Characterization of Recombinant CD45 Cytoplasmic Domain Proteins

EVIDENCE FOR INTRAMOLECULAR AND INTERMOLECULAR INTERACTIONS*

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CD45 is a transmembrane two-domain tyrosine phosphatase required for efficient signal transduction initiated by lymphocyte antigen receptors. As with most transmembrane two-domain phosphatases, the role of the second phosphatase domain is unclear. In this study, recombinant CD45 cytoplasmic domain proteins purified from bacteria were used to evaluate the function of the individual phosphatase domains. A recombinant protein expressing the membrane-proximal region, first phosphatase domain, and spacer region of CD45 (rD1) was catalytically active and found to exist primarily as a monomer. In contrast to this, a recombinant protein expressing the spacer region, the second phosphatase domain and the carboxy tail of CD45 (rD2) existed as a monomer and had no catalytic activity against any of the substrates tested. Comparison of rD1 with the recombinant protein expressing the entire cytoplasmic domain of CD45 (rD1/D2) indicated that rD1/D2 was 2–3-fold more catalytically active, was more thermostable, and existed primarily as a monomer. Limited trypsin digestion of rD1/D2 provided evidence for a noncovalent association between an N-terminal 27-kDa fragment and a C-terminal 53-kDa fragment, suggesting an intramolecular interaction. Furthermore, rD1 was found to specifically associate with rD2 in an in vitro binding assay. Taken together, these data provide evidence for an intramolecular interaction occurring in the cytoplasmic domain of CD45. In the absence of the C-terminal region containing the second phosphatase domain, intermolecular interactions occur, resulting in dimer formation.

CD45 is a transmembrane protein-tyrosine phosphatase (PTP)1 that is expressed on all nucleated hematopoietic cells.

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1 The abbreviations used are: PTP, protein-tyrosine phosphatase; GST, glutathione S-transferase; pNPP, para-nitrophenyl phosphate; rD1/D2, recombinant 6-His-tagged CD45 cytoplasmic domain protein; rD1, recombinant 6-His-tagged CD45 protein containing the membrane-proximal region, the first phosphatase domain, and the spacer region; rD2, recombinant 6-His-tagged CD45 protein containing part of the spacer region, the second phosphatase domain, and the carboxy tail; rD2.2, recombinant 6-His-tagged CD45 protein containing the membrane-proximal region, the second phosphatase domain, and the carboxy tail; PTP-D1, the first phosphatase domain; PTP-D2, the second

Its phosphatase activity is required for efficient signal transduction initiated by lymphocyte antigen receptors, which is essential for T cell development and T and B cell activation (reviewed in Refs. 1 and 2). In T cells, CD45 has been shown to dephosphorylate p56lck and p59fyn in vitro and to be responsible for their dephosphorylation in vivo (3–7). The role of CD45 in the dephosphorylation of p56lck and p59fyn is likely to be necessary in vivo. How substrate specificity is achieved in the cell is not yet understood. However, it is of interest to note that CD45 can associate with p56lck, a major substrate for CD45 in T cells, both in vitro (8, 9) and in T cells, albeit with low stoichiometry (10, 11).

It is also not known how the phosphatase activity of CD45 is regulated. By analogy with receptors possessing tyrosine kinase activity, it is possible that receptor tyrosine phosphatases may be regulated by dimerization. It has been suggested by cross-linking studies in T cells that CD45 may exist in dimeric form together with CD45AP, a 30-kDa CD45-associated protein (12). Induced dimerization of an epidermal growth factor receptor-CD45 chimera with epidermal growth factor has been shown to inhibit the restoration of T cell signaling events (13), suggesting the possibility that CD45 activity may be regulated by dimerization. In addition, the first phosphatase domain (PTP-D1) of a related phosphatase, RPTPa, crystallized as a dimer, with the membrane-proximal region of one PTP domain inserted into the catalytic region of the second PTP domain. It was predicted that if such an interaction occurred under more physiological conditions, phosphatase activity would be inhibited (14). However, to date, no direct evidence has been obtained for the formation of CD45 dimers.

CD45, like RPTPa, belongs to the growing family of protein-tyrosine phosphatases. For most members of the transmembrane, two-domain phosphatase family, the majority of catalytic activity has been shown to reside in PTP-D1 and the function of the second phosphatase domain (PTP-D2) is unclear. PTP-D2 of RPTPa has been shown to possess catalytic activity, albeit at much lower levels than PTP-D1 (15). In other transmembrane PTPs, no phosphatase activity has been detected for PTP-D2, which in some instances lack key residues involved in catalysis (Refs. 16–19 and reviewed in Refs. 20–22). In these cases, a regulatory role for PTP-D2 has been suggested, and in some two-domain PTPs, the presence of PTP-D2 has been shown to be required for optimal PTP-D1 activity (15, 17, 19). For CD45, contradictory data exists as to
whether CD45 is an active phosphatase in the absence of PTP-D2 (17, 18, 23). Recent data shows that CD45 PTP-D1 is active when coupled to a maltose-binding protein fusion partner but is not active when the fusion partner is proteolytically removed (24). Likewise, conflicting data exists as to whether CD45 PTP-D2 is an active phosphatase. No catalytic activity has been attributed to CD45 PTP-D2 when expressed in vitro as a recombinant protein (18) or after the essential cysteine in PTP-D1 has been mutated to a serine and expressed in vitro (16–18). Mutation of the critical cysteine in PTP-D1, but not in PTP-D2, prevented the restoration of CD45-mediated T cell signaling events (25), indicating that phosphatase activity residing in PTP-D1, and not PTP-D2, was essential for this function. However, expression in eukaryotic cells of an active, truncated form of CD45 lacking the catalytic cysteine present in PTP-D1 has led researchers to suggest that PTP-D2 has phosphatase activity (26). Thus the function of PTP-D2 of CD45 is unclear.

To clarify the function of the individual PTP domains of CD45 and to determine the role of these domains in the enzymatic function of CD45, recombinant CD45 cytoplasmic domain proteins were produced in Escherichia coli, purified, and analyzed. It was found that rD1 of CD45 was an active protein-tyrosine phosphatase in the absence of PTP-D2 and was present as a dimer. In contrast, no phosphatase activity was detected for rD2 of CD45. In the two-domain CD45 cytoplasmic domain protein, the presence of PTP-D2 enhanced the stability of the enzyme and was required for optimal enzymatic activity. Specific binding of rD1 to rD2 and the association of N- and C-terminal tryptic fragments of rD1/D2 provided evidence for an intramolecular interaction occurring in rD1/D2. In the absence of the C-terminal region including PTP-D2, intermolecular interactions occur, leading to the formation of rD1 dimers.

EXPERIMENTAL PROCEDURES

Materials—Nickel-NTA-agarose was from Qiagen Inc. (Santa Clarita, CA.), Sephadex G-25M PD-10, MonoQ and Superose 12 columns were from Amersham Pharmacia Biotech, p-Nitrophenyl phosphate (pNPP) was from Pierce, tyrosine-phosphorylated peptides (CD3-pY38, LGREEpYDVEKKRA; fn-pY531; TATEPpQpYQPpGpLN; src-pY416; LIEDEpYPYQRGC; cd2-pY15, KIGEGTpYGpVVyKA; and PDGF-R-pY1021; NKGNDpYIIpLpPD) were from Dr. F. Jirik and Dr. I. Clark-Lewis (Biomedical Research Center, University of British Columbia). GST-p56lck autophosphorylated GST-p56lck, and cdc2 (pY15; KIGEGTpYGpVVYKA; and polyvinylidene difluoride membrane (Immobilon P, Millipore Corp., Bedford MA.), and the amount of phosphorylated substrate remaining was determined by Western blot analysis with 4G10 antiphosphotyrosine antibody (Amer sham, Arlington Heights, IL). The decision to initiate the reaction was stopped by immersion in a dry ice/ethanol bath, samples were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon P, Millipore Corp., Bedford MA.), and the amount of phosphorylated substrate remaining was determined by Western blot analysis with 4G10 antiphosphotyrosine antibody diluted 1:1,000 as the primary antibody and horseradish peroxidase-labeled goat anti-mouse antibody diluted 1:5,000 (Southern Biotechnology Associates, Inc., Birmingham, AL) as the secondary antibody. The blot was developed using enhanced chemiluminescence (ECL) reagents (Amersham) and exposed to BioMax film from Eastman Kodak Co. Limited Trypsin Digestion—Trypsin was added to 1.5 μg of rD1/D2 in a total volume of 20 μl of substrate buffer (50 μM Tris, pH 7.5, 1 mM CaCl2) at ratios of 1:100, 1:50, 1:25, and 1:10 w/w. The digest was incubated at 37 °C for 1 h and terminated by the addition of 1 μl of 100 mM phenylmethylsulfonyl fluoride. 5 μl of each sample was run on a native polyacrylamide gel, and 15 μl was run on a 10% SDS-polyacrylamide gel under reducing conditions.

N-terminal Sequencing—12 μg of rD1/D2 was digested with trypsin for 1.5 h at 1:25 w/v rD1/D2:trypsin, reducing sample buffer was added at 37 °C for 15 min, and the sample was electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred to Immobilon P (polyvinylidenefluoride) membrane. The membrane was stained with 0.025% Coomassie Brilliant Blue R-250, the 53-KDa and 27-KDa bands were excised, and the N-terminal sequence was obtained from the Nucleic Acid-Protein Service (NAPS) unit (University of British Columbia, Vancouver, B.C., Canada).

Gel Filtration—A Superose 12 gel filtration column was equilibrated with 50 mM Tris, pH 7.5, 100 mM NaCl, 0.025% β-mercaptoethanol. The column was calibrated with globular protein gel filtration standards
Fig. 1. Recombinant murine CD45 cytoplasmic domain proteins. A, schematic representation of the 6-His-tagged CD45 cytoplasmic domain proteins expressed in E. coli (numbered according to Ref. 27). The numbers above construct 1 indicate the first and last residues of the protein and the various domains are named below the construct. MP is the membrane-proximal region, PTP-D1 is the first, N-terminal phosphatase domain, SP is the spacer region, PTP-D2 is the second, C-terminal phosphatase domain, and CT is the carboxy tail. The starred residue in construct 2 represents a point mutation of the catalytic cysteine in PTP-D1, which is altered to a serine, and the numbered residues in 3 and 4 denote the last and first residues, respectively. The deleted residues are indicated in 5. B, SDS-polyacrylamide gel electrophoresis analysis of the purified recombinant proteins. The proteins were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. Lanes 1 to 5 are constructs 1 to 5 depicted in A. Molecular mass standards are indicated on the left in kDa.

CD45 cytoplasmic domain antisera (data not shown), suggesting that the lower band is a proteolytic fragment. Equal molar amounts were assayed for phosphatase activity, and rD1 was found to be catalytically active using the CD3ζ pY83 phosphopeptide as a substrate, although it was about 2-fold less active than rD1/D2 (Fig. 2). Both rD2 and C817S had no detectable activity against this substrate. Kinetic analysis of rD1 and rD1/D2 activity using pNPP and other tyrosine-phosphorylated peptides as substrates indicated that the two-domain phosphatase (rD1/D2) was a 2–3-fold more efficient enzyme than rD1 (Table I). In contrast to this, the rD2 proteins expressing either the membrane-proximal region or the spacer region at the N terminus of the protein did not exhibit any detectable phosphatase activity for any of the substrates listed in Table I. Even when 100× more enzyme (2.6 μM) was assayed for up to 4 h with CD3ζ pY83 or pNPP substrates, no detectable activity was observed. The presence of PTP-D2 in the rD1/D2 protein did not affect the substrate specificity of CD45, at least when tested against the substrates indicated in Table I. rD1 and rD1/D2 were also able to dephosphorylate autophosphorylated recombinant GST-p56lck (Fig. 3). Comparison of the initial rates of dephosphorylation (Fig. 3B) indicated that the recombinant two domain phosphatase, rD1/D2, was again approximately 2-fold more efficient than rD1.

The Presence of Domain 2 Influences the Catalytic Activity and Enzyme Stability of CD45—To further investigate the differences between the single (rD1) and two-domain CD45 phosphatase (rD1/D2), enzymatic activity was compared at different pHs (Fig. 4). Both rD1 and rD1/D2 showed optimal activity at pH 7.2 using pNPP as the substrate and displayed a similar pattern of activity in the basic pH range. However, in the acidic pH range, between pH 5.5–7.0, rD1/D2 consistently showed a higher level of activity than rD1 alone, indicating that the presence of PTP-D2 creates a more favorable environment for an ionizable group involved in the catalytic reaction. Thermal stability of enzyme activity was also assessed by incubating rD1/D2 and rD1 at the indicated temperatures in Fig. 5 for 5 min then assessing equal molar concentrations at 30 °C using pNPP as a substrate. rD1 consistently lost phosphatase activity at lower temperatures than rD1/D2, indicating that it is less thermostable.

rD1/D2 and rD2 Are Primarily Monomers, rD1 Is Primarily a Dimer, and rD2.2 Aggregates in Solution—The molecular sizes of the native recombinant proteins were estimated by gel filtration (Fig. 6A). Interestingly rD1, but not rD2, eluted at a similar volume to rD1/D2, suggesting that the majority of rD1...
was present in a dimeric form, whereas the elution volume of rD2 suggested that it was primarily present as a monomer. rD1 eluted primarily as a single peak at this volume over a concentration range of 1–100 μM/ml, indicating that rD1 is primarily present in a dimeric form, whereas rD1/D2 eluted as a monomer over a similar concentration range. Although rD2 was present as a monomer, rD2/D2, containing the membrane-proximal region in place of the spacer region, was present in large aggregates and eluted in the void volume (data not shown).

To further confirm the presence of a dimeric form of rD1 and monomeric forms of rD1/D2 and rD2, the recombinant proteins were electrophoresed on both native and SDS-polyacrylamide gels. On the native gel (Fig. 6B), the majority of rD1/D2 and rD2 migrated to similar positions observed under denaturing, SDS-polyacrylamide gel electrophoresis conditions (Fig. 6C).

However, under native conditions, rD1 migrated to a similar apparent molecular weight as rD1/D2 (Fig. 6B), consistent with dimer formation, whereas it migrated as a monomer after SDS-polyacrylamide gel electrophoresis (Fig. 6C). Although rD1/D2 migrated primarily as a dimer under native conditions (Fig. 6B), some monomer was detected after Western blot analysis (data not shown). At 1 μM/ml (23 nM), rD1 was demonstrated to be active against tyrosine-phosphorylated peptides, indicating that rD1-rD1 dimer formation does not prevent catalytic activity. However, it was noted that this enzyme was approximately 2-fold less active than rD1/D2, which primarily

**TABLE I**

**Phosphatase activity of recombinant CD45 cytoplasmic domain proteins**

| Substrate                  | Enzyme       | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $V_{cat}/K_m$ (μM s$^{-1}$) |
|----------------------------|--------------|------------|----------------------|-----------------------------|
| pNPP$^a$                  | rD1/D2       | 0.15 ± 0.01| 1.33 ± 0.15          | 8.89 × 10$^3$               |
| CD3ζ pY83 (LGRRERpYDVEKKRA) | rD1/D2       | 0.15 ± 0.01| 0.56 ± 0.08          | 3.70 × 10$^3$               |
| src pY416 (LIEDNEpYTARQGA)  | rD1/D2       | 0.35 ± 0.04| 47 ± 11              | 1.34 × 10$^4$               |
| cdc2 pY15 (KIGEGTpYGVYKA)  | rD1/D2       | 0.36 ± 0.09| 98 ± 21              | 2.72 × 10$^4$               |
| PDGF-R pY1021 (NEGDNpYIIPLPD) | rD1/D2     | 0.31 ± 0.04| 87 ± 20              | 2.81 × 10$^4$               |
|                            | rD1          | 0.23 ± 0.06| 28 ± 13              | 1.21 × 10$^5$               |

$rD1/D2$ and $rD1$ were each assayed at 7.5 nM using pNPP as a substrate.

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**FIG. 3. Phosphatase activity of rD1/D2 and rD1 using recombinant GST-p56$^{lck}$ as the substrate.** A, 5 μl of 60 nM concentration of rD1/D2 or rD1 were incubated with 5 μl of 55 nM concentration of GST-p56$^{lck}$ fusion protein for the time points indicated. Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane, and the relative amount of phosphorylated tyrosine residues remaining in the GST-p56$^{lck}$ was detected by immunoblot analysis using an anti-phosphotyrosine antibody (4G10). Molecular mass standards are indicated on the left in kDa. B, graphical representation of GST-p56$^{lck}$ dephosphorylation by rD1/D2 and rD1. The bands in A were subjected to densitometry scan analysis, and the amount of phosphorylation at time 0 was taken as 100%.

**FIG. 4. pH optimum of rD1/D2 and rD1.** Phosphatase activity was measured using 13.5 nM pNPP as substrate and rD1/D2 and rD1 at equal molar concentrations ranging from 22 to 50 nM (see “Experimental Procedures”). The data is an average from three to six experiments.

**FIG. 5. Thermostability of rD1/D2 and rD1.** Equal molar concentrations of rD1/D2 and rD1 (233 nM) were incubated at the indicated temperatures for 5 min before phosphatase activity was measured at 30 °C using standard assay conditions with 2.5 mM pNPP as substrate; final enzyme concentration was 6 nM (see “Experimental Procedures”). The data was an average from two experiments. Similar results were obtained when the CD3ζ pY83 phosphopeptide was used as the substrate (data not shown).
Evidence for an Intramolecular Interaction in rD1/D2—The fact that rD1 forms a dimer in solution but rD1/D2 does not suggest that an intramolecular interaction may be occurring in the full-length cytoplasmic domain of CD45 between PTP-D1 and PTP-D2, thus preventing dimerization. This would also help to explain the effect of PTP-D2 on the catalytic activity and stability of PTP-D1 in the rD1/D2 protein. To try and establish whether intramolecular interactions were occurring in rD1/D2, limited trypsin digestion was performed, and the digested products were electrophoresed on both native and SDS-polyacrylamide gels. Fig. 7A shows that with increasing amounts of trypsin, rD1/D2 is digested primarily into two major fragments of approximately 27 and 53 kDa. Interestingly, these tryptic fragments electrophoresed as a single band on the native gel, indicating that both fragments associated with one another (Fig. 7B). Excision of this band from the native gel and subsequent electrophoresis on an SDS-polyacrylamide gel confirmed that both tryptic fragments were present (Fig. 7C). To further localize the regions involved in this interaction, N-terminal sequence was obtained for each tryptic fragment. From the N-terminal sequence, SSNLDE, the 27-kDa fragment was found to begin at residue 573, thus comprising the N-terminal portion of the CD45 cytoplasmic domain estimated to contain the majority of the membrane-proximal region and approximately two-thirds of the first phosphatase domain. The 53-kDa fragment was found to begin at residue 770 with the sequence ATGREV and thus can be estimated to consist of the last third of the first phosphatase domain (including the catalytic cysteine 817), the spacer region, the entire second phosphatase domain, and probably some of the carboxy tail. This indicates the presence of a noncovalent association between the N- and C-terminal regions of the cytoplasmic domain of CD45.

To further determine whether this intramolecular interaction involved the regions present in rD2, in vitro binding assays were performed with rD1 and rD2 proteins. rD2 was made as a GST fusion protein (GST-rD2) immobilized to beads, and recombinant 6-His-tagged proteins including rD1 and rD2 were added. As can be seen in Fig. 8, rD1 bound preferentially to immobilized GST-rD2 but not to GST alone or to another GST fusion protein. In addition, only rD1 and not other 6-His-tagged proteins, including rD2, associated with GST-rD2, indicating a specific association. However, only a small percentage (~5%) of rD1 bound to rD2. The reason for this is presently unknown but may reflect the fact that rD1 is already present as a dimer. The identification of a specific interaction between rD1 and rD2 provides further evidence for an intramolecular head to tail interaction occurring within the cytoplasmic domain of CD45.

**DISCUSSION**

This data establishes that PTP-D1 of CD45 does not require the presence of PTP-D2 to be catalytically active. This contradicts our previously published results, which did not detect any phosphatase activity from an in vitro translated and immunoprecipitated rD1 protein (18). Likewise, Streuli et al. (17) detected only 0.1% wild-type activity from a rD1 protein assayed from bacterial cell lysates, and Lorenzo et al. (24) recently found that a recombinant protein expressing CD45 PTP-D1 was not active when its maltose-binding protein fusion partner was cleaved (24). In this present study, several differences were evident, which may help explain why significant levels of activity were observed for the rD1 protein. First, the rD1 protein was produced in large amounts and was isolated and purified.
FIG. 8. Binding assay of soluble 6-His proteins to immobilized GST-rD2. A, 1 μg of each protein was incubated in 50 μl of 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, and 0.025% SDS for 2 h and washed (see “Experimental Procedures”). A, immobilized GST fusion proteins stained with Coomassie Blue. The first two lanes contain 50 and 100 ng of 6-His rD1, respectively, which are below the level of detection with Coomassie Blue. B, anti-6-His Western blot. The first two lanes indicate the signal obtained from 50 and 100 ng of rD1, respectively. The soluble 6-His proteins added to the immobilized GST proteins indicated in A are shown above the lanes. C is a control, a recombinant 6-His-glucosidase protein.

away from other potentially inhibitory compounds. Second, the isolation and purification procedure was optimized to minimize exposure to conditions that favor protein unfolding and degradation (see “Experimental Procedures”). Third, assays such as CD spectral analysis were performed, which helped to confirm the structural integrity of the protein (data not shown).

No catalytic activity was detected for rD2 or rD2.2. rD2 was found to be a monomer in solution, whereas rD2.2 was present as a large aggregate. rD2.2 contained the membrane-proximal region of CD45, which may have contributed to this aggregation. Alternatively, the splicing of this region to PTP-D2 may have produced an unstable or misfolded protein, although CD spectral analysis indicated that this molecule possessed a similar secondary structure to that observed in the rD1/D2 protein (data not shown). Under similar purification conditions that produced an active rD1, no detectable activity was observed for rD2, even when tested in a 100-fold excess for several h. Thus we conclude that PTP-D2 is not catalytically active against any of the substrates tested when expressed as a recombinant protein. This result supports previous data obtained from in vitro translated and immunoprecipitated recombinant PTP-D2-containing proteins (18). In addition, the sequence at the predicted catalytic region of CD45 PTP-D2 is lacking an arginine and aspartate residue, two residues that are thought to play a crucial role in the enzymatic reaction (reviewed in Refs. 20–22). Mutation of the presumed catalytic cysteine in PTP-D2 had no effect on the protein-tyrosine phosphatase activity of CD45 in recombinant systems and in the restoration of T cell function by a chimeric epidermal growth factor receptor/CD45 phosphatase (16, 18, 25), again implying that PTP-D2 is not an active phosphatase either as a recombinant protein or when expressed in T cells. However, one group isolated two truncated CD45 proteins lacking different regions of PTP-D1 from eukaryotic cells, which had catalytic activity, and this was attributed to an active PTP-D2 (26). In this study we have demonstrated that in the absence of any PTP-D1 sequence, two purified recombinant PTP-D2 proteins were not catalytically active against any of the substrates tested.

This present work establishes that CD45 is similar to other two domain phosphatases such as LAR, RPTPα, and RPTPμ, where PTP-D1 has been shown to be independently catalytically active (15, 17, 19). With the exception of LAR (17, 33), optimal catalytic activity was observed with these phosphatases when both PTP-D1 and PTP-D2 were present. Like CD45, no catalytic activity has been observed for the second domain of these phosphatases, except for RPTPα, where the observed catalytic activity for PTP-D2 was much lower than that observed with PTP-D1 (15). In other two domain phosphatases, PTP-D2 lacks key residues thought to be crucial for catalytic activity, making it very unlikely that these domains would be active phosphatases (reviewed in Refs. 20–22). Thus the second phosphatase domain in two-domain phosphatases has been shown to have little or no activity in comparison to their first phosphatase domain, and in many cases these domains are required for optimal activity of the first domain.

It has been suggested that PTP-D2 or phosphorylation of PTP-D2 can alter the substrate specificity of CD45 against certain artificial substrates (17, 34). In this study, using pNPP, a variety of peptide substrates, and the protein substrate GST-p56k, no evidence was obtained for a major role of PTP-D2 in influencing substrate specificity. Comparison of rD1 and rD1/D2 activities indicated that PTP-D2 is required for optimal enzymatic stability and optimal activity of the first phosphatase domain of CD45. The fact that the presence of PTP-D2 influences catalytic activity at acidic pH suggests that PTP-D2 can influence the catalytic environment of PTP-D1. Whether PTP-D2 is acting directly to modify the catalytic environment or indirectly by altering the conformation of PTP-D1 is not known. However, this and data indicating an interaction between rD1 and rD2 suggests that PTP-D1 and PTP-D2 may be in close proximity to one another.

Crystal structures of the first phosphatase domains of two transmembrane two-domain tyrosine phosphatases, RPTPα and RPTPμ, have been determined (14, 35). Although both crystallized as dimers, it was suggested that dimerization of the RPTPμ rD1 was an artifact of crystallization, as gel filtration indicated that the protein was a monomer in solution up to concentrations of 7.5 mg/ml (35). Dimers of RPTPα rD1 were formed by the membrane-proximal region of one rD1 molecule forming a “wedge” and binding to the active-site region of the second, an interaction that was predicted to block phosphatase activity. Dimers and higher oligomer formation occurred for RPTPα rD1 at concentrations between 0.1 and 5 mg/ml but did not occur for the rD2 protein (14). However, kinetic analysis of rD1 of RPTPα at lower concentrations was consistent with it being active as a monomer in solution (36). In this study, CD45 rD1 existed primarily as a dimer at the concentrations tested (1–100 μg/ml), whereas the two-domain phosphatase, rD1/D2, existed primarily as a monomer (5–1000 μg/ml). This suggests that the presence of PTP-D2 in the molecule prevents its dimerization. Contrary to the prediction from RPTPα studies (14), dimer formation of CD45 rD1 did not result in an inactive phosphatase; however, it was 2–3-fold less active than rD1/D2, raising the possibility that dimer formation may down-regulate activity. However, we cannot easily assess whether one or both of the phosphatase domains are active in the rD1/rD1 dimer.

Attempts to define the regions responsible for a potential intramolecular interaction in rD1/D2 identified a 27-kDa fragment containing the N-terminal region (the membrane-proximal region and two-thirds of PTP-D1) interacting with a 53-kDa fragment containing the C-terminal region (the last third of PTP-D1, the spacer region, PTP-D2, and the carboxyl tail) after limited trypsin digestion. This 53-kDa tryptic fragment is the same fragment identified and purified by Tan et al. (26) who concluded that the catalytic activity was derived from PTP-D2. However, in these studies, we found this tryptic frag-
ment noncovalently associated with a 27-kDa fragment derived from PTP-D1 and, despite finding no activity associated with rD2 proteins, found this complex to be catalytically active (data not shown).

Evidence for a noncovalent intramolecular interaction from tryptic digestion studies and for a rD1-rD2 association suggests an interaction between the N-terminal part of the protein containing PTP-D1 and the C-terminal part containing PTP-D2. It is tempting to speculate that in the rD1/rD2 protein, the membrane-proximal region interacts with PTP-D2 to promote the intramolecular interaction and prevent intramolecular dimer formation, resulting in rD1/rD2 monomers. In the absence of PTP-D2, the membrane-proximal region of rD1 may interact with another rD1 molecule, thereby promoting dimer formation. Thus, PTP-D2 may act to prevent dimerization. Regulation of this membrane-proximal region-PTP-D2 interaction would result in the formation of intra- or intermolecular interactions, which may act to regulate CD45 function. Consistent with this model is data from CD45-negative T cells expressing the membrane-proximal region-PTP-D2 interaction. Thus, PTP-D2 may act to prevent dimerization. Regulation noncovalently associated with a 27-kDa fragment derived from PTP-D1 and, despite finding no activity associated with rD2 proteins, found this complex to be catalytically active (data not shown).

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