Demonstration of a Direct Interaction between p56\textsuperscript{ck} and the Cytoplasmic Domain of CD45 in Vitro*  

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p56\textsuperscript{ck} is a potential in vivo substrate for the tyrosine-specific phosphatase, CD45. In this study, recombinant purified p56\textsuperscript{ck} was found to specifically associate with recombinant CD45 cytoplasmic domain protein, but not to the cytoplasmic domain of another related tyrosine phosphatase, receptor protein-tyrosine phosphatase α. Under equilibrium binding conditions, the binding was saturable and occurred at a 1:1 molar stoichiometry. A fusion protein containing only the amino-terminal region of p56\textsuperscript{ck} (residues 34–150) also bound to recombinant CD45, and further analysis of this region indicated that glutathione S-transferase fusion proteins of the unique amino-terminal region and the SH2 domain, but not the SH3 domain of p56\textsuperscript{ck}, bound to recombinant CD45. The SH2 domain protein bound with a higher affinity than the amino-terminal region, but both were able to compete for the binding of p56\textsuperscript{ck} to CD45, and when added together worked synergistically to compete for p56\textsuperscript{ck} binding. The SH2 domain interaction with CD45 was specific as glutathione S-transferase-SH2 fusion proteins from p85\subunit of phosphatidylinositol 3-kinase and SHC did not bind to CD45. In addition, this interaction occurred in the absence of any detectable tyrosine phosphorylation on CD45, suggesting a nonconventional SH2 domain interaction.

p56\textsuperscript{ck} and CD45 (see Ref. 1 for review) are both required to generate an effective T cell antigen receptor (TCR)\textsuperscript{1}–mediated signal, being required for the earliest detectable event to occur upon TCR stimulation, the tyrosine phosphorylation of cellular proteins (2, 3). p56\textsuperscript{ck} is a member of the Src family of tyrosine kinases and is thought to be regulated by tyrosine phosphorylation at the autophosphorylation and negative regulatory sites. Tyrosine phosphorylation at the carboxyl-terminal negative regulatory site is believed to result in an intramolecular interaction with its SH2 domain which results in an inactive or inaccessible kinase (4). In the presence of CD45 this negative regulatory tyrosine residue in p56\textsuperscript{ck} is dephosphorylated (5–9), and p56\textsuperscript{ck} is then thought to be able to participate in TCR-mediated signal transduction events (10–12). Exactly how dephosphorylation of p56\textsuperscript{ck} results in effective TCR-mediated signal transduction remains unclear as recent data indicate that this dephosphorylation event may not necessarily lead to increased kinase activity (13). Dephosphorylation of these kinases may also result in the release of the intramolecular binding of the carboxyl-terminal tyrosine to the SH2 domain, which would then allow the unoccupied SH2 domain to bind to other tyrosine-phosphorylated proteins. Hence CD45 may act to regulate the SH2 domain interactions of p56\textsuperscript{ck}, which itself could be crucial in mediating a TCR induced signal. Consistent with such a model is the demonstration that the SH2 domain of p56\textsuperscript{ck} also plays an active role in T cell activation (14).

CD45 is a major lymphocyte glycoprotein and a transmembrane two domain tyrosine-specific phosphatase (reviewed in Ref. 15). CD45 can dephosphorylate several protein substrates and phosphorylated peptides in vitro (6, 7, 16, 17), yet only p56\textsuperscript{ck} and another Src family kinase, p59\textsuperscript{vav}, have clearly been identified as potential in vivo substrates (5, 8, 9). Both p56\textsuperscript{ck} and, to a lesser extent, p59\textsuperscript{vav} were found to be hyperphosphorylated in the absence of CD45, whereas the tyrosine phosphorylation state of p60\textsuperscript{src} was reported to be unaffected (9). How the substrate specificity of CD45 is achieved in vivo is not known, although substrate accessibility may be a factor. There is some evidence indicating that p56\textsuperscript{ck} may be associated with CD45 in T cells (18–20), p56\textsuperscript{ck} and proteins of approximately 30 kDa can be co-precipitated with CD45 using a mild detergent to lyse the cells (18, 21, 22). CD4 and CD8 are strongly associated with p56\textsuperscript{ck} (reviewed in Ref. 1) and have been reported to associate with CD45 (23, 24), yet the interaction between p56\textsuperscript{ck} and CD45 was shown to occur independently of the expression of CD4, CD8, and the TCR (25). Hence in T cells, CD45 may associate directly with p56\textsuperscript{ck} or indirectly via the 30-kDa proteins.

Recently, an interaction between p56\textsuperscript{ck} and tyrosine-phosphorylated CD45 was observed in phenylarsate oxide-treated T cells (26). In addition, tyrosine phosphorylation of CD45 in vitro by p50\textsuperscript{ck} resulted in the binding of p56\textsuperscript{ck} to CD45. This binding was thought to be mediated by the SH2 domain of p56\textsuperscript{ck} as the interaction was prevented by the addition of excess recombinant p56\textsuperscript{ck} SH2 domain (26).

To further investigate the potential interaction of p56\textsuperscript{ck} and CD45, we chose to analyze this association using purified, recombinant forms of p56\textsuperscript{ck} and the cytoplasmic domain of CD45.

**EXPERIMENTAL PROCEDURES**  

Materials—Purified glutathione S-transferase (GST)-RPTP\textsubscript{α} fusion protein containing the entire cytoplasmic domain of human RPTP\textsubscript{α} was a gift from K. Harder (27). Purified GST-SH2 domain proteins from the bovine p85\subunit subunit of phosphatidylinositol 3-kinase (residues 612–722) (28) and from human SHC (residues 366–473) (29) were provided...
by S. Barbazuk and M. Gold. Anti-GST antisera was obtained from S. Robbins, G. W. Hooper Foundation, University of California, San Francisco. Rabbit antisera (R-49 and R-54) specific for p56^lck^ were raised against a purified Trp-p56^lck^ fusion protein containing residues 34-150 of p56^lck^. Two purified CD45 monoclonal antibodies, 13/2 (30) and Ly 5.2 (31) were directly conjugated to CNBr-activated Sepharose CL-4B. Rabbit antisera 4G10 anti-phosphotyrosine antibody was purchased from Upstate Biotechnology, Inc.

Expression of Recombinant Proteins—Recombinant p56^lck^ was produced using a baculovirus expression system and purified as described previously (32). Recombinant, cytoplasmic domain of CD45 containing a six-histidine tag (RPTP_lck^-SH2 domain) was expressed from plasmid pStu I fragment encoding amino acids 8-509, and the unique amino-terminal region of p56^lck^ (Stul-BsmAI fragment encoding amino acids 8-76) were both blunt-ended and cloned into pGEX 2T. The GST-p56^lck^ fusion protein was used in the competition binding assays. GST-p56^lck^ SH2 and GST-p56^lck^ SH3 domain constructs (encoding residues 67-122 and 122-234, respectively) were provided by A. Veillette, produced in E. coli, and purified as described in Peri al (35). The Trp-p56^lck^ fusion protein was produced in E. coli and purified as described previously (36).

Binding Assay—5ng of purified, recombinant cytoplasmic domain of CD45 was covalently coupled to 1 ml of packed CNBr-activated Sepharose CL-4B beads (Pharmacia). An aliquot of beads containing approximately 2.5 μg of recombinant CD45 cytoplasmic domain, 13/2, or Ly 5.2 was used in the binding assays. The GST fusion protein containing the cytoplasmic domain of RPTP_lck^ was bound to glutathione-Sepharose 4B beads as above, and approximately 2.5 μg of the immobilized protein was used as a control in the binding assays. Approximately 200 ng of p56^lck^, 200 ng of Trp-p56^lck^ fusion protein, or 2.0 μg of GST fusion protein was incubated for 2 h at 4°C with immobilized recombinant CD45 cytoplasmic domain in a total volume of 40 μl of binding buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, containing 0.2 mM phenylmethylsulfonyl fluoride, 1 mg/ml of aprotinin, leupeptin, pepstatin protease inhibitors and 0.5 mg/ml soybean protease inhibitors). The beads were then washed three times in RIPAbuffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 0.025% β-mercaptoethanol, plus protease and protein-tyrosine phosphatase inhibitors). The beads were then boiled in reducing SDS sample buffer and proteins separated by SDS-PAGE. Proteins were transferred to PVDF membrane (Immobilon P, Millipore) and detected by Western blotting with the appropriate antibody.

Equilibrium Binding Assay and Analysis—Recombinant p56^lck^ was radioactively labeled by autophosphorylation using γ-[32P]ATP (~3000 Ci/mmol, Amersham Corp.) in kinase buffer (20 mM PIPES, pH 7.2, 10 mM NaCl, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride, 1 mg/ml of aprotinin, leupeptin, pepstatin). At 10 min the reaction was stopped by the addition of the appropriate antibody.

FIG. 1. Binding of recombinant p56^lck^ to the cytoplasmic domain of CD45. Approximately 200 ng of p56^lck^ was incubated with 2.5 μg of various proteins coupled to beads at 4°C for 2 h in 40 μl of binding buffer. After washing in RIPAbuffer, the entire sample was subjected to SDS-PAGE, transferred to PVDF membrane, and bound p56^lck^ was detected by autoradiography. Lane C is a control containing 25 ng of recombinant p56^lck^. Lanes 1-5 show the amount of recombinant p56^lck^ remaining bound to Sepharose CL-4B beads alone (lane 1), recombinant CD45 cytoplasmic domain conjugated to Sepharose beads (lane 2), recombinant RPTP_lck^ cytoplasmic domain GST fusion protein coupled to glutathione-Sepharose beads (lane 3), 13/2 antibody conjugated to Sepharose beads (lane 4), Ly 5.2 antibody conjugated to Sepharose beads (lane 5).

CD45 and ~2000 cpm of bound p56^lck^ were then incubated in the presence of 1 × 10^13 M to 2 × 10^13 M (total volume 45 μl) of competing unlabeled GST fusion protein at 4°C for 12 h. The beads were then centrifuged, the supernatant removed, and the amount of [32P]-labeled p56^lck^ remaining bound to the beads determined using a scintillation counter. Nonspecific p56^lck^ binding was determined as described above and subtracted from each sample.

RESULTS

Recombinant CD45 cytoplasmic domain protein (34) was covalently coupled to CNBr-activated Sepharose CL-4B, and approximately 2.5 μg of the immobilized protein was incubated with approximately 200 ng of recombinant p56^lck^ (32) at concentrations of 700 and 900 nM, respectively. Under the binding conditions (the presence of phosphatase inhibitors, the absence of ATP and divalent cations, and with CD45 covalently coupled to beads) neither CD45 nor p56^lck^ was enzymatically active. After a 2-h incubation at 4°C, the CD45-conjugated beads were washed three times in RIPAbuffer, and any remaining proteins were subjected to SDS-PAGE, transferred to PVDF membrane, and probed by Western blotting for the presence of p56^lck^.

The results are shown in Fig. 1 and illustrate that p56^lck^ bound to CD45-coupled beads (lane 2), but did not bind to an equivalent amount of Sepharose beads alone (lane 1) or to equivalent amounts of Sepharose beads coupled to two irrelevant antibody proteins (lanes 4 and 5). Furthermore, p56^lck^ did not associate with a recombinant GST fusion protein of the cytoplasmic domain of a related protein-tyrosine phosphatase, RPTP_lck^, which had been immobilized on glutathione-Sepharose beads (Fig. 1, lane 3). It was verified by Coomasie Blue staining of recombinant CD45 and RPTP_lck^ proteins that similar amounts of protein had been taken and coupled to beads (data not shown). In addition, immobilized CD45 did not associate with a recombinant GST protein under similar binding conditions, and likewise, p56^lck^ did not bind to another immobilized recombinant protein with a 6-histidine tag (endoglucanase, CenA) (37) (data not shown), further indicating the specificity of the interaction.

To determine the stoichiometry of the interaction, increasing amounts of [32P]-labeled p56^lck^ were incubated with immobilized CD45. Under equilibrium binding conditions the specific binding of p56^lck^ to the cytoplasmic domain of CD45 was saturable (see Fig. 2). It was determined from double-reciprocal plot analysis of seven experiments that 1.1 ± 0.2 mol of p56^lck^ bound to 1 mol of the cytoplasmic domain of CD45 under saturating binding conditions. Attempts were made to determine the affinity of this interaction, but Scatchard analysis...
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**Fig. 2.** Graph showing amount of radioactively labeled \(p56^{\text{ck}}\) bound to immobilized cytoplasmic domain of CD45 at equilibrium. A, graph of total amount of radioactive \(p56^{\text{ck}}\) (ng) bound to 300 ng of immobilized cytoplasmic domain of CD45 after incubation in increasing concentrations (nanomolar) of radioactively labeled \(p56^{\text{ck}}\) (●). ○ is nonspecifically bound radioactive \(p56^{\text{ck}}\) (nanograms), and × is the amount of radioactive \(p56^{\text{ck}}\) that specifically bound. Specifically bound radioactive \(p56^{\text{ck}}\) was calculated from (total radioactivity added − amount free) − nonspecifically bound radioactivity.

generated a nonlinear plot which suggested a complex interaction from which the affinity constant could not be readily determined.

It was observed that when antisera raised against amino acid residues 34–150 of \(p56^{\text{ck}}\) was added together with recombinant \(p56^{\text{ck}}\), significant inhibition of the association occurred (data not shown). To confirm whether this region of \(p56^{\text{ck}}\) could mediate the binding to cytoplasmic CD45, approximately 200 ng of a purified TrpE fusion protein containing residues 34–150 of \(p56^{\text{ck}}\) was incubated with immobilized recombinant CD45 cytoplasmic domain. This TrpE-\(p56^{\text{ck}}\) fusion protein has a predicted molecular mass of 51 kDa and specifically bound to the recombinant cytoplasmic domain of CD45 immobilized on Sepharose beads (Fig. 3). To further localize which specific region of the TrpE-\(p56^{\text{ck}}\) fusion protein was mediating this interaction, the immobilized CD45 cytoplasmic domain was incubated with 2.0 \(\mu\)g of purified GST fusion proteins containing the unique amino-terminal region of \(p56^{\text{ck}}\), the SH3 domain of \(p56^{\text{ck}}\), or the SH2 domain of \(p56^{\text{ck}}\). From the Western blot probed with anti-GST antisera in Fig. 4A, it can be seen that GST fusion proteins containing the unique amino-terminal region and SH2 domain of \(p56^{\text{ck}}\), but not the SH3 domain of \(p56^{\text{ck}}\) or GST alone, specifically bound to the immobilized cytoplasmic domain of CD45. The GST-\(p56^{\text{ck}}\) unique amino-terminal region and SH2 fusion proteins did not associate with an immobilized irrelevant protein, an anti-CD45 monoclonal antibody. Since a similar detection system was employed to identify both the unique amino-terminal and SH2-GST fusion proteins, the results indicated that a greater amount of the SH2-GST fusion protein bound to recombinant CD45, implying that the SH2 domain had a higher affinity for CD45 than the unique amino-terminal region. It was verified by Coomassie Blue staining that similar amounts of recombinant GST fusion proteins were incubated with recombinant CD45 cytoplasmic domain-coupled Sepharose beads (Fig. 4B). Thus, two distinct regions of \(p56^{\text{ck}}\) can bind to the cytoplasmic domain of CD45.

**Fig. 3.** Binding of recombinant GST fusion proteins to the cytoplasmic domain of CD45. A, Western blot analysis of GST fusion proteins remaining bound to the immobilized cytoplasmic domain of CD45 (odd-numbered lanes) or to the control I3/2 antibody (even-numbered lanes). GST fusion proteins were detected using GST antiserum. 2.0 \(\mu\)g of the GST fusion proteins were added to 2.5 \(\mu\)g of immobilized CD45 cytoplasmic domain and incubated for 2 h at 4° C in 40 \(\mu\)l of binding buffer. The beads were washed three times in RIPA buffer and subjected to SDS-PAGE. Lanes 1 and 2, GST protein alone; lanes 3 and 4, GST \(p56^{\text{ck}}\) unique amino-terminal region; lanes 5 and 6, GST \(p56^{\text{ck}}\) SH3; lanes 7 and 8, GST \(p56^{\text{ck}}\) SH2. B, SDS-PAGE analysis of the purity of GST fusion proteins used in the binding assay, stained with Coomassie Blue. Lane 1, GST protein alone; lane 2, GST \(p56^{\text{ck}}\) unique amino-terminal region; lane 3, GST \(p56^{\text{ck}}\) SH3; and lane 4, GST \(p56^{\text{ck}}\) SH2.

Competition binding assays were performed to determine the role of these two regions in the binding of intact \(p56^{\text{ck}}\) to CD45. Both the unique amino-terminal region and the SH2-GST fusion protein could compete for the binding of \(p56^{\text{ck}}\) to CD45 (Fig. 5). The SH2 domain was approximately 5-fold more efficient at competing with \(p56^{\text{ck}}\) for binding to CD45 than the unique amino-terminal region. This supports the earlier observation that the SH2 domain of \(p56^{\text{ck}}\) bound with a higher affinity to CD45. Interestingly, when the two domains were used together, they acted synergistically to compete with \(p56^{\text{ck}}\) for binding to CD45, but were still not as efficient as the intact \(p56^{\text{ck}}\) protein.

The specificity of the interaction with the SH2 domain of \(p56^{\text{ck}}\) was further evaluated by determining if other GST-SH2 domain fusion proteins would bind to the immobilized recombinant CD45 cytoplasmic domain. It was found that neither the carboxy-terminal SH2 domain from the p85α subunit of phosphatidylinositol 3-kinase nor the SH2 domain of SHC bound to recombinant cytoplasmic CD45 protein (Fig. 6).

Protein-protein interactions involving SH2 domains have previously been shown to be mediated by the binding of the SH2 domain to a region of the protein containing a phosphorylated tyrosine residue (reviewed in Ref. 38). In this situation, the possibility that the SH2 domain of \(p56^{\text{ck}}\) was binding to a phosphorylated tyrosine residue in CD45 was considered unlikely for two reasons. First, recombinant CD45 had been generated and purified from E. coli, which is not known to contain any tyrosine kinase activity, and second, no tyrosine kinase or
remaining 32P-labeled p56<sup>ck</sup> was centrifuged, the supernatant removed, and the remaining 32P-labeled p56<sup>ck</sup> bound to CD45 determined.

Using GST fusion proteins to compete for the binding of p56<sup>ck</sup> to the cytoplasmic domain of CD45. 300 ng of immobilized recombinant cytoplasmic domain of CD45 was mixed with 32P-labeled p56<sup>ck</sup> for 8 h, washed, and then incubated with various concentrations of competing GST fusion protein (●, GST-p56<sup>ck</sup>; ○, GST-unique amino-terminal region; ×, GST-SH3 domain; ■, GST-SH2 domain; ▲, GST unique amino-terminal region and GST-SH2 domain) for 12 h. The immobilized CD45 was centrifuged, the supernatant removed, and the remaining 32P-labeled p56<sup>ck</sup> bound to CD45 determined.

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ATP was present in the incubation of recombinant cytoplasmic CD45 with the SH2-GST fusion proteins, which themselves do not possess tyrosine kinase activity. However, in order to evaluate this possibility experimentally, 2.5 μg of recombinant CD45 was incubated with approximately 200 ng of p56<sup>ck</sup> or 2.0 μg of GST-p56<sup>ck</sup> SH2 domain fusion protein in the binding buffer for 2 h at 4 °C. These proteins and aliquots of purified proteins that had not been incubated were separated by SDS-PAGE and then either transferred to PVDF membrane and Western blotted with 4G10, a monoclonal antibody specific for phosphotyrosine residues (Fig. 7A) or stained with Coomassie Blue (Fig. 7B). As can be seen in Fig. 7A (lanes 1 and 2) and 7B (lanes 1–5) or stained with Coomassie Blue (Fig. 7B).

DISCUSSION

This work demonstrates that recombinant p56<sup>ck</sup> can specifically associate in vitro with the recombinant cytoplasmic domain of CD45. The specificity of this interaction has been defined on the basis of the following criteria: 1) the interaction did not occur with a structurally related tyrosine phosphatase, RPTPα; 2) the binding was saturable and occurred at a stoichiometry of 1:1; 3) the binding could be localized to distinct protein domains of p56<sup>ck</sup> and CD45; and 4) the association was still detectable after washing in the presence of 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 150 mM NaCl (RIPA buffer).

The cytoplasmic region of RPTPα has a similar predicted domain structure to CD45, having two tandemly repeated phosphatase domains separated by a unique spacer region and flanked by unique membrane proximal and carboxyl-terminal sequences. The PTP domain 1 and domain 2 of CD45 share 47 and 33% sequence identity with PTP domain 1 and domain 2 of RPTPα, respectively (27, 33). The observation that p56<sup>ck</sup> associates with CD45 but not with RPTPα implies a specific interaction, but at present the significance of this interaction is not known. The in vitro interaction also occurred in the absence of PTP inhibitors ruling out the possibility that these inhibitors were mediating the interaction between p56<sup>ck</sup> and CD45 (data not shown). In addition, p56<sup>ck</sup> also bound to an immobilized recombinant CD45 protein in which the catalytic cysteine (C817) had been replaced with a serine, indicating that a phospho-tyrosine intermediate was not responsible for the observed tyrosine phosphorylation of p56<sup>ck</sup>, produced using a baculovirus expression system, was tyrosine-phosphorylated. However, even with 100 times the amount required to give a signal for p56<sup>ck</sup>, no bands at 95 kDa representing the cytoplasmic domain of CD45 (lanes 2, 3, 4, and 6) were observed using the anti-phosphotyrosine antibody. These data demonstrate that neither CD45 nor the GST-p56<sup>ck</sup> SH2 domain proteins were detectably tyrosine-phosphorylated and that the tyrosine phosphorylation state of p56<sup>ck</sup> and CD45 did not change during the course of the binding assay, indicating that under these conditions both p56<sup>ck</sup> and CD45 were not catalytically active. Thus, the SH2 domain of p56<sup>ck</sup> interacts with the cytoplasmic domain of CD45 by a mechanism that does not depend on the tyrosine phosphorylation of CD45.

Tyrosine phosphorylation state of recombinant p56<sup>ck</sup> and CD45 cytoplasmic domain proteins. A, Western blot of soluble recombinant proteins probed with the 4G10, an anti-phosphotyrosine monoclonal antibody. Proteins were incubated in 40 μl of binding buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, containing protease and phosphatase inhibitors) for 2 h at 4 °C. Lane 1, approximately 200 ng of p56<sup>ck</sup>; lane 2, approximately 200 ng of p56<sup>ck</sup>, and 2.5 μg of cytoplasmic CD45; lane 3, 2.5 μg of cytoplasmic CD45; lane 4, 2.0 μg of GST-p56<sup>ck</sup> SH2 domain and 2.5 μg of cytoplasmic CD45; lane 5, 2.0 μg of GST-p56<sup>ck</sup> SH2 domain; lane 6, 20 ng of p56<sup>ck</sup> and 2.5 μg of cytoplasmic CD45; and lane 7, 20 ng of p56<sup>ck</sup>. B, SDS-PAGE analysis of recombinant proteins detected by Coomassie Blue staining. Lanes 1–5 are identical to those in A.
interaction (data not shown). From the binding assay, only a small percentage of p56\textsuperscript{ck} (~3–4%) bound to recombinant CD45, yet under equilibrium binding conditions and using saturating amounts of p56\textsuperscript{ck}, a 1:1 interaction was indicated. These differences may reflect the fact that in the initial binding experiments, the amount bound was observed after washing three times in stringent conditions (RIPA buffer).

In an attempt to determine the affinity of the interaction, Scatchard analysis of the equilibrium binding data was performed. However, a nonlinear plot was obtained, indicating that it was not a simple single binding site interaction (data not shown). Interpretation of the plot as two distinct binding sites with different affinities was also precluded as the interaction at saturation resulted in a 1:1 stoichiometry. This indicated that the criteria for Scatchard analysis was not met and thus could not be used to determine the affinity of the interaction. Additional data using GST-fusion proteins indicated that two distinct regions of p56\textsuperscript{ck}, the unique amino-terminal region and the SH2 domain, could specifically bind to recombinant CD45. Competition analysis showed that the SH2 domain was approximately 5-fold more effective than the unique amino-terminal region in competing with intact p56\textsuperscript{ck}. Interestingly, together these two domains were able to compete with p56\textsuperscript{ck} more effectively than either domain alone, suggesting a synergistic effect. This strongly implies that both regions are involved in mediating the interaction between p56\textsuperscript{ck} and CD45. However, together, these two domains were still not as efficient as the intact p56\textsuperscript{ck} in these competition assays, suggesting that either these two domains need to be in a certain configuration for optimum binding or that additional regions within the COOH-terminal region of p56\textsuperscript{ck} are involved in binding. Since CD45 can dephosphorylate Tyr\textsuperscript{505} and Tyr\textsuperscript{394} residues, both of which are located in the COOH-terminal region, an interaction with this region must occur in order for CD45 to dephosphorylate these residues. Whether this region can interact stably with the cytoplasmic domain of CD45 in the absence of these two regulatory regions or whether these regulatory regions interact with CD45 to facilitate the dephosphorylation of p56\textsuperscript{ck} remains to be determined and is currently under investigation.

It was also shown that the association between p56\textsuperscript{ck} or the GST-p56\textsuperscript{ck}-SH2 fusion protein with the cytoplasmic domain of CD45 did not require the tyrosine phosphorylation of CD45. This is in contrast to previous studies by Autero et al. (26) where an interaction between p56\textsuperscript{ck} and CD45 was observed when CD45 was tyrosine-phosphorylated in vitro by p50\textsuperscript{ck}. It is possible that p56\textsuperscript{ck} may bind to both non-tyrosine-phosphorylated and phosphorylated forms of CD45. In support of this notion, another Src family kinase, p59\textsuperscript{yn}, has been reported to associate with both non-tyrosine-phosphorylated and phosphorylated forms of Ig\textsubscript{c}. Increased binding of p59\textsuperscript{yn} was observed upon tyrosine phosphorylation of Ig\textsubscript{c}, which resulted in an increase in the kinase activity of p59\textsuperscript{yn} (39).

Tyrosine phosphorylation-independent interactions with SH2 domains have been reported for three other tyrosine kinases; ABL, p59\textsuperscript{yn}, and p60\textsuperscript{src} (40, 41). In these examples, the SH2 domains were thought to bind to phosphoserine/threonine residues. It remains to be determined whether the interaction between the p56\textsuperscript{ck} SH2 domain and the cytoplasmic domain of CD45 requires serine/threonine phosphorylation. At present, this interaction has been observed with the SH2 domain of p56\textsuperscript{ck} but not with two other SH2 domains derived from two other signaling proteins, the p85 subunit of phosphatidylinositol 3-kinase and SHC.

Although we have demonstrated a direct interaction between p56\textsuperscript{ck} and the cytoplasmic domain of CD45 in vitro, it still remains to be established whether a direct interaction also occurs in vivo and whether such an interaction is regulated by the association of other proteins such as CD45AP. In T cells, p56\textsuperscript{ck} can be detected in immunoprecipitates of CD45 along with the presence of a 30-kDa protein (18, 25) and can be co-precipitated with CD45 under conditions where CD45 is not detectably tyrosine phosphorylated. Since p56\textsuperscript{ck} has not been co-precipitated in the absence of the 30-kDa protein (CD45AP or LPAP), it is not known whether p56\textsuperscript{ck} can directly associate with CD45 in T cells. In contrast, p30 has been shown to co-precipitate with CD45 in the absence of p56\textsuperscript{ck} (20), and recently, the transmembrane region of CD45 has been shown to be required for the association of p30 (42). However, as only a small percentage of p56\textsuperscript{ck} is co-precipitated with CD45, the physiological significance of this association remains to be established. In the in vitro assay conditions used to observe the binding of p56\textsuperscript{ck} to the cytoplasmic domain of CD45, neither enzyme was catalytically active and p56\textsuperscript{ck} but not CD45, was detectably tyrosine-phosphorylated. It is possible that these in vitro conditions favor or stabilize the interaction between these proteins whereas conditions in the cell may favor a more transient association.

Data from CD45 negative and positive cell lines have indicated that the absence of CD45 has a larger effect on the phosphorylation state of p56\textsuperscript{ck} than p59\textsuperscript{yn} and that the phosphorylation state of p60\textsuperscript{src} is unaffected (9). Since all these kinases are closely related, particularly at the dephosphorylation site, it is not clear why some members of this family appear to be preferred substrates in vivo. Recent data using a p56\textsuperscript{ck}/p59\textsuperscript{yn} chimera has shown that the unique amino-terminal region of p56\textsuperscript{ck}, but not that of p59\textsuperscript{yn}, was required for the dephosphorylation of Tyr\textsuperscript{505} in p56\textsuperscript{ck}, suggesting that the unique amino-terminal region of p56\textsuperscript{ck} may influence substrate specificity (43). In this paper we have demonstrated that both the unique amino-terminal region and SH2 domain of p56\textsuperscript{ck} can bind to the cytoplasmic domain of CD45 in vitro, and thus, it is tempting to speculate that an interaction between these noncatalytic domains of p56\textsuperscript{ck} and CD45 may act to facilitate the dephosphorylation of p56\textsuperscript{ck}.

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