A). An in vitro kinase assay performed on the CD2 immunoprecipitate generated prominent phosphorylated bands that comigrated with autophosphorylated p56<sup>lck</sup>. The presence of kinase activity in the CD2 immunoprecipitate required transfection with both the CD2 and p56<sup>lck</sup> cDNAs. There was no kinase activity in CD2 immunoprecipitates from COS cells trans-

fecting with p56<sup>lck</sup> alone, indicating that the CD2 mAb does not cross-react with a PTK. Similarly, in vitro kinase reactions on CD2 immunoprecipitates from COS cells transfected with CD2 alone failed to reveal bands corresponding to Src-family members. Taken together, these results indicate that rat CD2 and p56<sup>lck</sup> associate when coexpressed in COS cells. Comparable results were obtained when human CD2 was coexpressed with p56<sup>lck</sup> in COS cells (data not shown). CD2, therefore, does not require the expression of any other lymphoid specific molecules to associate with p56<sup>lck</sup>.

CD2 also binds p56<sup>lck</sup> when the latter is in the form of a purified GST fusion protein. After incubating GST/p56<sup>lck</sup> with lysate from CD2-expressing COS cells, we immunoprecipitated CD2 and then analyzed the immunoprecipitate for the presence of GST/p56<sup>lck</sup> by immunoblotting with an antiserum to GST. As shown in Fig. 2 B, GST/p56<sup>lck</sup> coprecipitates with CD2. This association requires the presence of the CD2 cytoplasmic domain, as evidenced by the failure to detect GST/p56<sup>lck</sup> in immunoprecipitates of a truncation mutant of CD2 (CY6) that lacks all but the first six amino acids of the cytoplasmic domain of CD2.

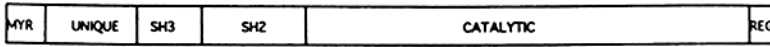
**The SH3 Domain of p56<sup>lck</sup> Mediates Association with CD2.** To determine the region of p56<sup>lck</sup> that binds to CD2, we constructed GST fusion proteins containing the following domains of p56<sup>lck</sup>: the NH<sub>2</sub>-terminal unique region, the SH3 domain, and the SH2 domain (Fig. 3). In these experiments, we used Sf9 cells (a blowfly salivary gland cell line) that expressed CD2 after gene transfer. Each of the fusion proteins was incubated with equal aliquots of CD2-expressing Sf9 lysate, and then immunoprecipitations with anti-CD2 mAb were performed. The immunoprecipitate was analyzed by immunoblotting with anti-GST. The GST/SH3 domain fusion protein coprecipitated with CD2, but GST alone, the GST/unique region fusion protein, and the GST/SH2 domain fusion protein did not (Fig. 4 A). Next we determined if the GST/SH3 domain fusion protein could competitively inhibit the association of CD2 with full-length GST/p56<sup>lck</sup> (we used the fusion protein rather than p56<sup>lck</sup> because the fusion protein could be easily purified, permitting better control of its concentration). In each of several experiments, the GST/SH3 domain fusion protein, but not GST/unique region or GST alone (data not shown), inhibited the binding of CD2 to GST/p56<sup>lck</sup> (Fig. 4 B). As was the case with GST/p56<sup>lck</sup>, the GST/SH3 domain fusion protein associated with full-length (F.L.)



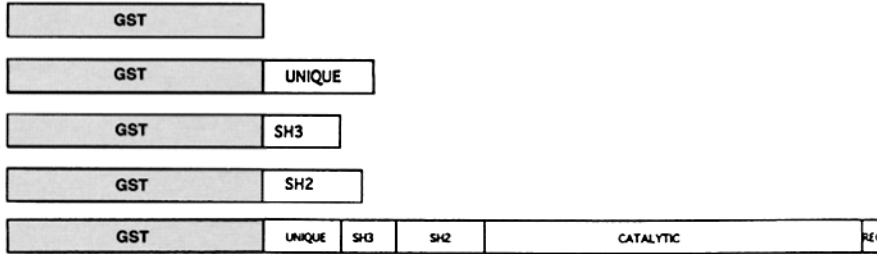
**Figure 2.** The association of CD2 with p56<sup>lck</sup> can be reproduced in nonlymphoid cells and requires the cytoplasmic domain of CD2. (A) p56<sup>lck</sup> coprecipitates with rat CD2 when expressed in COS cells. COS cells were plated onto 100-cm<sup>2</sup> dishes and transfected with 5  $\mu$ g of plasmid DNA encoding the molecules indicated (Tx). After 72 h, the cells were lysed in buffers containing 1% NP-40, and the precleared lysates were subjected to immunoprecipitation with the indicated antibodies (IP). The immune complexes were washed and incubated in a kinase reaction buffer containing 20  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP. Phosphoproteins were separated by SDS-PAGE on an 8% gel and evaluated by autoradiography. OX-55 (anti-rat CD2 mAb), OX-8 (isotype-matched control mAb recognizing rat CD8), and  $\alpha$ lck (rabbit antiserum recognizing p56<sup>lck</sup>) were used in the immunoprecipitations. (B) GST/p56<sup>lck</sup> fusion protein associates with full-length CD2 (F.L.) but not a tailless CD2 construct (CY6) produced in COS cells. 3  $\mu$ g of purified GST/p56<sup>lck</sup> was added to a COS cell lysate containing either F.L. CD2 or a truncation mutant lacking a cytoplasmic domain (CY6). CD2 was immunoprecipitated from the lysates, and the CD2 immunoprecipitates were then analyzed by immunoblotting with anti-GST antiserum.

the indicated antibodies (IP). The immune complexes were washed and incubated in a kinase reaction buffer containing 20  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP. Phosphoproteins were separated by SDS-PAGE on an 8% gel and evaluated by autoradiography. OX-55 (anti-rat CD2 mAb), OX-8 (isotype-matched control mAb recognizing rat CD8), and  $\alpha$ lck (rabbit antiserum recognizing p56<sup>lck</sup>) were used in the immunoprecipitations. (B) GST/p56<sup>lck</sup> fusion protein associates with full-length CD2 (F.L.) but not a tailless CD2 construct (CY6) produced in COS cells. 3  $\mu$ g of purified GST/p56<sup>lck</sup> was added to a COS cell lysate containing either F.L. CD2 or a truncation mutant lacking a cytoplasmic domain (CY6). CD2 was immunoprecipitated from the lysates, and the CD2 immunoprecipitates were then analyzed by immunoblotting with anti-GST antiserum.

## p56<sup>lck</sup> STRUCTURE



### GST FUSION PROTEINS



**Figure 3.** Schematic representation of p56<sup>lck</sup> and the GST/p56<sup>lck</sup> fusion proteins. The structure of p56<sup>lck</sup> can be divided into an NH<sub>2</sub>-terminal myristylation site (MYR), a unique region, SH3 and SH2 domains, a catalytic domain, and a COOH-terminal regulatory region (REG).

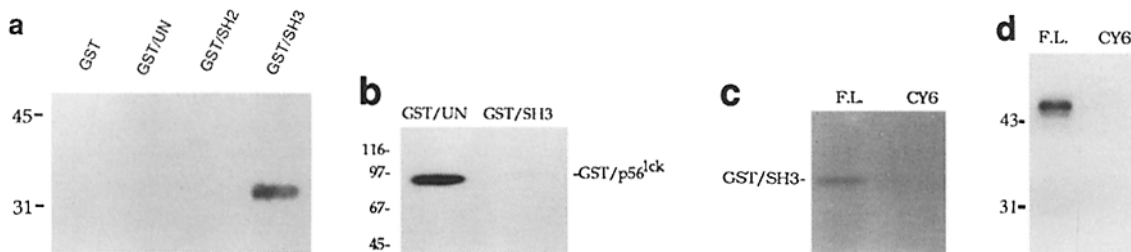
CD2 but not the tailless CY6 mutant of CD2 (Fig. 4 C). Similarly, immobilized GST/SH3 domain fusion protein precipitated F.L. CD2, but not CY6, from Sf9 lysates (Fig. 4 D).

*The Cytoplasmic Domain of CD2 Directly Associates with p56<sup>lck</sup>.* To determine if p56<sup>lck</sup> binds directly to the cytoplasmic domain of CD2, we constructed two nickel histidine T7 epitope-tagged fusion proteins: one of the full-length cytoplasmic domain of CD2 (T7-CY) and one containing the first 40 amino acids of the cytoplasmic domain of CD2 (T7-40). The fusion proteins were purified to homogeneity, immobilized onto Sepharose with an anti-T7 mAb, and used to affinity precipitate purified GST/SH3 fusion protein. The GST/SH3 fusion coprecipitated with the full-length cytoplasmic domain but not with the fusion protein containing the first 40 amino acids (Fig. 5 A). Similarly, immobilized GST/p56<sup>lck</sup> fusion protein precipitated T7-CY, but not T7-40 (Fig. 5 B). These studies indicate p56<sup>lck</sup> binds directly to the cytoplasmic domain of rat CD2 and that the first 40 amino acids of the cytoplasmic domain of CD2 are not sufficient for this binding.

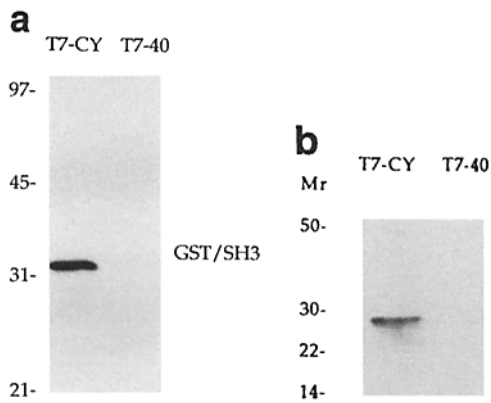
*Identification of the Minimal Cytoplasmic Domain of CD2 Required for Binding to p56<sup>lck</sup>.* To determine the minimal

cytoplasmic domain of CD2 needed for binding to p56<sup>lck</sup>, we constructed a series of CD2 truncations in addition to CY6. Mutants containing 40, 78, 85, and 105 of the 116 amino acids in the cytoplasmic tail of rat CD2 were generated by PCR and were designated CY40, CY78, CY85, and CY105, respectively (Fig. 6).

We expressed F.L. CD2 and each of the mutants in Sf9 cells and then determined their ability to associate with p56<sup>lck</sup>. Lysates from the CD2-expressing Sf9 cells were combined either with wild-type p56<sup>lck</sup> produced in Sf9 cells (Fig. 7 A) or with purified GST/p56<sup>lck</sup> (Fig. 7 B). F.L. CD2 and the CD2 mutants were immunoprecipitated, and the immunoprecipitates were analyzed either by *in vitro* kinase reaction (Fig. 7 A) or by immunoblotting with anti-GST antiserum (Fig. 7 B). In both experiments, equivalent activities or amounts of p56<sup>lck</sup> coprecipitated with F.L. and CY105 (Fig. 7). As the cytoplasmic tail of CD2 was shortened from 105 amino acids to 85 amino acids, there was a decrease in the associated p56<sup>lck</sup> to ~30% of that seen with F.L. (determined by densitometry of Fig. 7 B). There was no change in the associated p56<sup>lck</sup> as the cytoplasmic tail was shortened from 85 to 78 amino acids (Fig. 7 B). As the



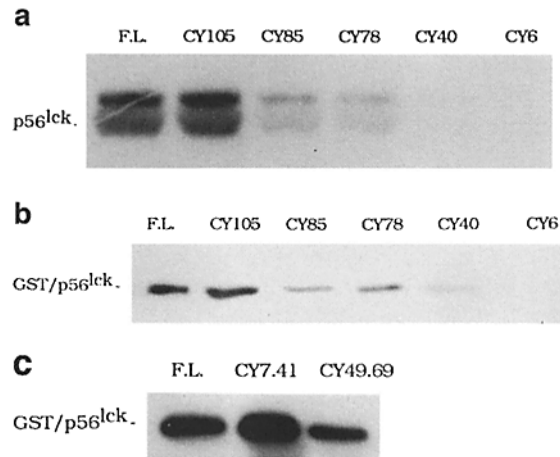
**Figure 4.** The association between CD2 and p56<sup>lck</sup> requires the cytoplasmic domain of CD2 and the SH3 domain of p56<sup>lck</sup>. (a) After the addition of 1.5  $\mu$ g of GST, GST/p56<sup>lck</sup> unique region, GST/p56<sup>lck</sup> SH2 domain, or GST/p56<sup>lck</sup> SH3 domain to Sf9 lysate containing F.L. CD2, CD2 was immunoprecipitated, and the immunoprecipitate was analyzed for associated fusion protein by anti-GST immunoblotting. (b) 5  $\mu$ g of GST/p56<sup>lck</sup> unique region or GST/p56<sup>lck</sup> SH3 domain (final concentration ~333 nM each) was incubated in 500  $\mu$ l Sf9 lysate containing F.L. CD2 for 30 min before the addition of 3  $\mu$ g GST/p56<sup>lck</sup> (66 nM). The lysates were precleared and then immunoprecipitated with mAb directed against rat CD2 and analyzed for associated fusion protein by immunoblotting with anti-GST. Binding of the GST/p56<sup>lck</sup> fusion protein (31 kD) to CD2 is not detected because of its lower molecular mass. (c) 1.5  $\mu$ g of GST/p56<sup>lck</sup> SH3 domain was added to Sf9 lysate containing either F.L. CD2 or CY6, which was then immunoprecipitated with mAb directed against rat CD2 and analyzed for associated fusion protein by immunoblotting. (d) To equimolar amounts of F.L. CD2 and CY6, sepharose bound GST/p56<sup>lck</sup> SH3 was added and incubated for 1.5 h. The affinity precipitations were separated by SDS-PAGE and immunoblotted with anti-rat CD2 mAb. Molecular masses are indicated on the left. Each figure is representative of at least three independent experiments.



**Figure 5.** The cytoplasmic tail of CD2 binds directly to p56<sup>lck</sup>. (a) T7-CY (lane 1) and T7-40 (lane 2) purified fusion proteins were immobilized onto anti-T7 mAb coupled to Sepharose bound rabbit anti-mouse antiserum and used to affinity precipitate 0.5  $\mu$ g of GST/SH3 fusion protein in 1% NP-40 lysis buffer. The precipitates were washed three times in lysis buffer, eluted with Laemmli sample buffer, separated by SDS-PAGE, and immunoblotted with anti-GST antiserum. The presence of equimolar amounts of T7-CY and T7-40 was confirmed by immunoblotting with anti-T7 mAb (data not shown). (b) To equimolar amounts of T7-CY and T7-40 fusion protein, Sepharose bound GST/p56<sup>lck</sup> SH3 was added and incubated for 1.5 h. The GST fusion protein was eluted in 10 mM glutathione, and the affinity precipitations were separated by SDS-PAGE and immunoblotted with anti-T7 mAb. Molecular masses are indicated on the left. Each figure is representative of at least three independent experiments.

cytoplasmic domain was truncated to 40 amino acids, virtually all p56<sup>lck</sup> binding was eliminated (Fig. 7). Although a small amount of GST/p56<sup>lck</sup> was seen in the CY40 immunoprecipitate, this was not a consistent finding in other experiments. Immunoblots of these samples with a mAb to extracellular CD2 revealed that there was severalfold more CY40 than F.L. (data not shown). When corrected for the amount of CD2 in the immunoprecipitates in Fig. 7, the GST/p56<sup>lck</sup> in CY40 immunoprecipitates represents  $\sim$ 2% of that in F.L. CD2 immunoprecipitates.

The data in Fig. 7 are consistent with two possibilities. First, there may be two binding sites for p56<sup>lck</sup> in CD2: one between amino acids 40 and 78 and another between amino acids 85 and 105. Alternatively, there may be a proximal binding site that is secondarily affected by the truncation such that its ability to bind is impaired. To distinguish between these possibilities, we generated two internal deletion mutants of CD2: one lacking amino acids 49–69 and the other lacking amino acids 7–41 of the cytoplasmic domain (designated CY49.69 and CY7.41, respec-



**Figure 7.** Association of p56<sup>lck</sup> with F.L. rat CD2 and truncation and internal deletion mutants of rat CD2. Lysates of Sf9 cells expressing F.L. or the indicated CD2 mutants were mixed with either p56<sup>lck</sup> produced in Sf9 cells (a) or purified GST/p56<sup>lck</sup> fusion protein (b and c). The lysates were precleared, immunoprecipitated with anti-rat CD2, and subjected to in vitro kinase reactions (a) or immunoblotted with anti-GST antiserum (b and c). Expression of the CD2 constructs was analyzed by flow cytometry, and anti-CD2 blotting of the lower half of the immunoblots confirmed the presence of equimolar concentrations of each of the constructs (data not shown).

tively; Fig. 6). We expressed F.L. CD2 and each of the internal deletion mutants in Sf9 cells and determined their ability to associate with purified GST/p56<sup>lck</sup>. Immunoprecipitates of F.L. CD2 and the CD2 mutants were analyzed by immunoblotting with anti-GST antiserum (Fig. 7 C). In each of several experiments, CY7.41 consistently bound more GST/p56<sup>lck</sup> than F.L. indicating that the membrane-proximal portion of the cytoplasmic domain of CD2 is not required for binding to p56<sup>lck</sup>. CY49.69 bound GST/p56<sup>lck</sup>, but not as well as F.L., CD2, consistent with a possible p56<sup>lck</sup> binding site between amino acids 40 and 78. Taken together with the truncation studies, these data indicate that CD2 has two discrete regions that bind p56<sup>lck</sup>: one between amino acids 40 and 78 and the other between amino acids 85 and 105 of the cytoplasmic tail of rat CD2.

*Proline-rich Sequences from the Cytoplasmic Domain of CD2 Bind p56<sup>lck</sup>.* Studies by several investigators indicate that the binding sites for SH3 domains are rich in proline residues (32–35). The cytoplasmic tail of CD2 has several proline-rich domains. Two of these (amino acids 92–95 and

#### AMINO ACID SEQUENCE OF THE CYTOPLASMIC DOMAIN OF F.L. CD2 AND EACH OF THE CD2 MUTATIONS

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F.L.      KRKKRNRNRKGELEIKASRMSTVERGPKPHSTQASAPASQNPVASQAPPPGGHLLQTPGHRPLPPSHRNREHQPKRPPPSGTQVHQKQGPPLRPRVQPKPPCGSGDVSLEPPN
CY105    KRKKRNRNRKGELEIKASRMSTVERGPKPHSTQASAPASQNPVASQAPPPGGHLLQTPGHRPLPPSHRNREHQPKRPPPSGTQVHQKQGPPLRPRVQPKPPC
CY85     KRKKRNRNRKGELEIKASRMSTVERGPKPHSTQASAPASQNPVASQAPPPGGHLLQTPGHRPLPPSHRNREHQPKRPPPSGTQVHQKQGPPLRPRVQPKPPC
CY78     KRKKRNRNRKGELEIKASRMSTVERGPKPHSTQASAPASQNPVASQAPPPGGHLLQTPGHRPLPPSHRNREHQPKRPPPSGTQVHQKQGPPLRPRVQPKPPC
CY40     KRKKRNRNRKGELEIKASRMSTVERGPKPHSTQASAPASQNPVASQAPPPGGHLLQTPGHRPLPPSHRNREHQPKRPPPSGTQVHQKQGPPLRPRVQPKPPC
CY6      KRKKRN
CY49.69 KRKKRNRNRKGELEIKASRMSTVERGPKPHSTQASAPASQNPVASQAPPPGGHLLQTPGHRPLPPSHRNREHQPKRPPPSGTQVHQKQGPPLRPRVQPKPPCGSGDVSLEPPN
CY7.41   KRKKRN
  
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**Figure 6.** Amino acid sequence of the cytoplasmic domain of F.L. rat CD2 and the various CD2 mutants. Proline-rich regions are underlined in the F.L.

101–104; Fig. 6) lie in the putative binding regions indicated above between amino acids 85 and 105. Two other proline-rich regions lie between amino acids 49 and 66, the putative binding region deleted in the CY49.69 mutant. A fifth proline-rich sequence (amino acids 79–81) lies in a region whose truncation had no detectable effect on the ability of CD2 to associate with p56<sup>lck</sup>.

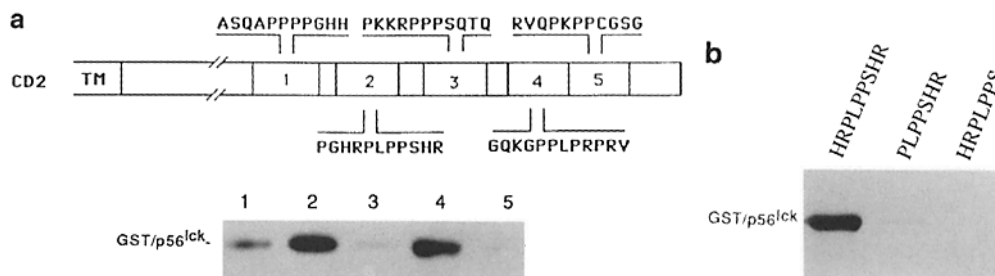
To investigate the possibility that one or more of the CD2 proline-rich sequences could bind p56<sup>lck</sup>, we synthesized 11-mer peptides corresponding to the five proline-rich regions between amino acids 49 and 105. The peptides were coupled to activated Sepharose and then incubated in lysis buffer with full-length GST/p56<sup>lck</sup>. Eluates from the immobilized peptides were assayed for the presence of GST/p56<sup>lck</sup> by anti-GST immunoblotting. Two peptides (designated 2 and 4) consistently bound GST/p56<sup>lck</sup> (Fig. 8 A). Peptide 2 corresponds to amino acids 59–69 and thus lies within the putative binding site between amino acids 40 and 78. Peptide 4 represents amino acids 89–99 and is within the putative binding site between amino acids 85 and 105. Peptide 1, which also lies within the amino acid 40–79 site, bound GST/p56<sup>lck</sup> poorly (relative to peptides 2 and 4) in two experiments (Fig. 8 A) or not at all in eight experiments (data not shown). Peptide 3 (which resides between amino acids 75 and 85) and peptide 5 (which resides between amino acids 98 and 108) failed to bind in every experiment (Fig. 8 A). There also was no binding to a control peptide corresponding to a CD2 cytoplasmic sequence that was not rich in prolines (data not shown).

Because peptide 2 bound GST/p56<sup>lck</sup> best, we determined the effect on binding of truncating this peptide. The following peptides were synthesized, immobilized on Sepharose, and assayed for their ability to bind GST/p56<sup>lck</sup>: HRPLPPSHR, PLPPSHR and HRPLPPS. Only HRPLPPSHR bound GST/p56<sup>lck</sup> (Fig. 8 B). This peptide differed from peptide 2 by the deletion of PG on the NH<sub>2</sub> terminus. The removal of the HR from either the NH<sub>2</sub> terminus or the COOH terminus resulted in loss of binding (Fig. 8 B), suggesting either that the flanking basic amino acids are required for binding of this peptide to the SH3 domain of p56<sup>lck</sup> or that the peptide length must exceed seven residues. Although our studies do not distinguish between these possibilities, the sequences of the first and second proline-rich regions

are quite similar, the major difference being that the second has basic amino acids at both flanks of the prolines, whereas the first has basic residues at the carboxy terminus only. The absence of NH<sub>2</sub>-terminal basic residues may explain the decreased ability of the peptide derived from the first proline-rich region (peptide 1) to bind GST/p56<sup>lck</sup>.

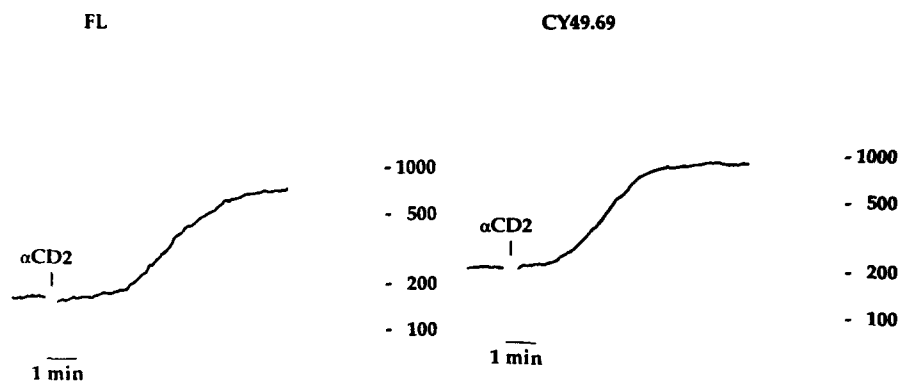
*The First Two Proline-rich Regions in the Cytoplasmic Tail of CD2 Are Not Required for Coupling to Signaling Pathways.* Previous studies examining the structural requirements for signal transduction by human CD2 indicated that the first two proline-rich regions in the cytoplasmic domain of human CD2 were required for signal transduction by CD2 (21, 36, 37). Chang et al. (21) demonstrated that a truncation mutant retaining 77 amino acids, but not one retaining 43 amino acids, of the cytoplasmic domain of human CD2 could couple to a rise in free intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>) and IL-2 production, suggesting that an essential sequence for human CD2 signal transduction lies between amino acids 43 and 77. Point mutations on either side of the second proline-rich domain did not affect IL-2 production, but a substitution of DE for the HR after the first proline-rich region abrogated signaling, consistent with a key role of the first proline-rich region in CD2 signal transduction (37). This finding could not be corroborated by Hahn and Bierer (36), who found that the same HR to DE substitution failed to have an effect on CD2 signaling. They did note that deletion of either the first or second proline-rich region resulted in a reduction of CD2-mediated IL-2 production, and elimination of both regions completely abrogated CD2 signaling (36). Therefore, although the studies differed on the relative importance of the first and second proline-rich regions, both studies clearly defined this region as essential for CD2-mediated signaling.

Our CY78 mutant, which retains the first two proline-rich regions in the cytoplasmic domain of CD2, can bind p56<sup>lck</sup> in vitro and, thus, is consistent with the notion that the binding of p56<sup>lck</sup> by CD2 is important in CD2-mediated signaling. On the other hand, based on the earlier functional studies, our CY49.69 would not be expected to couple to signaling pathways even though we find that it binds p56<sup>lck</sup> in vitro (36, 37). Therefore, we investigated the possibility that CY49.69 could couple to signaling pathways in T cells. To this end, we expressed F.L. rat



**Figure 8.** Binding of GST/p56<sup>lck</sup> by proline-rich peptides. (a) Proline-rich peptides in the cytoplasmic domain of CD2 can bind p56<sup>lck</sup>. 11-mer peptides corresponding to five proline-rich regions (1–5) in the cytoplasmic domain of CD2 were synthesized (peptide 4 is a 12-mer; a glycine was added to the NH<sub>2</sub> terminus to prevent cyclization of the NH<sub>2</sub>-terminal

glutamine). The locations and sequences of the peptides are indicated in the diagram. The peptides were coupled to Sepharose and used to precipitate purified GST/p56<sup>lck</sup>. (b) The effect of truncations on the ability of peptide 2 to bind p56<sup>lck</sup>. Peptides indicated above the lanes in Fig. 8 b were synthesized, conjugated to Sepharose, incubated with equivalent concentrations of purified GST/p56<sup>lck</sup>, and immunoblotted with anti-GST antiserum.



**Figure 9.** Anti-CD2 mAb (OX-54 and OX-55) trigger an increase in  $[Ca^{2+}]_i$  in F.L. CD2 and CY49.69 when expressed in Jurkat. The calcium-sensitive fluorescence of a suspension of Jurkat cells expressing F.L. rat CD2 and CY49.69 was monitored over time. At the indicated times, the cells were stimulated with 5  $\mu$ g of OX-54 and 5  $\mu$ g of OX-55 for every  $10^6$  cells. Values for  $[Ca^{2+}]_i$  (nM) were calculated as described and are indicated on the righthand side of the figure (31). These data are representative of at least three separate experiments.

CD2 and the CY49.69 mutant in the human T cell line, Jurkat, and assessed the ability of each to couple to a rise in  $[Ca^{2+}]_i$  and to produce IL-2 after CD2 stimulation.

As previously described, F.L. CD2 can stimulate a rise in  $[Ca^{2+}]_i$  when activated with a pair of anti-rat CD2 mAb (OX-54 and OX-55; Fig. 9). Similarly, the CY49.69 construct stimulated a rise in  $[Ca^{2+}]_i$  comparable to that seen with F.L. CD2 when activated with OX-54 and OX-55 (Fig. 9). To determine whether these constructs are capable of producing IL-2 in response to anti-CD2 stimulation, we stimulated F.L. CD2 Jurkat and CY49.69 with PMA alone, the combination of OX-54, OX-55, and PMA, or the combination of C305 (anti-TCR) and PMA. As previously demonstrated, stimulation of F.L. rat CD2 results in IL-2 production comparable to that seen after TCR stimulation (Table 1). CY49.69 also produced IL-2 in response to CD2 stimulation, and the amount of IL-2 produced was comparable to that elicited by TCR stimulation (Table 1). These data demonstrate that the first two proline-rich regions in the cytoplasmic tail of CD2 are not required for CD2 to transduce signals.

## Discussion

In this report we have examined the mechanism by which CD2 and p56<sup>lck</sup> associate. The interaction between these does not require the coexpression of other leukocyte-specific molecules, as evidenced by the ability of CD2 and p56<sup>lck</sup> to associate in COS cells and Sf9 cells. Therefore, al-

though CD2 coprecipitates with a number of lineage-restricted molecules, including the TCR, CD4, CD45, and CD53, none of these need be present for CD2 to associate with p56<sup>lck</sup> (11, 12). The association requires the cytoplasmic domain of CD2, to which the SH3 domain of p56<sup>lck</sup> binds. Truncation mutants of CD2 reveal two regions whose deletion leads to a substantial decrease in the amount of p56<sup>lck</sup> that coprecipitates with CD2. When the cytoplasmic domain is shortened from 105 to 85 amino acids, there is a 70% decrease in the amount of p56<sup>lck</sup> binding, relative to full length CD2. As the cytoplasmic tail of CD2 is further shortened from 78 to 40 amino acids, virtually all binding to p56<sup>lck</sup> is lost. Each of these regions contains a proline-rich sequence that, in the form of an immobilized, synthetic peptide, binds p56<sup>lck</sup>. Thus proline-rich sequences, which are a distinctive feature of the CD2 cytoplasmic domain, allow this transmembrane receptor to couple to p56<sup>lck</sup> by binding its SH3 domains.

SH3 domains, which are found primarily in enzymes and other molecules involved in signal transduction, mediate protein-protein interactions (38). A striking feature of the known binding sites for SH3 domains is the frequency of proline residues (32). The sequence requirements for interactions with the SH3 domains of most proteins, including p56<sup>lck</sup>, are not known. Yu et al. (39) recently screened a 9-mer combinatorial peptide library in order to identify amino acid sequences that can bind the SH3 domain of p60<sup>Src</sup>. The library, which was in the form of XXXPPX-PXX, was biased to include a common PXP motif. Two classes of peptides bound to the Src SH3 domain. All mem-

**Table 1.** IL-2 Production (pg/ml) in Full-Length CD2 and CY49.69 Jurkat Clones after Stimulation with Anti-Rat CD2 (OX-54 and OX-55) or Anti-TCR mAb (C305)

Clone	Experiment 1			Experiment 2		
	PMA	CD2 + PMA	TCR + PMA	PMA	CD2 + PMA	TCR + PMA
Full-length	<10	392	641	<10	>1,500	1,570
CY49.69	<10	835	707	<10	407	848

$10^6$  cells were stimulated overnight with PMA (10 ng/ml) alone, anti-rat CD2 mAb (25 mg/ml of OX-54 and OX-55) plus PMA, or anti-TCR mAb (1:500 dilution from C305 ascites) plus PMA. Culture supernatants were assayed for IL-2 as described in Materials and Methods. Data presented are representative of at least four independent experiments. At least two separate clones for each construct were evaluated.



bers of the first group had an RXL motif at the amino terminus and a leucine at position 6; the majority had a basic amino acid, usually arginine, at the penultimate position and a hydrophobic amino acid at the carboxy terminus. Peptide 2 from the CD2 cytoplasmic domain also has an amino terminal RXL motif and an arginine at the equivalent of the penultimate position of the library peptide. In the CD2 sequence this arginine (which is the COOH terminus of peptide 2) is followed by a hydrophobic residue. Peptide 4 contains the motif PPLPR, which is the common feature of the second class of Src SH3 domain-binding peptides. Thus, both CD2 sequences that bind p56<sup>lck</sup> share key features with peptides selected for their ability to bind to the SH3 domain of a closely related PTK, p60<sup>Src</sup>. In contrast, analysis of the other proline-rich sequences in CD2 fails to reveal similarities between these sequences and the Src SH3 domain-binding peptides.

By interacting with the SH3 domain of p56<sup>lck</sup>, CD2 may influence the enzymatic activity of this PTK. Phosphorylation of a COOH-terminal tyrosine residue (tyrosine 505 in p56<sup>lck</sup>) down regulates the activity of Src-like PTKs (40). The phosphorylated COOH-terminal tyrosine likely binds to the SH2 domain of the kinase, causing the PTK to fold back on itself and rendering the kinase inactive. It has been proposed that an interaction between the SH3 domain and a proline-rich region in the COOH terminus of p56<sup>lck</sup> further stabilizes this "closed" conformation (41). Indeed, mutations in the SH3 domain of Src-like PTKs, which would prevent this interaction, increase kinase activity (40). When p56<sup>lck</sup> binds to CD2, its SH3 domain is occupied, preventing it from interacting with the COOH-terminal proline-rich region of p56<sup>lck</sup>. This may destabilize the inactive conformation of p56<sup>lck</sup> and favor an "open" or active form of the kinase. It is possible, therefore, that there is an important qualitative difference between CD2-associated p56<sup>lck</sup> and the p56<sup>lck</sup> associated with receptors, such as CD4 or CD8, that, by interacting with the unique region of p56<sup>lck</sup>, should not affect the stability of the inactive form of the enzyme (42, 43).

Previous studies by several investigators have focused on the potential importance of proline-rich sequences for signaling by human CD2 (21, 36, 37). Mutants with as few as the first 77 amino acids of the cytoplasmic domain of human CD2 were capable of coupling to a rise in  $[Ca^{2+}]_i$  and IL-2 production, whereas truncations to  $\leq 43$  amino acids failed to couple to signaling pathways, suggesting that the region between amino acids 43 and 77 was required for CD2 signal transduction (21). This region contains the first two proline-rich domains. Mutational analysis of this region has led to conflicting results. Chang et al. (37) found that substitution of the HR with DE after the first proline-rich region, but not the second, resulted in a loss of signaling by CD2. Alternatively, when Hahn and Bierer (36) made the same mutation, they failed to detect a change in IL-2 production after CD2 stimulation. Internal deletion mutants excising either of the PPPGHR motifs in the first and second proline-rich regions reduced CD2-mediated IL-2 production, and deletion of this motif in both regions

resulted in complete abrogation of CD2-mediated IL-2 production (36). Consistent with the findings presented above, an internal deletion lacking amino acids 19–77 of the cytoplasmic tail of human CD2 also failed to produce IL-2 in response to CD2 stimulation (36).

In our experiments, deletion of the first and second proline-rich regions (CY49.69) had little if any effect on the ability of CD2 to couple to a rise in  $[Ca^{2+}]_i$  or to IL-2 production. Although the rat and human sequences differ slightly in the first two proline-rich regions, they are identical in the fourth and fifth proline-rich regions (amino acids 80–103 in the human), suggesting that a construct comparable to our CY49.69 in the human would signal. The reason for the discrepancy between our results and those of Hahn and Bierer (36) may lie in the way the constructs were made. One of Hahn and Bierer's constructs retained the sequences between the proline-rich domains and the other excised a larger portion of the cytoplasmic domain than our CY49.69. This may have resulted in secondary structural changes that prevented the fourth proline-rich region from binding p56<sup>lck</sup>.

Our studies indicate that specific proline-rich regions in the COOH-terminal half of the cytoplasmic domain of CD2 mediate the direct interactions of CD2 and p56<sup>lck</sup> by binding the SH3 domain of p56<sup>lck</sup>. Carmo et al. (13), however, have shown that p56<sup>lck</sup> is present in CD2 immunoprecipitates from the CY40 mutant expressed in Jurkat. These immunoprecipitations were performed using Brij 96 lysates. It is possible that the proximal 40 amino acids in the cytoplasmic domain of CD2 may associate with other molecules that mediate an association with p56<sup>lck</sup> and that these associations are not maintained in the conditions we used for our studies (1% NP-40 lysis buffer). Our studies indicate that the first 40 amino acids do not participate in the direct binding to p56<sup>lck</sup>. Neither our CY40 truncation mutant nor our T7-40 fusion protein coprecipitated with GST/p56<sup>lck</sup>. Additionally, our 7.41 internal deletion mutant, which lacks amino acids 7–41 of the cytoplasmic tail of CD2, consistently bound p56<sup>lck</sup> better than F.L. CD2.

Because the CD2 sequences that bind the p56<sup>lck</sup> SH3 domain resemble the consensus sequences for binding the p60<sup>Src</sup> SH3 domain, interactions between CD2 and the Src family may not be limited to p56<sup>lck</sup> but may involve other members as well. Several observations indicate that this is likely the case. Both p59<sup>lyn</sup> and p56<sup>lck</sup> coimmunoprecipitate with CD2 from T cells and NK cells, raising the possibility that p59<sup>lyn</sup> directly interacts with CD2 (13, 14, 27). We also have found that another Src-like kinase, p54<sup>lyn</sup>, coimmunoprecipitates with CD2 when the latter is expressed in RBL, a rat basophilic leukemia line that does not express p56<sup>lck</sup> (G. Bell; unpublished observations). We are currently investigating whether the SH3 domains of p59<sup>lyn</sup> and p54<sup>lyn</sup> mediate these interactions with CD2 and whether they bind the same CD2 sequences as p56<sup>lck</sup>.

Although our results are consistent with a role for p56<sup>lck</sup> in CD2-mediated signaling, we cannot conclude that the association with p56<sup>lck</sup> is sufficient or even necessary for CD2 signaling. An interesting possibility, not addressed in

our study, is that signaling molecules other than the Src-like PTKs interact with CD2 using a mechanism similar to the one described here. Of note in this regard, the peptide corresponding to the first proline-rich region (peptide 1) binds p56<sup>lck</sup> poorly and does not resemble the Src SH3 domain-binding peptides. Nevertheless, deletion of this region in human CD2 results in a 10-fold reduction of CD2-mediated IL-2 production (36). One possibility is that this proline-rich region binds to the SH3 domain of signaling molecule, other than a Src-like PTK, which may be required for CD2-mediated signal transduction.

CD2-mediated signaling requires the presence of the TCR or of receptors that contain members of the TCR  $\zeta$  chain family (16, 44). Perturbation of CD2 on T cells leads

to the tyrosine phosphorylation of the TCR- $\zeta$  chain, and it is likely that this event is crucial for CD2 signaling (20, 26). Tyrosine phosphorylation triggers an association between TCR- $\zeta$  and ZAP70, a PTK thought to play a critical role in TCR signaling (45). As noted above, CD2 appears to be in physical proximity with the TCR and, under certain conditions, TCR- $\zeta$  coimmunoprecipitates with CD2 (12). The demonstration that CD2 associates with p56<sup>lck</sup> suggests that, like the CD4 and CD8 coreceptors, CD2 may bring p56<sup>lck</sup> into proximity with potential substrates within the TCR complex. Furthermore, the mechanism by which CD2 associates with p56<sup>lck</sup> may make CD2 uniquely suited to stimulate the phosphorylation of TCR components.

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This work was supported by the Arthritis Foundation, United States Public Health Service grants K11-AI00999, R29-AI34911, RO1-CA52669, RO1-AI26644, and the Department of Veterans Affairs.

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Received for publication 16 May 1995 and in revised form 1 September 1995.

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