CD45-associated protein inhibits CD45 dimerization and upregulates its protein tyrosine phosphatase activity

Running head: CD45-AP INHIBITS CD45 DIMERIZATION

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Abstract

CD45, a receptor-like protein tyrosine phosphatase (PTP), plays an essential role in lymphocyte development and immune responses. Recent evidence suggests that dimerization of CD45 downregulates its function. However, the mechanisms by which CD45 dimerization is regulated remain unclear and there is no direct evidence that the PTP activity of CD45 dimers is less than that of monomers. CD45 in lymphocytes associates with CD45-AP (CD45-associated protein). Here we show that T cells from CD45-AP-null mice have a much higher level of CD45 dimers than those of wild-type mice, suggesting that CD45-AP inhibits CD45 dimer formation. This was confirmed using a novel CD45-AP-null T cell line, ALST-1, that we established from a spontaneous thymic tumor found in a CD45-AP-null mouse. Transfected CD45-AP inhibited CD45 dimer formation in ALST-1 cells in proportion to the amount of CD45-AP expressed. Finally, using microsomal fractions from both mouse thymocytes and ALST-1 transfectants, the PTP activity of CD45 was found to be significantly lower in CD45-AP-negative cells than in CD45-AP-positive cells. Therefore, our results support a model in which binding of CD45-AP to inactive CD45 dimers converts them to active monomers.
Introduction

CD45 is a leukocyte-specific receptor-like PTP consisting of an extracellular domain whose structure varies among different isoforms, a single-span transmembrane segment, and a cytoplasmic portion with two tandem PTP domains \(^1\). Antigen-receptor-mediated signal transduction in lymphocytes requires CD45 PTP activity \(^2-^4\). One of the earliest events in T cell receptor (TCR) signaling is tyrosine phosphorylation of the cytoplasmic domain of the TCR \(\zeta\) chain by Lck, a Src family protein tyrosine kinase (PTK) \(^5,^6\). It is generally thought that CD45 activates Src family PTKs by dephosphorylating their downregulatory tyrosyl residues and enabling them to assume an active conformation \(^7,^8\). However the mechanisms by which CD45 function is regulated remain poorly understood. In principle, the function of CD45 could be regulated by modulating its PTP activity and/or by regulating its access to substrates.

CD45 exists in both monomeric and dimeric forms \(^9,^10\), and several lines of evidence suggest that dimerization of CD45 downregulates its function. A recombinant chimeric CD45 protein that contains the ligand-binding domain of the epidermal growth factor receptor (EGFR) loses its ability to support TCR signaling upon forced dimerization by EGF \(^11\). Further the crystal structure of RPTP\(\alpha\), a PTP related to CD45, revealed a conserved wedge structure that inhibits function in dimers by interacting with the catalytic site \(^12\). Mutation of the corresponding wedge in CD45 appears to promote its signaling activity \(^13,^14\). Finally, different CD45 isoforms produced by alternative splicing have different homodimerization efficiencies that appear to be inversely proportional to their ability to support TCR signaling \(^15\).
CD45-AP is a transmembrane protein that consists of a short extracellular segment, a single-span transmembrane segment, and a cytoplasmic domain of 144 amino acid residues\textsuperscript{16,17}. Approximately 75\% of the total CD45 and CD45-AP in lymphocytes exist in a complex with each other\textsuperscript{9}. The interaction between the two proteins is mediated through their respective transmembrane segments\textsuperscript{18,19}. Since the transmembrane domain of CD45 may play an essential role in CD45 homodimerization\textsuperscript{15,20}, CD45-AP may hinder CD45 homodimerization and thereby increase its PTP activity. Indeed, data from CD45-AP-null mice suggest that CD45-AP promotes CD45-mediated signal transduction\textsuperscript{21}. In the present work, we show that cells from wild-type mice have a reduced amount of CD45 dimers compared to cells from CD45-AP-null mice. CD45 PTP activity was lower in cells from CD45-AP-null mice. To confirm these results, a CD45-AP-negative T cell line, ALST-1, was established from a CD45-AP-null mouse. When CD45-AP was transfected into ALST-1 cells, there was a reduction in CD45 dimers with a concomitant increase in CD45-PTP activity. These results are consistent with the notion that CD45-AP upregulates CD45 PTP activity by inhibiting CD45 homodimerization.
Materials and methods

Mice

CD45-AP-null mice \(^{21}\) were backcrossed into C57BL/6 mice for 8 generations and raised in our facility at Roger Williams Medical Center and Northwestern University. The wild-type C57BL/6 mice were purchased from Taconic (Germantown, NY) and Charles River (Wilmington, MA). Mice at 6 - 10 wk old were used.

Cell lines

ALST-1 cell line was established by continuous culture of cells obtained from a large spontaneous thymic tumor found in a CD45-AP-null mouse in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% fetal calf serum (FCS), 5 x 10^{-5} M 2-ME (2-mercaptoethanol), 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. ALST-1 cells were characterized by flow cytometric analysis after staining with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies (BD Sciences, SanDiego, CA) against CD3ε (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD19 (1D3), CD28 (37.51), CD45 (30-F11), CD45RA (14.8), CD45RB (16A), CD45RC (DNL-1.9), CD90 (30-H12), TCRβ (H57-597), IgM (R6-60.2), IgD (11-26c.2a), Ly6G (RB6-8C5), and panNK (DX5). The mouse T cell line YAC-1 (ATCC, Manassas, VA) \(^{22}\) was cultured in the same media as described above. To determine total cellular expression of CD45-AP, ALST-1 or YAC-1 cells were lysed at 50 x 10^6
cells/ml in BRIJ 97 (polyoxyethylene 10 oleyl ether) containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EGTA (ethyleneglycol-bis [beta-aminoethyl ether]-N, N', N', N'-tetraacetic acid), 3 mM MgCl₂, 2.5 mM thioglycolic acid, 1 mM PMSF (phenylmethylsulfonyl fluoride), 5 µg/ml aprotinin, and 1 µM bestatin. CD45 was immunoprecipitated from the postnuclear supernatants of the lysates by anti-CD45 antibody (M1.89.18.7) cross-linked to Protein G-Sepharose 4 beads with dimethyl pimelimidate. The lysates and the immunoprecipitates were then analyzed by immunoblotting with antibodies against CD45-AP followed by horseradish peroxidase-conjugated secondary antibodies and detection using the SuperSignal substrate system (Pierce, Rockford, IL).

**Stable transfection of ALST-1**

To construct the hemagglutinin A (HA)-tagged CD45-AP expression vector for transfection, the entire coding sequence of CD45-AP cDNA flanked with short linker sequences was ligated downstream from the Ig κ–chain leader sequence and the coding sequence for HA epitope. The resulting construct included sequences for 7 additional amino acids (GAQPARS) between HA epitope and the first amino acid of CD45-AP and was subcloned in pcDNA3.1/Hygro (Invitrogen, Carlsbad, CA). Transfection of 20 x 10⁶ cells was carried out with 40 µg DNA by electroporation using Bio-Rad Gene Pulser at 270 V and 960 µF. After 24 h, the cells were placed in limiting dilution culture in the presence of hygromycin B (0.8 mg/ml) to select stable transfectants. Surface expression of HA-CD45-AP was determined by flow cytometry of
ALST-1 transfectants stained with FITC-labeled anti-HA antibody (12CA5 from Roche, Indianapolis, IN). Cells expressing the highest level of CD45-AP were derived by cell sorting of the HA-CD45-AP transfectants with anti-HA antibody and collecting brightly stained cells using MoFlo (Dako-Cytomation, Fort Collins, CO). Unsorted cells had an intermediate level of CD45-AP expression. Cells expressing the lowest level of CD45-AP were the result of a spontaneous reduction of transfected CD45-AP after a few months of culture. To determine total cellular expression of HA-CD45-AP, ALST-1 transfectants were lysed at 50 x 10^6 cells/ml in the BRIJ 97 buffer described above. Anti-CD45 immunoprecipitates were prepared also as described above. The lysates and CD45 immunoprecipitates were then analyzed by immunoblotting with antibodies against CD45 (provided by J. Marth of UCSD, San Diego, CA or clone 69 purchased from BD Sciences), CD45-AP, HA (3F10 from Roche), and actin (I-19 from Santa Cruz Biotech., Santa Cruz, CA) followed by horseradish peroxidase-conjugated secondary antibodies and the SuperSignal substrate system.

**Biosynthetic labeling and chemical cross-linking**

Mouse splenic T cells were purified by two rounds of nylon-wool fiber columns. Typical splenic T cell populations obtained were approximately 90% pure by flow cytometric analysis of surface markers. Thymocytes from mice (3.5 x 10^6 cells/ml) or ALST-1 transfectants (1.0 x 10^6 cells/ml) were labeled with 15 µCi/ml [35S]-Tran35S-label (ICN, Irvine, CA) for 16 h in Dulbecco's minimum essential medium (DMEM) lacking cystine and methionine, but containing 25 mM Hepes (N-2-hydroxyethylpiperazine-N'2-ethane...
sulfonic acid)-NaOH pH 7.4, 10% dialyzed FCS, 5 x 10^{-6} M 2-ME, 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Splenic T cells were labeled likewise for 8.5 h. In addition, cultures for ALST-1 transfectants contained 0.4 mg/ml hygromycin B, 50 µg/ml cysteine, and 15 µg/ml methionine. After labeling, cells were washed in Hank’s balanced salt solution (HBSS) containing 25 mM Hepes-NaOH pH 7.4 and lysed (at 50 x 10^6 cells/ml for thymocytes and splenic T cells or at 7.5 x 10^6 cells/ml for ALST-1 transfectants) by 0.8% BRIJ 58 (polyoxyethylene 20 cetyl ether) or BRIJ 97 in HBSS containing 25 mM Hepes-NaOH pH 8.0, 2 mM EGTA, 3 mM MgCl_2, 2.5 mM thioglycolic acid, 1 mM PMSF, 5 µg/ml aprotinin, and 1 µM bestatin. DSP (dithiobis succinimidyl propionate) (Pierce) dissolved at 10 mg/ml in DMSO (dimethylsulfoxide) was added to the postnuclear supernatant of lysates at 1:100 (v/v). Mock cross-linking was carried out in the same fashion but without DSP. After 1 h at 4°C, 1 M Tris-HCl pH 8.0 was added to a final concentration of 25 mM followed by further incubation at 4°C for 30 min to quench the cross-linkers. CD45 was immunoprecipitated as described above and subjected to SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis (PAGE) under non-reducing conditions. Densitometric analysis of autoradiograms was carried out using Scan Analysis program (Biosoft, Ferguson, MO). For 2-dimensional diagonal SDS-PAGE, samples under non-reducing conditions were first subjected to 3.5 – 8% acrylamide gradient gel, and the sample lane was cut out. The gel strip was then equilibrated in SDS sample buffer containing 5% 2-mercaptoethanol for 30 min at room temperature and place onto the second dimension of SDS-PAGE in 8 – 15% acrylamide gradient.
Preparation of enzyme samples for PTP assays

To prepare microsomal fractions, thymocytes from mice (1 x 10^8 cells/ml) or ALST-1 transfectants (4 x 10^7 cells/ml) were suspended in cold hypotonic buffer consisting of 25 mM Hepes-NaOH pH7.4, 5 mM KCl, 1 mM MgCl₂, 2.5 mM thioglycolic acid, 1 mM PMSF, 5 µg/ml aprotinin, and 1 µM bestatin and were disrupted with a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 4,000 x g for 15 min at 4°C and the 4,000 x g supernatant was then centrifuged at 30,000 x g for 40 min at 4°C. The 30,000 x g pellet (microsomal fraction) was washed once in the hypotonic buffer and dissolved in 0.8% BRIJ 58, 25 mM Hepes-NaOH, pH 7.4, 5 mM EDTA (ethylenediaminetetraacetic acid), 150 mM NaCl, 2.5 mM thioglycolic acid, 0.2 mM PMSF, 5 µg/ml aprotinin, and 1 µM bestatin. CD45 was removed from the microsomal preparations by immunoprecipitating with anti-CD45 antibody-conjugated Protein G-Sepharose 4 beads as described above. Microsomal preparations before and after immunoprecipitation were used for PTP assays. The amounts of CD45 present in microsomal samples before and after immunoprecipitation were determined by immunoblotting with anti-CD45 antibody and densitometric analysis of the immunoblots as described above.

PTP assays

Substrates for PTP assays were prepared by labeling Raytide (a modified gastrin analog of 2116 daltons, from Calbiochem, San Diego, CA), myelin basic protein (MBP,
from bovine brain, Sigma, St. Louis, MO), and recombinant glutathione S-transferase-tagged Lck (GST-Lck)\textsuperscript{24} with [\gamma^{32}\text{P}]ATP (adenosine triphosphate) as described before\textsuperscript{9}. Briefly, Raytide at 0.33 mg/ml in 50 mM Hepes-NaOH, pH 7.4, 0.1 mM EDTA, 0.015\% BRIJ 35 (polyoxyethylene 23 lauryl ether), 33 \mu g/ml bovine serum albumin (BSA), 0.067\% 2-ME, 10 mM MgCl\textsubscript{2}, 16.7 \mu M ATP, and 1 mCi/ml [\gamma^{32}\text{P}]ATP (4500 Ci/mmol from ICN) was incubated with p60\textsuperscript{c-src} (Calbiochem) at room temperature overnight. The peptides were then precipitated in trichloroacetic acid (TCA), washed several times in TCA and acetone, and dissolved in PTP assay buffer consisting of 0.4\% BRIJ 58, 25 mM Hepes-NaOH, pH 7.4, 5 mM EDTA, 150 mM NaCl, 2.5 mM thioglycolic acid, and 0.2 mM PMSF. PTP assays were carried out by incubating the enzyme samples described above with one of the \textsuperscript{32}P-labeled substrates in PTP assay buffer at 30\degree C for 20 min as described before\textsuperscript{9}. The reaction was terminated by adding a chilled charcoal suspension consisting of 4\% (v/v) Norit A (acid-washed activated charcoal, from ICN), 0.9 N HCl, 90 mM sodium pyrophosphate, and 2 mM NaH\textsubscript{2}PO\textsubscript{4}. After a 5 min incubation at 4\degree C and a brief centrifugation, the amount of released phosphate present in the supernatant was determined by scintillation counting. The amount of phosphate released spontaneously during reactions without enzyme was subtracted as background. The amount of enzyme preparation used for the assay was adjusted so that no more than 15\% of the substrate was dephosphorylated.
Results

CD45 dimerization is increased in CD45 AP-null T cells

Recent data suggest that the transmembrane segment of CD45 is essential for homodimerization \(^{15,20}\). On the other hand, CD45 specifically interacts with CD45-AP through its transmembrane segment \(^{18,19}\). Therefore, it seems likely that the interaction with CD45-AP might modulate CD45 dimer formation. To examine this possibility, the relative proportions of monomeric and dimeric CD45 in thymocytes from CD45-AP-null and wild-type mice were compared (Fig. 1A).

Cells were biosynthetically labeled with \(^{35}\)S-labeled amino acids, lysed in BRIJ 97 which preserves the interaction between CD45 and CD45-AP, and subjected to chemical crosslinking by DSP which introduces covalent S-S bonds between closely bound peptides. CD45 immunoprecipitates obtained from these lysates were analyzed by SDS-PAGE under non-reducing conditions. A substantially larger population of CD45 dimers (estimated molecular mass of 360 kDa) existed in the CD45-AP-null cell than in the wild-type cells. CD45 dimer/monomer ratios obtained by densitometric analysis were 1.87 for the wild-type cells and 0.781 for the CD45-AP-null cells. Analysis of the DSP-crosslinked sample of CD45-AP-null thymocytes by two-dimensional diagonal SDS-PAGE, where the cross-linker was cleaved after the first dimension of electrophoresis, revealed only a protein corresponding to CD45 (180 kDa), indicating that the 360 kDa band represents CD45 dimers and not a complex of CD45 with other proteins (Fig. 1B). The band just above the major CD45 monomer band in the DSP-
crosslinked wild-type sample represents a complex of CD45 monomer and CD45-AP as determined by our earlier studies using two-dimensional diagonal SDS-PAGE \(^9\). These results suggest that CD45-AP inhibits CD45 dimer formation.

A similar analysis was carried out using splenic T cells from CD45-AP-null and wild-type mice (Fig. 1C). The CD45-AP-null cells appeared to form CD45 dimers more readily than the wild-type cells, but the difference was less striking than that seen with thymocytes. CD45 dimer/monomer ratios obtained by densitometric analysis were 0.616 for the wild-type cells and 0.807 for the CD45-AP-null cells. This may be due to the differences in CD45 isoforms expressed in mature splenic T cells versus those expressed in thymocytes \(^{25}\).
Establishment of a CD45-AP-null T cell line

Figure 1. CD45 dimerization is increased in the absence of CD45-AP. Thymocytes (A) and (B) or splenic T cells (C) obtained from wild-type (+) or CD45-AP-null (-) mice were biosynthetically labeled with 35S-labeled amino acids. The cell lysates were treated with or without DSP and immunoprecipitated with anti-CD45 antibody. In (A) and (C), the immunoprecipitates were analyzed by non-reducing SDS-PAGE. In (B), the immunoprecipitate of CD45-AP-null thymocytes was analyzed by 2-dimensional diagonal SDS-PAGE.
To test the effect of CD45-AP on CD45 dimerization more directly, a CD45-AP-null T cell line named ALST-1 was established from a large spontaneous thymic tumor found in a CD45-AP-null mouse. This cell line grows with an average doubling time of 15 h in IMDM with 10% FCS without any special supplement. Analysis of ALST-1 surface markers by flow cytometry demonstrated that ALST-1 is positive for the T cell markers TCRβ, CD3ε, CD8, CD90; and negative for CD4, B lymphocyte markers (IgM, IgD, CD19), monocyte/myeloid markers (Ly6G, CD11b), and the NK cell lineage marker (panNK). Therefore, the cell line is clearly of T cell lineage and at a mature single-positive stage. As is typical for most thymocytes, ALST1 cells are positive for CD45RB and negative for CD45RA and CD45RC. As expected, no CD45-AP was detected in either the cell lysate or the anti-CD45 immunoprecipitate of ALST-1 by immunoblotting with anti-CD45-AP antibody (Fig. 2). In contrast, the same procedure detected strong CD45-AP signals in samples obtained from the CD45-AP-positive YAC-1 cell line. To our knowledge, ALST1 is the only CD45-AP-negative cell line currently available.

Figure 2. CD45-AP is not expressed in ALST-1 cells. Cell lysates or anti-CD45 immunoprecipitates of ALST-1 and YAC-1 cells were immunoblotted with anti-CD45-AP antibody. The CD45-AP band is indicated by arrows. The prominent band indicated by * that is seen in immunoprecipitates of both cell lines represents Ig light chains.

**Expression of CD45-AP in stably transfected ALST-1**
To determine the effect of CD45-AP on CD45 homodimerization, ALST-1 was transfected with HA-tagged CD45-AP by electroporation and a stable transfectant was obtained by limiting dilution culture in the presence of hygromycin B. Expression of the transfected protein on the cell surface was measured by flow cytometry of intact cells with FITC-conjugated anti-HA antibody in comparison with a control sample transfected with empty pcDNA3.1 vector. As shown in Fig. 3, the majority of the HA-CD45-AP transfectant cells express the epitope tag on their cell surface. Since the N-terminus of native CD45-AP is on the extracellular side of the plasma membrane\(^2\), these results show that transfected HA-CD45-AP is targeted to the plasma membrane in the proper orientation.

![Figure 3. Transfected HA-CD45-AP is expressed on the surface of ALST-1 cells.](image)

ALST-1 cells stably transfected with either HA-CD45-AP (solid line) or empty vector (dotted line) were stained with FITC-conjugated anti-HA antibody and analyzed by flow cytometry.

Total expression of CD45-AP protein in transfected ALST1 was compared to that in an equal number of wild-type thymocytes by immunoblotting of cell lysates with anti-CD45-AP antibody (Fig. 4A, lanes 1 - 4). ALST-1 transfected with HA-CD45-AP
exhibited two populations of CD45-AP. Only the slower migrating dominant population was detected by immunoblotting of the same lysate with anti-HA antibody (Fig. 4A, lane 5). Therefore, the slower migrating protein represents intact HA-CD45-AP whereas the faster migrating, minor population is probably the result of an N-terminal cleavage resulting in loss of the HA tag. Immunoblotting with anti-actin antibody was also performed as a control for protein loading. These data demonstrate that the amount of CD45-AP expressed in transfected ALST-1 cells is comparable to the level expressed in normal thymocytes. Immunoblotting with anti-CD45 antibody showed that ALST-1 cells express CD45 at a level comparable to that seen in normal thymocytes (Fig. 4A, lanes 1 - 4). To determine whether the transfected HA-CD45-AP associates with endogenous CD45 in ALST-1, cell lysates were immunoprecipitated with anti-CD45 antibody and immunoblotted with anti-CD45 antibody and anti-HA antibody. As shown in Fig.4B, transfected HA-CD45-AP coimmunoprecipitated with CD45 as previously described for native CD45-AP. 
Expression of CD45-AP reduces CD45 dimerization in ALST-1 cells

To test whether expression of CD45-AP inhibits CD45 dimer formation in ALST-1, the CD45 dimer to monomer ratio was determined in transfectants expressing CD45-AP and cells transfected with empty vector. Lysates of biosynthetically labeled cells were subjected to either mock treatment or DSP crosslinking. CD45 was immunoprecipitated and analyzed by non-reducing SDS-PAGE (Fig. 5). Dimer/monomer ratios of CD45 were quantitated by densitometric analysis of autoradiograms from three independent experiments and summarized in Table 1. Expression of CD45-AP in ALST-1 cells clearly reduced the ratio of CD45 dimers to monomers.
To confirm that the reduction in CD45 dimer formation is due to the expression of CD45-AP rather than clonal variability, ALST-1 cells expressing three different levels of CD45-AP and two independent control transfectants with empty vector were analyzed in the same manner as described above. SDS-PAGE patterns of CD45 obtained from these cells are shown in Fig. 6A. Expression of HA-CD45-AP in the transfectants was determined by anti-HA immunoblotting of cell lysates (Fig. 6B) and also by flow cytometry of intact cells with FITC-conjugated anti-HA antibody (Fig. 6C). Dimer/monomer ratios of CD45 were quantitated by densitometric analysis of autoradiogram regions corresponding to dimers and monomers (Fig. 6A) and summarized in Table 2 along with the expression levels of HA-CD45-AP and CD45 determined by flow cytometry. The two empty vector transfectants exhibited the highest
levels of CD45 dimer formation. Increasing levels of CD45-AP expression resulted in proportional decreases in CD45 dimerization. There was no correlation between the amount of CD45 expressed and the CD45 dimer/monomer ratio. These results, in conjunction with the results obtained from comparing CD45-AP-null mice to their wild type counterparts (Fig. 1), clearly demonstrate that CD45-AP inhibits CD45 dimer formation.
Figure 6. Increasing expression of CD45-AP proportionally reduces CD45 dimerization in ALST-1 cells.

(A) ALST-1 cells expressing three different levels of HA-CD45-AP and two independent control transfectants with empty vector were biosynthetically labeled with $^{35}$S-labeled amino acids. The cell lysates were treated with or without DSP and immunoprecipitated with anti-CD45 antibody. The immunoprecipitates were analyzed by SDS-PAGE under non-reducing conditions. Scanned regions for monomers and dimers are indicated by brackets. (B) Cell lysates of ALST-1 transfectants (1 x $10^6$ cells) were immunoblotted with anti-HA antibody. (C) ALST-1 transfectants were stained with FITC-conjugated anti-HA antibody and analyzed by flow cytometry.
CD45 PTP activity is lower in CD45-AP-negative cells

Recent evidence suggests that CD45-mediated signaling is downregulated by homodimerization of CD45 \(^{11,13-15}\). Our results described above show that CD45-AP-negative cells have a much higher level of CD45 dimers than wild-type cells. Therefore, we sought to determine whether the absence of CD45-AP results in reduced CD45 PTP activity.

In the past, CD45 PTP activity was usually determined using CD45 prepared by immunoprecipitation with anti-CD45 antibodies \(^{9,21}\). However, immunoprecipitation may force CD45 dimer/multimer formation and is thus not appropriate for the present study. Moreover, any attempt to dissociate CD45 from anti-CD45 antibody after immunoprecipitation will risk conformational changes and disruption of physiologically relevant associations such as the one between CD45 and CD45-AP. Therefore, in this study, CD45 PTP activity was estimated by directly measuring the PTP activity of microsomal fractions before and after CD45 removal by immunoprecipitation. The PTP activity removed from microsomal fractions by CD45 immunoprecipitation represents CD45 PTP activity and was determined by subtracting the PTP activity of the post-immunoprecipitation samples from that of the corresponding pre-immunoprecipitation samples. This approach is feasible because most of the PTP activity associated with the lymphocyte membrane is due to CD45 \(^{26}\) and because CD45 is efficiently depleted from microsomal fractions by immunoprecipitation as shown in Fig. 7.
The PTP activity of thymocyte microsomes from CD45-AP-null and wild-type mice was determined by measuring the amount of phosphate released from $^{32}$P-labeled Raytide. The amount of CD45 present in microsomal fractions before and after immunoprecipitation was determined by scanning densitometry of anti-CD45 antibody immunoblots (Fig. 7); the difference between the two represents the amount of CD45 removed by immunoprecipitation. Finally, the specific activity of CD45 PTP was obtained by dividing the CD45 PTP activity by the amount of CD45. The results of 3 independent experiments are summarized in Table 3 and show a significant reduction of CD45 PTP specific activity in thymocytes from CD45-AP-null mice as compared to their wild-type counterparts. The mean of the relative specific activities of CD45-AP-null thymocytes, expressed as a percentage of the specific activity of wild-type counterparts was 76.2%. Similar results were obtained using MBP and Lck as substrates with average relative specific activities of 64.9% and 81.3%, respectively (data not shown).

Figure 7. The bulk of CD45 is removed from microsomal fractions by immunoprecipitation with anti-CD45 antibody. Microsomal fractions of thymocytes from wild-type or CD45-AP-null mice and of ALST-1 cells transfected with either CD45-AP or empty vector were subjected to immunoprecipitation with anti-CD45 antibody -conjugated beads. Fractions before and after immunoprecipitation were analyzed by immunoblotting with anti-CD45 antibody.
These data show that the absence of CD45-AP results in reduced CD45 PTP specific activity.

To confirm that CD45-AP increases CD45 PTP specific activity, the ALST-1 cell line, transfected either with empty vector or with vector expressing CD45-AP, was used. As shown in Table 4, ALST-1 cells transfected with empty vector exhibited lower CD45 PTP specific activity than those expressing CD45-AP. The mean of the relative specific activities of CD45-AP-null cells was 58.2%. Similar results were obtained using MBP and Lck as substrates with average relative specific activities of 63.8% and 77.0%, respectively (data not shown). These data confirm that the PTP activity of CD45 is higher in the presence of CD45-AP.
Discussion

We have previously detected the presence of a dimeric form of CD45 in lymphocytes using chemical crosslinkers and separation through sucrose gradients. Spontaneous dimerization of recombinant CD45 fragments was subsequently observed in vitro. Recent experiments using fluorescence energy resonance transfer demonstrated the presence of CD45 dimers and monomers on the cell surface indicating that these two forms exist in equilibrium. Different CD45 isoforms are expressed in a cell-differentiation and activation-dependent manner by alternative splicing of exons A, B, and C. There is evidence that the homodimerization efficiency of CD45 varies among the different isoforms, suggesting that modulation of CD45 dimerization may be achieved by isoform switching. In the present study, we showed that CD45-AP-negative T cells have much higher levels of CD45 dimers than cells expressing CD45-AP, suggesting that CD45-AP inhibits CD45 homodimerization. Further, the effect of CD45-AP appears to differ between thymocytes and splenic T lymphocytes (Fig. 1), suggesting differential regulation of CD45 dimerization depending on the level of T cell maturation. Our findings provide another potential mechanism for regulation of CD45 dimerization and activity as discussed below.

Several lines of evidence have emerged recently suggesting that dimerization of CD45 downregulates its function. A recombinant chimeric CD45 protein (EGFR-CD45) that contains the ligand-binding domain of EGFR is capable of restoring TCR signaling to a CD45-deficient cell line, but loses this capacity upon EGF binding and receptor dimerization. These results suggest that CD45 PTP activity may be regulated by
ligand-induced dimerization. This concept gained support from the elucidation of the crystal structure of RPTPα, a receptor-like PTP related to CD45 \(^ {12}\). The study identified a wedge containing conserved acidic residues that can specifically interact with the catalytic site in symmetrical dimers resulting in the inhibition of RPTPα function. Consistent with this notion, chimeric EGFR-CD45 with a point mutation that inactivates the inhibitory wedge of CD45 PTP was resistant to downregulation by EGF-induced dimerization \(^ {13}\). Furthermore, mice with a genetically engineered point mutation that inactivates the inhibitory wedge of CD45 were found to develop lymphoproliferation and severe autoimmunity, suggestive of hyperactive CD45 \(^ {14}\). In these studies downstream biological events were taken as an indirect measure of CD45 PTP activity. In the present study, on the other hand, a correlation was made between increased CD45 dimerization and reduced specific activity of CD45 PTP, providing evidence that CD45 homodimerization indeed reduces its PTP activity.

In our previous studies \(^ {9,21}\), CD45 for PTP assays was isolated from cell lysates by immunoprecipitation with anti-CD45 antibody and elution from the immunoprecipitates by a brief exposure to high pH. Such procedures for isolating CD45 may introduce artificial dimer/multimer formation as well as changes in interactions between CD45 and its associated proteins and thus are not suited to study the effect of CD45 dimerization on its PTP activity. In fact, we found no difference in specific activities of CD45 PTP when CD45-AP-null and wild-type lymphocytes were compared in our earlier study \(^ {21}\). Likewise, another group using CD45 immunoprecipitates prepared from cells lysed in Triton X-100, a disruptive detergent known to abolish the interaction between CD45 and CD45-AP \(^ {16}\), detected no effect of CD45-AP on CD45 PTP activity when CD45-AP-
decifient Jurkat variants were compared to the wild-type Jurkat cells. In the present study, in order to preserve the physiological interactions among proteins as much as possible, PTP activity present in microsomal fractions dissolved in a non-disruptive detergent, BRIJ 58, was determined before and after removing CD45 by immunoprecipitation. The specific activity of CD45 PTP obtained by this procedure was significantly reduced in CD45-AP-null thymocytes compared to their wild-type counterparts (Table 3). Similarly, the specific CD45 PTP activity was significantly lower in the CD45-AP-null cell line ALST-1 than in its CD45-AP-expressing counterpart (Table 4).

Mutational studies of RPTPα demonstrated that the extracellular domain and the transmembrane domain are independently capable of homodimerization while each of the two catalytic domains affects dimerization but is not essential by itself. Thus, a “zipper” model was proposed in which RPTPα dimers are stabilized by interactions through multiple interfaces. This model suggests that PTP homodimerization can be regulated via multiple domains by different mechanisms. CD45-AP is known to interact with the transmembrane domain of CD45, and our data show that CD45-AP inhibits CD45 dimer formation (Figs. 1, 5, and 6). Thus, the mechanism by which CD45-AP inhibits CD45 dimerization may involve interference with the interaction between the transmembrane domains of two CD45 molecules. Alternatively, since the CD45 dimer complex of wild-type cells appears to contain CD45-AP, and since CD45-AP may directly interact with some of CD45 substrates, both CD45-AP and a CD45 substrate may be required for disrupting CD45 dimers.
As discussed above, different CD45 isoforms homodimerize with different efficiencies. In response to activation or differentiation signals, some lymphocytes switch CD45 isoforms by a process that typically takes several days \(^{1,31}\). The CD45 isoform switch, therefore, has been proposed as one of the mechanisms by which CD45 PTP can be regulated \(^{15}\). Since variation among CD45 isoforms is restricted to the extracellular domains, regulation by isoform switching may be mediated by interactions at the extracellular interface. On the other hand, the mechanism proposed in the present study involves the interaction of the transmembrane domain of CD45 with CD45-AP. Since the interactions between CD45 and CD45-AP do not vary among different CD45 isoforms \(^{9,18}\), the two mechanisms are likely to operate independently of each other. It is possible, for example, that long-term regulation occurs through the isoform switch mechanism whereas CD45-AP interaction is important in short-term regulation. However, the mechanisms that regulate the interaction between CD45-AP and CD45 remain to be elucidated.

CD45 is believed to play a key positive role at the onset of TCR signaling. Since signals would be amplified as they are transduced through signaling cascades, even a small difference at the onset may have a profound effect at the end of the cascade. Thus the 20 – 40% reduction of CD45 PTP activity that we observed in CD45-AP-negative cells (Tables 3 and 4) could exert a significant effect on signaling. Indeed, our data from CD45-AP-null mice demonstrated that lymphocyte signaling is diminished in the absence of CD45-AP \(^{21}\). The substrates of CD45 during T lymphocyte signaling are thought to include Lck \(^{32,33}\), JAK family PTKs \(^{34}\), the TCR \(\zeta\)-chain \(^{35}\), and ZAP-70 \(^{36}\).
Modulation of CD45 PTP activity by CD45-AP may have an effect on the phosphorylation and function of these substrates.

In addition, CD45 function may be modulated by regulating its access to substrates. We and others have previously demonstrated that CD45-AP directly binds Lck through its intracellular segment and coordinates the interaction between CD45 and Lck. These results, in conjunction with the current study, suggest that CD45-AP plays a pivotal role in CD45 function by regulating its access to substrates as well as modulating its PTP activity through dimerization.
Acknowledgements

We thank A. Maizel and J. Stoeckler for helpful discussions, P. Johnson and J. Marth for generously providing reagents, and M. Dooner and the Flow Cytometry Laboratory of Roger Williams Hospital for technical help.
References


tyrosine protein kinase in murine lymphoma T-cell lines. Proc Natl Acad Sci U S A. 1989;86:8959-8963


Table 1. CD45-AP expression reduces the CD45 dimer/monomer ratio

<table>
<thead>
<tr>
<th>CD45-AP expression</th>
<th>CD45 dimer/monomer ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>0.381 ± 0.076</td>
</tr>
<tr>
<td>–</td>
<td>1.15 ± 0.075</td>
</tr>
</tbody>
</table>

The dimer/monomer ratios of CD45 were obtained by densitometric analysis of autoradiograms from three independent experiments. Scanned regions for monomer and dimer quantification are indicated in Fig. 5. Data are expressed as an arithmetic mean ± SD.
Table 2. Increasing expression of CD45-AP proportionally reduces CD45 dimerization

<table>
<thead>
<tr>
<th>Fig. 6A sample lane</th>
<th>HA-CD45AP expression</th>
<th>CD45 expression</th>
<th>Dimer/monomer ratio of CD45</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37.2</td>
<td>404</td>
<td>0.530 ± 0.059</td>
</tr>
<tr>
<td>2</td>
<td>32.5</td>
<td>392</td>
<td>0.583 ± 0.019</td>
</tr>
<tr>
<td>3</td>
<td>20.8</td>
<td>480</td>
<td>0.679 ± 0.030</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
<td>382</td>
<td>0.993 ± 0.012</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
<td>484</td>
<td>1.17 ± 0.083</td>
</tr>
</tbody>
</table>

The surface expression levels of HA-CD45-AP and CD45 were determined by staining the same ALST-1 transfectants as those described in lanes 1-5 of Fig. 6 with FITC-conjugated anti-HA or anti-CD45 antibody. The numbers shown are mean fluorescence intensities above background. The dimer/monomer ratios of CD45 were obtained by densitometric analysis of three different autoradiograms of Fig. 6A. Scanned regions for monomer and dimer quantification are indicated in Fig. 6A. Data are expressed as an arithmetic mean ± SD.
Table 3. Specific activity of CD45 PTP is reduced in CD45-AP-null thymocytes

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>Cell type</th>
<th>Before IP</th>
<th>After IP</th>
<th>Amount removed by IP</th>
<th>Specific activity</th>
<th>Relative specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild-type</td>
<td>PTP activity 12,783</td>
<td>2,494</td>
<td>10,289</td>
<td>23.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45 amount 520.4</td>
<td>81.0</td>
<td>439.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CD45-AP-null</td>
<td>PTP activity 5,649</td>
<td>991</td>
<td>4,658</td>
<td>17.2</td>
<td>73.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45 amount 338.6</td>
<td>68.3</td>
<td>270.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Wild-type</td>
<td>PTP activity 3,465</td>
<td>959</td>
<td>2,506</td>
<td>17.87</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45 amount 140.2</td>
<td>0</td>
<td>140.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CD45-AP-null</td>
<td>PTP activity 1,766</td>
<td>456</td>
<td>1,310</td>
<td>14.92</td>
<td>83.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45 amount 87.8</td>
<td>0</td>
<td>87.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Wild-type</td>
<td>PTP activity 15,758</td>
<td>4,708</td>
<td>11,050</td>
<td>74.8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45 amount 190.8</td>
<td>43.1</td>
<td>147.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>CD45-AP-null</td>
<td>PTP activity 15,300</td>
<td>3,693</td>
<td>11,607</td>
<td>53.5</td>
<td>71.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45 amount 249.0</td>
<td>32.1</td>
<td>216.9</td>
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<td></td>
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</tbody>
</table>

The PTP activity present in microsomal fractions of wild-type and CD45-AP-null thymocytes was determined using Raytide as a substrate, before and after removal of CD45 by immunoprecipitation. The PTP activity value shown for each experiment is an average of duplicate or triplicate assays. PTP activity removed by immunoprecipitation
was obtained by subtracting the activity present after immunoprecipitation from the activity present before immunoprecipitation; this represents PTP activity attributable to CD45. Similarly, the amount of CD45 before and after immunoprecipitation was obtained by scanning densitometry of immunoblots; the difference between the two amounts represents the amount of CD45 removed by immunoprecipitation. The specific activity of CD45 PTP was then calculated by dividing the CD45 PTP activity removed by immunoprecipitation by the amount of CD45 removed by immunoprecipitation. The relative specific activity of CD45-AP-null thymocytes is expressed as a percentage of the specific activity of wild-type counterparts. The arithmetic mean of relative specific activities ± SD of the three experiments is 76.2 ± 6.4. The difference between wild-type and CD45-AP-null thymocytes was statistically significant as determined by Student’s t-test (p < 0.005).
Table 4. Specific activity of CD45 PTP is reduced in CD45-AP-negative ALST-1 cells

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>Cell type</th>
<th>Before IP</th>
<th>After IP</th>
<th>Amount removed by IP</th>
<th>Specific activity</th>
<th>Relative specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD45-AP-positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CD45-AP-positive</td>
<td>PTP activity</td>
<td>18,484</td>
<td>12,783</td>
<td>5,701</td>
<td>70.4</td>
</tr>
<tr>
<td></td>
<td>CD45-AP-positive</td>
<td>CD45 amount</td>
<td>88.8</td>
<td>7.8</td>
<td>81.0</td>
<td>49.4</td>
</tr>
<tr>
<td>2</td>
<td>CD45-AP-positive</td>
<td>PTP activity</td>
<td>7,685</td>
<td>4,318</td>
<td>3,367</td>
<td>39.6</td>
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<td></td>
<td>CD45-AP-positive</td>
<td>CD45 amount</td>
<td>134.1</td>
<td>49.1</td>
<td>85.0</td>
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<td>3</td>
<td>CD45-AP-positive</td>
<td>PTP activity</td>
<td>30,079</td>
<td>17,417</td>
<td>12,662</td>
<td>186</td>
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<td></td>
<td>CD45-AP-positive</td>
<td>CD45 amount</td>
<td>68.2</td>
<td>0</td>
<td>68.2</td>
<td>134</td>
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<td></td>
<td>CD45-AP-negative</td>
<td>PTP activity</td>
<td>45,750</td>
<td>29,583</td>
<td>16,167</td>
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<tr>
<td></td>
<td>CD45-AP-negative</td>
<td>CD45 amount</td>
<td>120.4</td>
<td>0</td>
<td>120.4</td>
<td></td>
</tr>
</tbody>
</table>

The PTP activity present in microsomal fractions of CD45-AP-positive and negative ALST-1 cells was determined using Raytide as a substrate, before and after removal of CD45 by immunoprecipitation. The PTP activity value shown for each experiment is an average of duplicate or triplicate assays. PTP activity removed by immunoprecipitation
was obtained by subtracting the activity present after immunoprecipitation from the activity present before immunoprecipitation; this represents PTP activity attributable to CD45. Similarly, the amount of CD45 before and after immunoprecipitation was obtained by scanning densitometry of immunoblots; the difference between the two amounts represents the amount of CD45 removed by immunoprecipitation. The specific activity of CD45 PTP was then calculated by dividing the CD45 PTP activity removed by immunoprecipitation by the amount of CD45 removed by immunoprecipitation. The relative specific activity of CD45-AP-null cells is expressed as a percentage of the specific activity of CD45-AP-positive ALST-1 cells. The arithmetic mean of the relative specific activities ± SD of the three experiments is 58.2 ± 22.4. The difference between CD45-AP-null ALST-1 cells and CD45-AP-positive transfectants was statistically significant as determined by Student’s t-test (p < 0.05).
Figure Legends

**Figure 1. CD45 dimerization is increased in the absence of CD45-AP.** Thymocytes (A) and (B) or splenic T cells (C) obtained from wild-type (+) or CD45-AP-null (-) mice were biosynthetically labeled with $^{35}$S-labeled amino acids. The cell lysates were treated with or without DSP and immunoprecipitated with anti-CD45 antibody. In (A) and (C), the immunoprecipitates were analyzed by non-reducing SDS-PAGE. In (B), the immunoprecipitate of CD45-AP-null thymocytes was analyzed by 2-dimensional diagonal SDS-PAGE.

**Figure 2. CD45-AP is not expressed in ALST-1 cells.** Cell lysates or anti-CD45 immunoprecipitates of ALST-1 and YAC-1 cells were immunblotted with anti-CD45-AP antibody. The CD45-AP band is indicated by arrows. The prominent band indicated by * that is seen in immunoprecipitates of both cell lines represents Ig light chains.

**Figure 3. Transfected HA-CD45-AP is expressed on the surface of ALST-1 cells.** ALST-1 cells stably transfected with either HA-CD45-AP (solid line) or empty vector (dotted line) were stained with FITC-conjugated anti-HA antibody and analyzed by flow cytometry.

**Figure 4. HA-CD45-AP is expressed at a normal level and binds CD45 in stably transfected ALST-1 cells.** (A) Cell lysates were immunoblotted with anti-CD45, anti-CD45-AP, and anti-actin antibodies (lanes 1-4), or with anti-HA antibody (lanes 5-6).
Wild-type thymocyte lysate (3.75 x 10^5 cells). 2; CD45-AP-null thymocyte lysate (3.75 x 10^5 cells). 3 and 5; Lysates of ALST-1 transfected with HA-CD45-AP (3.75 x 10^5 cells). 4 and 6; Lysates of ALST-1 transfected with empty vector (3.75 x 10^5 cells). (B) Anti-CD45 immunoprecipitates of ALST-1 cells transfected with HA-CD45-AP (lane 1) or with empty vector (lane 2) were immunoblotted with anti-CD45 and anti-HA antibodies.

**Figure 5. Expression of CD45-AP reduces CD45 dimerization in ALST-1 cells.**
ALST-1 cells stably transfected with HA-CD45-AP or empty vector were biosynthetically labeled with 35S-labeled amino acids. The cell lysates were treated with or without DSP and immunoprecipitated with anti-CD45 antibody. The immunoprecipitates were analyzed by SDS-PAGE under non-reducing conditions. Scanned regions for monomers and dimers are indicated by brackets.

**Figure 6. Increasing expression of CD45-AP proportionally reduces CD45 dimerization in ALST-1 cells.** (A) ALST-1 cells expressing three different levels of HA-CD45-AP and two independent control transfectants with empty vector were biosynthetically labeled with 35S-labeled amino acids. The cell lysates were treated with or without DSP and immunoprecipitated with anti-CD45 antibody. The immunoprecipitates were analyzed by SDS-PAGE under non-reducing conditions. Scanned regions for monomers and dimers are indicated by brackets. (B) Cell lysates of ALST-1 transfectants (1 x 10^6 cells) were immunoblotted with anti-HA antibody. (C) ALST-1 transfectants were stained with FITC-conjugated anti-HA antibody and analyzed by flow cytometry.
Figure 7. The bulk of CD45 is removed from microsomal fractions by immunoprecipitation with anti-CD45 antibody. Microsomal fractions of thymocytes from wild-type or CD45-AP-null mice and of ALST-1 cells transfected with either CD45-AP or empty vector were subjected to immunoprecipitation with anti-CD45 antibody - conjugated beads. Fractions before and after immunoprecipitation were analyzed by immunoblotting with anti-CD45 antibody.
CD45-associated protein inhibits CD45 dimerization and upregulates its protein tyrosine phosphatase activity

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