Subdomain X of the Kinase Domain of Lck Binds CD45 and Facilitates Dephosphorylation*

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CD45 is a transmembrane, two-domain protein-tyrosine phosphatase expressed exclusively in nucleated hematopoietic cells. The Src family kinase, Lck, is a major CD45 substrate in T cells and CD45 dephosphorylation of Lck is important for both T cell development and activation. However, how the substrate specificity of phosphatases such as CD45 is achieved is not well understood. Analysis of the interaction between the cytoplasmic domain of CD45 and its substrate, Lck, revealed that the active, membrane-proximal phosphatase domain of CD45 (CD45-D1) is involved in substrate binding of Lck kinase domain, the SH2 domain, and the unique N-terminal region of Lck. The second, inactive phosphatase domain (CD45-D2) bound only to the kinase domain of Lck. CD45-D2 was unable to bind phosphotyrosine, and its interaction with the kinase domain of Lck was independent of tyrosine phosphorylation. The binding of CD45-D2 was localized to subdomain X (SD10) of Lck. CD45-D2 bound similarly to Src family kinases but bound Csk to a lesser extent and did not bind significantly to the less related kinase, Erk1. CD45 dephosphorylated Lck and Src at similar rates but dephosphorylated Csk and Erk1 at lower rates. Replacement of Erk1 SD10 with that of Lck resulted in the binding of CD45-D2 and the conversion of Erk1 to a more efficient CD45 substrate. This demonstrates a role for CD45-D2 in binding substrate and identifies the SD10 region in Lck as a novel site involved in substrate recognition.

CD45, a transmembrane, two-domain protein-tyrosine phosphatase (PTP)† expressed exclusively on nucleated hematopoietic cells, and Lck, a Src family protein-tyrosine kinase expressed predominantly in T lymphocytes, are both required for effective signal transduction from the T cell receptor (TCR) when it engages major histocompatibility molecules-presenting antigen (reviewed in Refs. 1 and 2). They are necessary for Lck activation of the tyrosine phosphorylation cascade, which is the earliest detectable event occurring upon TCR engagement (reviewed in Ref. 1). CD45 is thought to be required for T cell activation by constitutively dephosphorylating the negative regulatory tyrosine of Lck, Tyr505. This creates a “primed” Lck molecule that can become activated by autophosphorylation at Tyr394. Lck is thought to be activated upon TCR encounter with antigen, which then initiates the signal transduction cascade by phosphorylating downstream substrates such as CD3ζ and Zap-70 (reviewed in Refs. 1, 3, and 4). There is accumulating evidence that CD45 can also dephosphorylate the activating autophosphorylation site of Lck, thereby down-regulating Lck activity (Refs. 5–7, and reviewed in Ref. 8). Thus, by regulating the phosphorylation state of Lck, CD45 can up- or down-regulate Lck activity.

Like all members of the Src kinase family, Lck has a unique N-terminal region, a Src homology 3 (SH3) domain, an SH2 domain, a catalytic kinase domain and a short C-terminal region. Biochemical and structural evidence indicates that Src family members are regulated by an intramolecular interaction that occurs when the negative regulatory tyrosine in the C-terminal region is phosphorylated and interacts with the SH2 domain, resulting in a closed, inactive conformation (reviewed in Refs. 4 and 9). In contrast to the Src kinase family, very little is understood about how CD45 PTP activity is regulated or how substrate specificity is achieved. In the cytoplasmic domain of CD45, the membrane proximal PTP domain has been shown to be the active PTP domain, whereas most evidence indicates that the second PTP domain possesses no catalytic activity (10–13). However, the C-terminal region containing the second PTP domain, CD45-D2, is required for optimal activity and stability of the active PTP domain (13, 14). CD45 activity, like that of Zap-70, may also be regulated by intra- and inter-molecular interactions (13–16). There is good evidence that Lck and, to a lesser extent, Fyn, are physiological substrates of CD45 in T cells (17–20). In B cells, Lyn appears to be the major CD45 substrate (21–24) and in macrophages, the phosphorylation state and activity of Hck and Lyn, but not Fgr, are affected by CD45 (25). Therefore, Src family kinases appear to be the preferred substrates for CD45 in leukocytes, although CD3ζ, Zap-70, and Jak kinases have also been implicated as potential CD45 substrates (26–28). Why Src family kinases are preferred substrates for CD45 or how the substrate specificity of signal-regulated kinase 1; PVDF, polyvinylidene difluoride; Csk, C-terminal Src kinase; KAP, kinase-associated phosphatase.
CD45 is achieved at the molecular level is not well understood. To gain insight into these questions, we analyzed the enzyme-substrate interaction between CD45 and Lck using purified recombinant Lck and CD45 cytoplasmic domain proteins. We have determined that the second inactive PTP domain (CD45-D2) interacts with substrate and have identified a distinct region in the kinase domain of Lck, away from the catalytic site, involved in docking mutations in CD45. The interaction region in CD45 with this region was sufficient to promote substrate dephosphorylation, implicating a role for this region in substrate recognition and in contributing toward the determination of Lck as a preferred substrate for CD45.

EXPERIMENTAL PROCEDURES

Reagents—Glutathione-Sepharose 4B beads, CNBr-activated Sepharose CL-4B beads, and Sepharose CL-4B beads, were from Amersham Biosciences and O-phospho-tyrosine was from Sigma Aldrich. Antibodies used were: 4G10, anti-phosphotyrosine monoclonal antibody from Upstate Biotechnology Inc., rabbit anti-Erk1 polyclonal IgG (C-16) from Santa Cruz Biotechnology, R02.2 rabbit antiserum generated against the cytoplasmic domain of recombinant CD45 (29), and rabbit anti-Lck antisera, R4–3B (30).

Recombinant DNA Constructs—The plasmid encoding GST-human Erk1 was from S. Pelech (31). GST-SH3-SH2 was from T. Pawson (32). GST-Grb2 (33) was obtained from M. Gold and pGEX-3X used to express GST-kinase, containing a NcoI-StuI fragment of Lck encoding the Lck kinase domains (amino acids 232–509) subcloned into pGEX-4T-2 vector (Amersham Biosciences). The cDNA for murine Src, modified by removal of six unique amino acids found in the neuronal form of Src was provided by T. Hunter (35) and the cDNA for rat Csk (36) was obtained from F. Jirik. These were subcloned into pGEX-2T (Amersham Biosciences). The GST-Lck fusion protein encompassing the whole murine Lck sequence (37) and GST-N, containing the unique N terminus of Lck (residues 5–6) were, as described previously (30). GST-SH3 containing the Lck SH3 domain (residues 67–122) and GST-SH2 containing the Lck SH2 domain (residues 122–234) were provided by A. Veillette (38). GST-N32, containing a Stul-Ncol fragment of Lck encoding the non-catalytic regions of Lck (residues 8–233) in pGEX-2T and GST-kinase, containing a Ncol-StuI fragment of Lck encoding the Lck catalytic domain and residues C-terminal to it (residues 225–509) in pGEX-4T-3 were as described previously (39).

The full-length Lck phosphorylation mutant constructs, Tyr505 mutated to Phe (Y505F), Tyr516 mutated to Phe (Y516F), and Lys273 mutated to Phe (Y505F), Tyr394 mutated to Phe (Y394F), and Lys273 mutated to Phe (Y273F), were suggested (43). The truncation mutant (6His-C817S), were as described (43). The truncated mutant (6His-D1), encoding residues 565–927, containing the membrane proximal region, PTP-D1, and the spacer region, and 6His-D2 (residues 903–1268) containing part of the spacer region, PTP-D2, and the carboxyl tail were also as described (13). A 6His-D2 protein (6His-D2ΔAcidic) lacking the acidic region, residues 958–978, a GST-D2 protein (residues 903–1268) and a GST-D2 (GST-D2-ΔCT, residues 903–1190) lacking 78 amino acids from the carboxyl tail, were also constructed in pGEX-3X (Amersham Biosciences). D2 and D2–ΔCT were cleaved from GST by incubation overnight at 4 °C with Factor Xa (Roche Applied Science). Expression and purification of the Histagged and GST fusion proteins were as described (13).

In Vitro Binding Assays of Immobilized GST Fusion Proteins with Soluble 6His-CD45 Proteins—Either equimolar amounts or equal amounts of protein (as indicated in each individual experiment) of soluble 6His-CS17S, 6His-D2, or 6His-D1 were added to 2 μg of washed, immobilized GST fusion proteins (bound to glutathione-Sepharose 4B beads). Immobilized GST alone or irrelevant GST fusion proteins were included as negative controls. The assays were incubated shaking for 2 h at 37 °C in 40 μl of binding buffer (20 mM Tris, pH 7.5, 7.5 M NaCl, 0.05% β-mercaptoethanol) with protease inhibitors (1 mM β-glycerophosphate, 1 μM leupeptin, 1 μM pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride). In 6His-D1 binding assays, 500 μM sodium orthovanadate was included in the buffer to inhibit PTP activity. After incubation, the samples were washed four times by adding 1 ml of radioimmuno precipitation assay buffer (20 μl Triton X-100, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 0.05% β-mercaptoethanol) and vortexing for 30–60 s. Reducing sample buffer was added, and the samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore Corp.), and the amount of added soluble protein remaining bound to the beads was determined by densitometric analysis with 1/5,000 anti-CD45 antibody (R02.2) as the primary, and 1/10,000 horserasherd peroxidase (HRP)-labeled protein A (Bio-Rad) as the secondary. Membranes were subsequently stained with Coomassie Blue to visualize the immobilized proteins and subjects to densitometry scan analysis using AlphamagerTM software (Alpha Innotech Corp.). In experiments where tyrosine phosphorylation levels of the immobilized proteins were determined, the samples were divided into two, and one membrane was probed with R02.2 and the other with anti-phosphotyrosine antibody, 4G10, diluted 1/5,000 as the primary antibody and 1/10,000 dilution of HRP-labeled goat anti-mouse antibody as the secondary antibody (Southern Biotechnology Associates, Inc.).

CD45 Binding Assays Using Immobilized Phosphotyrosine and Soluble 6His-CD45 Proteins—O-Phospho-tyrosine (10 μmol) was covalently coupled to 1 ml of packed CNBr-activated Sepharose CL-4B beads according to manufacturer’s instructions. Approximately 65% coupling efficiency was achieved. 1 μg of 6His-CS17S or 6His-D2 (300 nm and 555 nm, respectively) were added to ~1 μmol phosphotyrosine bound to Sepharose CL-4B beads (2.5 μl beads) in 40 μl of binding buffer plus protease inhibitors. These were incubated and shaken for 2 h at 4 °C, then washed as described above. Reducing sample buffer was added, and the samples were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-CD45 antibody (R02.2). Sepharose CL-4B beads were included as a negative control.

Phosphorylation Assays—Phosphorylation assays were performed at 37 °C in 10 μl of PTP buffer (50 mM Tris, pH 7.2, 1 mM EDTA, 0.1% β-mercaptoethanol, with or without 0.01% Triton X-100) essentially as described (29). 60 μM of purified recombinant 6His-CD45 was incubated with autophosphorylated recombinant GST fusion proteins: either (a) 245 nm GST-Lck or GST-Src or (b) 25 nm GST-Lck, 130 nm GST-Csk, and 380 nm GST-Erk1 or GST-Erk1-LSDL10 for increasing amounts of time. The concentrations of the latter were determined by densitometric analysis using AlphamagerTM software to contain approximately equal levels of phosphorytrosine. The reactions were stopped by immersion in a dry ice/ethanol bath, and samples were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane, and the amount of phosphorylated substrate remaining was determined by Western blot analysis. The tyrosine-phosphorylated bands were analyzed by densitometric analysis using AlphamagerTM software and subsequently gapped. The amount of tyrosine phosphorylation at time zero was taken as 100% for all of the substrates.

RESULTS

CD45-D1 Binds the Unique N-terminal, SH2, and Kinase Domains of Lck, Whereas CD45-D2 Binds Only the Kinase Domain of Lck—To analyze the interaction between CD45 and its substrate, Lck, we first determined which regions of the cytoplasmic domain of CD45 were interacting with the different regions of Lck. Purified, soluble, recombinant CD45 proteins, 6His-D1, 6His-D2, and 6His-CS17S, were added to various immobilized GST-Lck domain proteins (Fig. 1), and a
binding assay was performed (see “Experimental Procedures” for details). The full-length, inactive CD45 cytoplasmic domain protein (6His-C817S) interacted with both the catalytic kinase domain of Lck, GST-kinase, and the non-catalytic portion of the protein, GST-N32 (Fig. 2A). 6His-C817S bound at low levels to GST-N and GST-SH2, but it did not bind to GST-SH3, as reported previously for inactive CD45 cytoplasmic domain protein (30). The highest level of binding was observed between 6His-C817S and GST-kinase (Fig. 2A). 6His-D1 bound to the same regions of Lck as the full-length CD45 cytoplasmic domain protein, 6His-C817S, although less binding was observed to GST-kinase (Fig. 2B). This may be partly due to the inclusion of sodium orthovanadate in the 6His-D1 binding assays, added to inhibit 6His-D1 PTP activity. Attempts to make a catalytically inactive 6His-D1 recombinant protein did not result in sufficient protein for analysis. In contrast, the catalytically inactive 6His-D2 did not bind to any of the non-catalytic regions of Lck but bound significantly to GST-kinase (Fig. 2C). Even upon further exposure, no binding was observed between 6His-D2 and any of the non-catalytic domains above the background level. This indicates that the interaction between the enzyme, CD45, and the substrate, Lck, involves more than just the catalytic site of CD45 and the phosphorylated tyrosine of Lck. It implicates the non-catalytic regions of both CD45 and Lck in this interaction. In particular, it suggests that the second, non-catalytic PTP domain of CD45 is involved in binding substrate.

A comparison of equimolar amounts of 6His-D2 binding to Lck with the inactive CD45 cytoplasmic domain (6His-C817S) revealed that ~5-fold less 6His-D2 bound compared with 6His-C817S (data not shown). This indicates that 6His-D2 can interact with full-length Lck. It also suggests that the binding of 6His-C817S to Lck might be a product of multiple interactions involving both the catalytic and non-catalytic regions of CD45. Binding of CD45-D2 to the Lck Kinase Domain Is Phosphorylation-independent and Does Not Require the Acidic Region or C-terminal Region of CD45-D2—Because 6His-D2 bound to the kinase domain of Lck and has significant sequence identity to active PTP domains, it was possible that the CD45-D2 PTP domain interacted with the phosphorylated tyrosines on Lck. To test this possibility, binding assays were performed between 6His-D2 and specific phosphorylated and non-phosphorylated forms of the Lck kinase domain expressed as GST fusion proteins. In E. coli, the active Lck kinase domain is normally phosphorylated at the autophosphorylation site, Tyr394, as is a phosphorylation mutant where the negative regulatory tyrosine, Tyr505, has been mutated (Y505F). Mutation of Tyr394 (Y394F) prevents its autophosphorylation and co-expression of this construct with Csk (C-terminal Src kinase) promotes the phosphorylation at the negative regulatory site, Tyr505. Mutation of the catalytic lysine, Lys272, generates an inactive Lck mutant (K273R) that is not phosphorylated. Fig. 3A indicates that 6His-D2 bound to all Lck kinase domain proteins, irrespective of their tyrosine phosphorylation status (Fig. 3B). Fig. 3C confirms that equivalent amounts of GST-kinase domain proteins were used in each assay. The results show no significant difference in the binding of 6His-D2 to the various phosphorylated and non-phosphorylated forms of the Lck kinase domain.

To investigate whether the acidic region unique to CD45-D2 (residues 958–978) was involved in binding to the Lck kinase domain, a 6His-D2 protein lacking the acidic region, 6His-D2-ΔAcidic, was made and purified. Equimolar amounts of 6His-D2 and 6His-D2-ΔAcidic were compared in their ability to bind to the GST-kinase phosphorylation mutants. As can be seen in the right panel of Fig. 3, the binding of CD45-D2 to the Lck kinase domain did not require the acidic region. In fact, more 6His-D2-ΔAcidic bound to the kinase domain than 6His-D2, suggesting that the acidic residues have a negative effect on binding. The binding of 6His-D2-ΔAcidic is also independent of the tyrosine phosphorylation state of the Lck kinase domain. To determine if the C-terminal 78 amino acids of CD45-D2...
were involved in binding to Lck, a GST-D2 protein lacking these amino acids, GST-D2ΔCT, was made. Both GST-D2 and GST-D2ΔCT were cleaved with factor Xa from GST and used in a binding assay with immobilized GST-Lck. The C-terminal deletion of 78 amino acids did not have any significant effect on the binding of CD45-D2 to Lck (data not shown). Therefore, the C-terminal region and the unique acidic region are not required for CD45-D2 to bind to Lck, indicating that the interaction with Lck is mediated by amino acid residues close to or within the D2 PTP domain.

**CD45-D2 Does Not Bind Phosphotyrosine**—The phosphorylation-independent binding of 6His-D2 to the differentially phosphorylated GST-Lck kinase domain proteins suggested that CD45-D2 may not be binding to Lck via its proposed catalytic pocket, or if it is, the highly substituted CD45-D2 catalytic pocket may not bind to phosphotyrosine. To investigate whether CD45-D2 can bind tyrosine phosphate, phosphotyrosine was immobilized to CNBr-activated Sepharose CL-4B beads and soluble 6His-D2 or 6His-C817S was added to the beads in an in vitro binding assay. The full-length inactive cytoplasmic domain, 6His-C817S, bound to the phosphotyrosine beads, but 6His-D2 did not (Fig. 4). In a total of eight experiments, with varying concentrations of proteins, phosphotyrosine beads, and buffer components, 6His-C817S consistently bound to phosphotyrosine, whereas 6His-D2 did not. Upon longer exposure of the film, 6His-D2 was sometimes detectable in the phosphotyrosine bead sample relative to the Sepharose bead control, but this was insignificant relative to the binding of 6His-C817S. Therefore, unlike the catalytic PTP domain of CD45, CD45-D2 does not bind significantly to phosphotyrosine.

**CD45-D2 Binding to the Lck Kinase Domain Is Not Hindered by the Non-catalytic Regions or Conformational State of Lck**—To determine if the non-catalytic regions of Lck affected the binding of CD45-D2 to the Lck kinase domain, binding of 6His-D2 to the isolated kinase domain was compared with binding to full-length Lck (Fig. 5A). Quantitative densitometric analyses from seven experiments, correcting for the amount of immobilized fusion protein and standardizing the binding to GST-Lck to 1, indicated that the relative binding of 6His-D2 to GST-kinase was 1.1 ± 0.3. The similar levels of binding indicate that the non-catalytic domains of Lck do not interfere with this interaction. In this case, full-length Lck is active, phosphorylated primarily at the autophosphorylation site, Tyr394, and predicted to be in an open, unconstrained conformation (reviewed in Ref. 9). When Src family kinases are phosphorylated at their negative regulatory site (Tyr505 for Lck), the phosphorylated tyrosine binds to the SH2 domain causing the kinase domain to adopt a constrained position with the autophosphorylation loop blocking the active site (44, 45). Thus it was possible that CD45-D2 may bind to the unconstrained kinase domain but not to the constrained form. To evaluate this possibility, 6His-D2 binding to GST-Lck Y505F (not phosphorylated at Tyr505 and therefore predicted to be in the open conformation) and Csk-phosphorylated GST-Lck Y394F (phosphorylated at Tyr505 and predicted to be in the closed or constrained conformation) was assessed (Fig. 5B). 6His-D2 bound equally well to both GST-Lck proteins, implying that CD45-D2 can bind to the Lck kinase domain independent of the phosphorylation or conformational state of Lck.

**6His-D2 Does Not Discriminate Between Binding to Lck, Fyn or Src**—Previous work indicates that the absence of CD45-D2 does not affect substrate specificity at the peptide level (13). However, it is possible that an effect of CD45-D2 on substrate specificity may only be observed at the protein level. To determine if the binding ability of CD45 or CD45-D2 reflected the substrate preference observed in the T cell where Lck is preferred over Fyn, and Src is not a substrate when retrovirally expressed in T cells (46, 47), in vitro binding assays were performed. Soluble 6His-D2 or 6His-C817S was incubated with immobilized GST fusion proteins of Lck, Fyn, and Src. Both CD45 proteins bound to all three Src family kinases (Fig. 6). Although there appears to be a slight preference for the binding of 6His-D2 to Lck in this experiment, when immobilized protein amounts are taken into account and densitometric analysis performed and averaged from at least three experiments, overlapping standard deviations indicated that the differences in binding were not significant. This demonstrates that both CD45 and CD45-D2 do not discriminate between different members of Src family kinases in an in vitro binding assay, suggesting that the substrate preference observed in T cells for particular Src family members is dictated by cellular factors.
6His-D2 Preferentially Binds to GST-Lck Compared with GST-Csk and GST-Erk1—To correlate the binding preference of CD45-D2 to substrate specificity, the *in vitro* dephosphorylation of autophosphorylated Lck by active recombinant CD45 cytoplasmic domain protein (6His-CD45) was compared with the dephosphorylation rate of autophosphorylated Src. Consistent with the 6His-D2 binding assay, no significant difference was observed (Fig. 8A). As previously reported, both GST-Csk and GST-Erk1 were autophosphorylated to low stoichiometry on tyrosine residues when produced from *E. coli* (31, 48, 49). Tyrosine phosphorylation of Csk has also been reported to occur *in vitro* (50) and in HeLa cells (51). The tyrosine phosphorylation level of each kinase was assessed by Western blotting. Dephosphorylation of approximately equivalent levels of tyrosine phosphorylated Lck, Csk, and Erk1 by CD45 demonstrated that Lck was preferentially dephosphorylated compared with Csk and Erk1 (Fig. 7). Therefore, although CD45-D2 did not show preferential binding between the Src family kinases, it did discriminate between Src family kinases and less related kinases, and may therefore contribute toward the preference of CD45 for Src family kinases over other less related kinases in the cell.

**CD45 Preferentially Dephosphorylates Src Family Kinases over the More Distantly Related Kinases, Csk and Erk1**—To determine if CD45-D2 showed preferential binding to Lck over non-Src family kinases, *in vitro* binding assays were performed between 6His-D2 and GST-Lck and compared with 6His-D2 binding to a related tyrosine kinase, GST-Csk, and to a less-related serine/threonine kinase, GST-Erk1. 6His-D2 did bind to GST-Csk, but to a much lesser extent than to GST-Lck (Fig. 7). Densitometric analysis from four separate experiments, correcting for the amount of immobilized protein, indicated that the level of binding of 6His-D2 to GST-Csk, relative to GST-Lck, is 24 ± 15%. 6His-D2 did not bind significantly to the more distantly related kinase, GST-Erk1 (Fig. 7). Therefore, although CD45-D2 did not show preferential binding between the Src family kinases, GST-Src family kinases present. GST-Src was always heavily degraded, however, GST-Lck degradation varied between different purification preparations.

6His-D2 Preferentially Binds to GST-Lck Compared with GST-Csk and GST-Erk1 Kinases—To determine if CD45-D2 showed preferential binding to Lck over non-Src family kinases, *in vitro* binding assays were performed between 6His-D2 and differentially phosphorylated GST-Lck proteins. A, 1 µg of 6His-D2 was added to 2 µg of GST-Lck or GST-kinase in a binding assay as described in Fig. 2. GST alone was used as a negative control, and 20 ng of 6His-D2 was included for reference. The top panel is an anti-GST Western blot (R02.2) showing the amount of 6His-D2 remaining bound to the immobilized GST fusion proteins, and the bottom panel shows the membrane after it was stained with Coomassie Blue to assess the relative amount of GST fusion proteins present. Prestained molecular mass standards are indicated on the right. B, 1 µg of 6His-D2 was added to 2 µg of full-length GST-Lck fusion proteins in a binding assay as previously described. Y394F*P is phosphorylated by Csk, and Y505F is autophosphorylated. These are indicated above the bottom panel. GST alone and the GST-SHC SH2 domain (SHC-2) were used as negative controls, and 20 ng of 6His-D2 was included for reference. The top and bottom panels are as in A. Locations of 6His-D2, GST-Lck, GST-SHC-2, and GST are indicated on the right. Prestained molecular mass standards are indicated on the left in kDa. Approximately equal amounts of phosphotyrosine levels on Tyr394 and Tyr505 were determined by an anti-phosphotyrosine Western blot (data not shown).

**Fig. 5.** Relative binding of 6His-D2 to GST-Lck, GST-kinase, and differentially phosphorylated GST-Lck proteins. A, 1 µg of 6His-D2 was added to 2 µg of GST-Lck or GST-kinase in a binding assay as described in Fig. 2. GST alone was used as a negative control, and 20 ng of 6His-D2 was included for reference. The top panel is an anti-GST Western blot (R02.2) showing the amount of 6His-D2 remaining bound to the immobilized GST fusion proteins, and the bottom panel shows the membrane after it was stained with Coomassie Blue to assess the relative amounts of 6His-D2 and 6His-C817S remaining bound. The lanes are as indicated above the gels. GST alone was included as a negative control. Prestained molecular mass standards are indicated on the right. B, 1 µg of 6His-D2 was added to 2 µg of full-length GST-Lck fusion proteins in a binding assay as previously described. Y394F*P is phosphorylated by Csk, and Y505F is autophosphorylated. These are indicated above the bottom panel. GST alone and the GST-SHC SH2 domain (SHC-2) were used as negative controls, and 20 ng of 6His-D2 was included for reference. The top and bottom panels are as in A. Locations of 6His-D2, GST-Lck, GST-SHC-2, and GST are indicated on the right. Prestained molecular mass standards are indicated on the left in kDa. Approximately equal amounts of phosphotyrosine levels on Tyr394 and Tyr505 were determined by an anti-phosphotyrosine Western blot (data not shown).

**Fig. 6.** Binding assay of 6His-D2 and 6His-C817S to Src family GST fusion proteins. 1 µg of soluble 6His-D2 or 6His-C817S was incubated with 2 µg of immobilized GST fusion proteins in 50 µl of 20 mM Tris, pH 7.5, 150 mM NaCl, and 0.025% β-mercaptoethanol for 2 h at 4 °C, and washed with radioimmunoprecipitation assay buffer (see "Experimental Procedures"). The samples were separated by 10% SDS-PAGE and transferred to PVDF membrane. A, 6His-C817S added to immobilized GST-Src family kinases. B, 6His-D2 added to immobilized GST-Src family kinases. The top panels in both A and B are anti-GST-CD45 Western blots (R02.2) to detect relative amounts of 6His-D2 and 6His-C817S remaining bound. The lanes are as indicated above the gels. GST alone was included as a negative control. Prestained molecular mass standards are indicated on the left in kDa. The bottom panels are the membranes after Coomassie Blue staining to show the relative amounts of immobilized GST fusion proteins present. GST-Src was always heavily degraded, however, GST-Lck degradation varied between different purification preparations.

**Fig. 7.** Relative binding of 6His-D2 to GST-Lck, GST-Csk, and GST-Erk1. 0.54 µg of 6His-D2 was added to 2 µg of immobilized GST fusion protein in a binding assay as described in Fig. 2. The immobilized proteins are indicated above. GST-Grb2 and GST alone were included as negative controls. After the beads were washed, reducing sample buffer was added to each assay, and the samples were electrophoresed on an SDS-polyacrylamide gel and transferred to PVDF membrane for immunoblot analysis. A, anti-CD45 Western blot (R02.2) showing the amount of 6His-D2 remaining bound to the GST fusion proteins. B, Coomassie Blue stain. The membrane from A was stained with Coomassie Blue to show the relative amounts of immobilized proteins. Prestained molecular mass standards are indicated on the left in kDa.
CD45-D2 to substrate facilitates substrate dephosphorylation and contributes towards the observed substrate specificity for Src family kinases.

**The SD10 Region of Lck Binds 6His-D2 and Is a Contributing Factor in Determining Substrate Specificity**—To determine the region in Lck involved in mediating binding to 6His-D2, we used SWISS-MODEL (52) to model CD45 and Lck (based on the *in vitro* data and the three-dimensional structures of the two-domain PTP, LAR (53) and the Src family kinases (54–56)) to look for possible exposed sites of interaction. Because the catalytic domains of tyrosine and serine/threonine kinases are highly conserved in three-dimensional structure and in the existence of 12 subdomains (57, 58), the overall structures of Lck, Csk, and Erk1 are quite similar. Given the 6His-D2 binding data, the interacting region was predicted to be conserved within the Src family kinases, Lck, Fyn, and Src, to be less conserved in Csk and not conserved in Erk1. In addition, many kinases are regulated by phosphorylation (59), thereby implicating interactions with other regulatory protein kinases and phosphatases. It is possible that a specific region within the kinase domain may have evolved as a substrate recognition domain, to mediate the regulatory interaction with other kinases and phosphatases. Such a region would be predicted to be divergent between distantly related kinases. This rationale focused our attention on SD10 (57, 58). This subdomain is located and exposed at the base of the large kinase lobe. It is the most poorly conserved subdomain in the protein kinase superfamily, and its function is not known (57, 58). Comparison of this region in Lck with Src, Fyn, Csk, and Erk1 revealed 60% identity with Src and Fyn, 40% with Csk, and essentially no identity with Erk1 (Fig. 9). This was consistent with the observed binding of 6His-D2 to these kinases.

To determine if the SD10 of Lck played a role in binding 6His-D2, a chimeric protein was made in which the Erk1 SD10 region was replaced by that of Lck (see “Experimental Procedures” for details). This chimeric protein was expressed in *E. coli* as a soluble GST fusion protein (GST-Erk(L-SD10)). Like GST-Erk1 (31, 49), GST-Erk(L-SD10) was capable of *in vitro* tyrosine autophosphorylation, indicating that the chimeric kinase was active. Fig. 10A shows that significantly more 6His-D2 bound to the chimeric GST-Erk(L-SD10) protein than to the GST-Erk protein, and Fig. 10B shows that equal amounts of GST-Erk1 and GST-Erk(L-SD10) were present. Calculation of the amount of 6His-D2 binding to equivalent amounts of kinase (Fig. 10D) indicated that the increased binding of 6His-D2 to GST-Erk(L-SD10) over GST-Erk approached the level of binding of 6His-D2 to GST-Lck. Specifically, the binding of 6His-D2 to GST-Erk (L-SD10) was approximately two-thirds the level of 6His-D2 binding to GST-Lck. This demonstrates that Lck SD10 is sufficient to mediate binding to 6His-D2 as transfer of this region to a distantly related kinase confers binding to a kinase that does not normally bind to 6His-D2.

Given the binding of 6His-D2 to the Erk(L-SD10) chimera but not to Erk1, it was next investigated whether the presence of Lck SD10 in the Erk(L-SD10) chimera also affected its dephosphorylation by CD45. GST-Erk1 and GST-Erk(L-SD10) were phosphorylated to approximately equal levels but were not autophosphorylated as efficiently as GST-Lck. To compare the rate of dephosphorylation of Erk and the Erk chimera with equivalent amounts of phosphorylated Lck (Fig. 11), one tenth the amount of Lck was used. Unlike GST-Erk1, the GST-Erk(L-SD10) chimera was dephosphorylated at a faster rate, similar to the dephosphorylation rate of GST-Lck (Fig. 11), indicating that the exchange of Erk SD10 with Lck SD10 is sufficient to convert Erk1 into an efficient substrate for CD45. This indicates that the SD10 region of Lck plays a significant role in binding CD45 and in facilitating substrate dephosphorylation. Identification of this region as a substrate recognition domain for CD45 helps explain why Src family kinases, but not more distantly related kinases, are preferred substrates for the tyrosine phosphatase, CD45.

**DISCUSSION**

We have identified multiple interactions occurring between Lck and the cytoplasmic domain of CD45. CD45-D1 containing the active catalytic domain interacted with the phosphorylated kinase domain as well as the SH2 domain and unique N-terminal region. CD45-D2 containing the inactive phosphatase domain bound to the SD10 region of the kinase domain in a
CD45 Western blot

Erk Western blot

Coomassie blue

D Relative D2 binding

FIG. 10. Binding of 6His D2 to GST-Lck, GST-Erk1, and GST-Erk(L-SD10). A, 0.54 μg of 6His-D2 was added to 2 μg of immobilized GST fusion protein in 40 μl of 20 mM Tris, pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, and 0.05% Triton X-100 and incubated for 2 h at 4 °C in a binding assay as described in Fig. 2. After the beads were washed, reducing sample buffer was added to each assay, and the samples were electrophoresed on an SDS-polyacrylamide gel and transferred to PVDF membrane for immunoblot analysis. A, anti-CD45 Western blot (R02.2) showing the amount of 6His-D2 remaining bound to the GST fusion proteins. GST fusion proteins used in the binding assay are shown in an Erk Western blot (B), and in a Coomassie Blue protein stain (C). Prestained molecular mass standards are indicated on the left in kDa. D, graph showing the relative binding of 6His-D2 to equivalent amounts of GST-kinase (indicated at the top of panel A). Relative binding was determined by dividing the level of 6His-D2 binding by the amount of GST-kinase (derived from the Coomassie Blue stain (C)). The data are the average from four experiments, and the error bars represent S.E.

manner that was independent of Lck phosphorylation and conformation.

The reason for the presence of two tandem repeated PTP domains in several transmembrane phosphatases has been enigmatic, particularly as the second domain often has a less well conserved catalytic cleft and many have no detectable PTP activity (reviewed in Ref. 60). One possibility is that, like STYX domains (61), an inactive PTP domain may have evolved to bind phosphotyrosine-containing proteins. However, CD45-D2 bound to Lck independently of its tyrosine phosphorylation status and did not bind detectable levels of phosphotyrosine, making this mode of binding unlikely for the second domain of CD45. Two proteins, a CD45-associated protein, CD45-AP, and the β chain of the interleukin 2 receptor, have been shown to interact with the Lck kinase domain (62, 63). In both these cases an acidic region was shown to mediate their interaction with Lck. Because the acidic region of CD45-D2 was not required for its interaction with Lck, the mode of binding to CD45 must be distinct from the interaction between Lck and these two molecules.

CD45 is a very potent tyrosine phosphatase, and over time it will dephosphorylate all tyrosine-phosphorylated proteins in a pervanadate-stimulated T cell lysate.2 In this study, differences in the rate of dephosphorylation of certain protein substrates by CD45 indicated that CD45 does exhibit substrate specificity in vitro. Evidence was presented for a role of the SD10 region of Lck and the non-catalytic PTP domain of CD45 in facilitating the enzyme-substrate interaction and in promoting substrate dephosphorylation. Accumulating evidence indicates that PTPs are not as promiscuous as originally thought, and cytosolic PTPs, PTP1B and T cell-PTP, exhibit very selective substrate specificity in vivo. This is thought to be achieved by a combination of intrinsic catalytic domain specificity and by phosphatase targeting of the non-catalytic C-terminal region of PTP1B and the alternatively spliced forms of T cell-PTP to specific regions in the cell (reviewed in Ref. 64). Here we provide evidence of a non-catalytic interaction between Lck and CD45 that helps to explain the preferred dephosphorylation of Src family kinases by CD45 observed in leukocytes.

The data reported here demonstrate that one function of the second phosphatase domain of CD45 (CD45-D2) is to bind substrate. Consistent with this, CD45-D2 has also been implicated in binding of CD3ζ, a potential CD45 substrate in T cells, because exchange of CD45-D2 for LAR-D2 resulted in the loss of binding of CD3ζ to a CD45 substrate-trapping mutant (26). Recently, another kinase family, the Jak kinases, was implicated as CD45 substrates. In this example, CD45-D2 was shown to bind Jak2 kinase (28). Comparison of the SD10 region of Jak2 with Lck shows 35% sequence identity, making it similar to that of Csk. It will be of interest to compare the relative binding of CD45-D2 to Lck and Jak2. CD45-D2 has previously been implicated in influencing substrate specificity, because serine phosphorylation of D2 can enhance CD45 activity for certain artificial substrates (65, 66) and CD45-D2 deletions can differentially influence enzymatic activity, depending upon the substrate used (11, 12).

We determined that a 23-amino acid sequence from the SD10 region of Lck is sufficient to mediate the interaction with CD45-D2. This region is located at the bottom of the large lobe of the kinase domain and contains Helix G and the connecting loop to Helix H and is exposed in both the active and inactive conformations (54–57). This region is well conserved between

2 J. Felberg and P. Johnson, unpublished observations.
Src family kinases (–60% sequence identity), is less conserved for Csk (40% sequence identity), and is not conserved for more distantly related kinases such as Erk1 (no significant sequence identity; see Fig. 9). The crystal structure of an Erk family kinase, Erk2, indicates that the larger SD10 region of Erk kinases contains two additional small helices in addition to Helix G (67), making this region quite distinct from Lck SD10. This report identifies the SD10 region of Lck as an important docking site for CD45. The binding of CD45 to this site facilitates substrate dephosphorylation implying that this region plays a significant role in optimizing the enzyme-substrate interaction and thereby facilitating substrate dephosphorylation. The more divergent the sequence of SD10 was from that of Src family kinases, the less CD45-D2 bound and the slower the kinase was dephosphorylated by CD45. Because many protein kinases are regulated by phosphorylation and must therefore interact with protein phosphatases, it will be interesting to determine if the SD10 region of other kinases influences the binding and substrate selection of other protein phosphatases. Interestingly, the crystal structure of the serine/threonine kinase, Cdk2, complexed with its regulatory phosphatase, KAP, revealed an extensive interaction away from the catalytic site between the C-terminal region of the kinase (encompassing the SD10 region) and the C-terminal helix of the phosphatase (68). The structural and biochemical data suggest that this interaction provides the dominant specificity site for recognition of the kinase by KAP, which also provides the correct alignment for the dephosphorylation of the activation segment of the kinase by KAP (68). This, together with the data reported here, raises the intriguing possibility that the SD10 region of protein kinases may have evolved to be a general recognition/specificity domain that binds regulatory phosphatases.

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Subdomain X of the Kinase Domain of Lck Binds CD45 and Facilitates Dephosphorylation
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