Interaction between CD45-AP and Protein-tyrosine Kinases Involved in T Cell Receptor Signaling*

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CD45-AP associates specifically with CD45, a protein-tyrosine phosphatase essential for antigen receptor-mediated signal transduction. CD45 modulates the activity of Src family protein-tyrosine kinases involved at the onset of antigen receptor-mediated signaling by dephosphorylating their regulatory tyrosyl residues. We have shown that lymphocyte responses to antigen receptor stimulation are impaired in CD45-AP-null mice. To examine the possibility that CD45-AP coordinates the interaction between CD45 and its substrates, we investigated the associations of CD45-AP with several protein-tyrosine kinases. Endogenous CD45-AP coimmunoprecipitated with Lck and ZAP-70 in both CD45-positive T cells and their CD45-negative variants after stimulation by antigen receptor ligation. Concomitantly, CD45 coimmunoprecipitated with Lck and ZAP-70 after T cell receptor-mediated stimulation of CD45-positive cells. Recombinant CD45-AP exhibited specific binding to Lck and ZAP-70 protein-tyrosine kinases, but not to Fyn or Csk, in lysates of both CD45-positive and –negative T cells. Specific interactions were demonstrated between the respective recombinant proteins as well. These results demonstrate that CD45-AP associates directly and selectively with Lck and ZAP-70 in response to T cell receptor-mediated stimulation. The associations of CD45-AP with Lck and ZAP-70 may mediate the functional interactions of these kinases with CD45 during antigen receptor stimulation.

Protein-tyrosine phosphatase activity residing in the cytoplasmic portion of CD45 (1) is essential for antigen receptor-mediated signal transduction in lymphocytes (2). The T cell receptor (TCR)† and its coreceptors recruit Src family protein-tyrosine kinases (such as Lck and Fyn) and Syk family protein-tyrosine kinases (such as ZAP-70) by forming specific associations via their cytoplasmic segments (3–7). Tyrosine phosphorylation of cellular proteins by these protein-tyrosine kinases is one of the earliest events of TCR signaling (8–10). There is strong evidence supporting the notion that CD45 activates Src family protein-tyrosine kinases by dephosphorylating their down-regulatory tyrosine residues (2, 11–14). On the other hand, considerable data indicate that the effect of CD45 on antigen receptor-mediated signaling can be inhibitory rather than stimulatory under certain circumstances. For example, CD45 has been shown to specifically interact with and dephosphorylate the tyrosine-phosphorylated ζ chain of the TCR (15). Since the tyrosine-phosphorylated ζ chain recruits Syk family protein-tyrosine kinases for TCR-mediated signal amplification (3, 4), dephosphorylation of the ζ chain by CD45 will diminish the stimulatory signal. In addition, it has been suggested that CD45 directly or indirectly inactivates ZAP-70 by dephosphorylating its key tyrosyl residues (16). It has also been reported that CD45 can down-regulate Lck by dephosphorylating its up-regulatory tyrosyl residue in some cells (17). It remains unknown whether CD45 indeed exercises both stimulatory and inhibitory effects on lymphocyte signaling and, if so, how these opposing activities of CD45 are regulated.

Most of the CD45 in lymphocytes is associated with a predominantly lymphocyte-specific protein, CD45-associated protein (CD45-AP), with an estimated stoichiometry of 1 to 1 (18–20). Expression of CD45-AP is limited to certain subsets of leukocytes, whereas CD45 is expressed in all subsets of leukocytes. CD45-AP is expressed strongly in T, preB, and B cells, very weakly in mast cells, and not at all in plasma cells or cells of the monococyte/macrophage lineage (20). A human homologue of CD45-AP, named LPAP, is also expressed in comparable subsets of leukocytes (21, 22). Two forms of CD45-AP mRNA exist, and the resulting two CD45-AP proteins are identical in their capacity for specific binding to CD45 but employ different mechanisms for endoplasmic reticulum membrane translocation (20). The potential transmembrane segment of CD45-AP binds specifically to the transmembrane segment of CD45, and this physical association does not require the presence of other leukocyte-specific proteins, such as Lck, Fyn, and ZAP-70 (23). Amino acid sequence analysis and protease susceptibility analysis of CD45-AP indicate that only a short segment at the amino terminus of CD45-AP protrudes extracellularly, whereas the bulk of the protein is located intracellularly (23).

We have previously suggested that CD45-AP plays an adapter-like role for CD45 (19). Evidence in support of this hypothesis was provided recently by investigation of CD45-AP-null mice created by gene targeting (24). CD45-AP-null mice do not exhibit any detectable abnormality in lymphocyte differentiation in contrast to CD45-deficient (25) or -null (26) mice that show a profound block in T cell differentiation. However, similar to CD45-deficient or -null mice, responses of CD45-AP-null mice to TCR-mediated or B cell receptor-mediated stimuli are markedly reduced (24). Interestingly, the interaction between CD45 and Lck is significantly decreased in CD45-AP-null T
cells, indicating that CD45-AP directly or indirectly mediates the interaction of CD45 with Lck. Therefore, the present study was carried out to examine the possibility that CD45-AP specifically interacts with potential CD45 substrates. Specific interactions of CD45-AP with Lck and ZAP-70, but not with Fyn or Csk, were detected in both CD45-positive T cells and their CD45-negative variants in response to TCR ligation. Moreover, a specific association of CD45-AP with Lck and ZAP-70 was demonstrated by using the respective recombinant proteins. In CD45-positive cells, TCR ligation resulted in association of CD45 with Lck and ZAP-70. These results demonstrate that direct and specific interactions of CD45-AP with Lck and ZAP-70 are triggered by TCR ligation and support the notion that CD45-AP plays an adapter-like role by coordinating the interaction between CD45 and specific protein-tyrosine kinases.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The mouse T cell line YAC-1 (WT) and its CD45-negative variant (N1) (27) were provided by Dr. J. Ashwell (National Cancer Institute, Bethesda). The cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

**Preparation of Cell Lysates**—YAC-1 or N1 cells, 40 × 10^6 cells/ml in Iscove's modified Dulbecco's medium containing 2 mM sodium orthovanadate, were incubated first with anti-CD3 ε antibody (145-2C11) (28) on ice for 3 min. Following the addition of anti-hamster IgG (12.5 μg/ml), cells were further incubated either on ice for 60 min for negative controls or at 37 °C for 5 or 60 min for stimulation. After a wash in cold Dulbecco's phosphate-buffered saline, the cells were lysed at 20 × 10^6 cells/ml in 0.8% polyoxyethylene 10 oleyl ether (BRIJ 96) containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 0.1 mM EGTA, 2.5 mM thioglycolic acid, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 25 μM phenylarsine oxide, and 10 μM sodium pyrophosphate. Postnuclear supernatants of the lysates were used for binding assays and immunoprecipitations as described below.

**Binding Assay of Recombinant CD45-AP with Cell Lysates**—Recombinant full-length (F) and deleted (C) form of CD45-AP fused to the carboxyl terminus of glutathione S-transferase (GST) have been described previously (5). The C form begins with amino acid position 47 and continues to the carboxyl terminus of the CD45-AP cDNA. Approximatively equimolar amounts of full-length or C-form of recombinant CD45-AP proteins bound to glutathione-Sepharose 4B beads were incubated at 37 °C for 1 h with lysates of YAC-1 or N1 cells prepared as described above for binding, followed by extensive washing to remove unbound material. Total cell lysates and material bound to the beads were analyzed by SDS-PAGE followed by Western blotting.

**Immunoprecipitation**—Anti-CD45 antibody (28) cross-linked with dimethyl pimelimidate (30) to protein G-Sepharose 4B beads (Amersham Pharmacia Biotech) was used for anti-CD45 immunoprecipitation. Rabbit antiserum against CD45-AP (23) was used for immunoprecipitation in combination with protein G-conjugated Sepharose 4B beads. Postnuclear supernatants of cell lysates prepared as described above were precleared with protein G-Sepharose 4 and then used for immunoprecipitation. Material bound to the beads was analyzed by SDS-PAGE followed by Western blotting.

**Western Blotting**—Western blotting was carried out with antibodies against CD45-AP, CD45 (provided by Drs. J. Marsh, University of California, San Diego, La Jolla, CA, and I. Trowbridge, The Salk Institute, San Diego, CA), Fyn (Oncogene Research Products and Transduction Laboratories), Lck (Transduction Laboratories and Upstate Biotechnology Incorporated), ZAP-70 (Transduction Laboratories), or Csk (Transduction Laboratories) followed by horseradish peroxidase-conjugated protein A, anti-mouse Ig antibody, or anti-rat Ig antibody. The signals were detected with either the ECL Western blotting system (Amersham Pharmacia Biotech) or Supersignal substrate system (Pierce) quantitated by densitometric analysis (Scan Analysis version 2.56 by Biosoft).

**Binding Assay of Recombinant CD45-AP with Recombinant Protein Tyrosine Kinases**—The CD45-AP cDNA (19) was subcloned into the pQE30 expression vector (Qiagen) downstream from six histidine residues using the BamHI site. Mouse ZAP-70 cDNA (provided by Dr. A. Weiss, University of California, San Francisco) (4) was excised by XhoI and subcloned into the pQE32 expression vector (Qiagen) downstream from six histidine residues at the BamHI sites by using a BamHI linker. The respective histidine-tagged recombinant proteins were produced in the JM109 strain of *Escherichia coli* by isopropylthio-β-D-galactoside induction and purified by binding to nickel-nitrilotriacetate resin (Qia- gen). GST-tagged recombinant histidine-tagged CD45-AP construct (a mouse Lck cDNA subcloned into pGEM-2T expression vector) provided by Dr. P. Johnson, University of British Columbia, Vancouver, Canada (31), was purified by binding to glutathione-Sepharose 4B beads. GST-tagged recombinant CD45-AP has been described before (23). GST-tagged Lck eluted from glutathione-Sepharose 4B beads was incubated with various amounts of GST-tagged CD45-AP elute at 37 °C for 30 min prior to addition of histidine-tagged CD45-AP bound to nickel-nitrilotriacetate beads and further incubation at 37 °C for 1 h for binding. Likewise, histidine-tagged ZAP-70 eluted from nickel-nitrilotriacetate beads was incubated with various amounts of histidine-tagged CD45-AP elute prior to addition of GST-tagged CD45-AP bead to glutathione-Sepharose 4B beads and further incubation at 37 °C for 1 h for binding. Binding reactions were carried out in 0.4% Brij 96, 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM thioglycolic acid, 1 mM phenylmethylsulfonyl fluoride, and 10 μM ovalbumin. GST or histidine tags without a fusion partner were isolated in the same way as described above for fusion proteins and were used as negative controls for competition experiments. Material bound to the beads was analyzed by SDS-PAGE followed by Western blotting.

**RESULTS**

**Lck and ZAP-70 Interact with Recombinant CD45-AP Independently of CD45**—The earliest stages of TCR signaling are mediated by protein-tyrosine kinases such as Lck, Fyn, ZAP-70, and Syk (8–10). Lck and Fyn are most likely in vivo substrates of CD45, whereas ZAP-70 may also be regulated directly or indirectly by CD45 (2, 11–14, 16). To examine the possibility that CD45-AP directs the interaction between CD45 and its substrates, the interactions of recombinant CD45-AP with several protein-tyrosine kinases were investigated.

Wild-type CD45-positive YAC-1 cells (WT) and their CD45-negative variant cells (N1) (27) were stimulated by TCR cross-linking with anti-CD3 ε antibody (28), and cell lysates were prepared at various time points after stimulation. Whole cell lysates were analyzed by Western blotting with anti-CD45, Fyn, Lck, ZAP-70, or Csk antibodies. As shown in Fig. 1A, there is no detectable CD45 in N1 cells. The absence of CD45 in N1 cells was also confirmed by Northern hybridization (20), anti-CD45 immunoprecipitation combined with Western blotting, and flow cytometry (data not shown). The wild-type and variant cells expressed comparable amounts of Fyn, Lck, ZAP-70, and Csk, and the amounts remained unchanged by stimulation. Syk is not expressed in YAC-1 cells (not shown). Immunoblotting performed with anti-ZAP-70 antibodies tested two bands with different mobilities as has been reported previously by others (32). The structural difference between these two bands is not known. However, the fast-migrating band indicated by the arrow appears to represent the subpopulation that interacts with CD45-AP and CD45 as described below.

For binding assays, either full-length recombinant CD45-AP (F) or the bulk of its cytoplasmic portion (C) (Fig. 1B) bound to glutathione-Sepharose 4B beads were incubated with the same cell lysates. Material bound to the beads was analyzed by
FIG. 1. Specific interaction of Lck and ZAP-70 with recombinant CD45-AP. A, expression levels of CD45 and protein-tyrosine kinases. Wild-type CD45-positive YAC-1 cells (WT) and their CD45-negative variant cells (N1) were stimulated by TCR cross-linking with anti-CD3 ε antibody for 0, 5, or 60 min. Cell lysates were analyzed by Western blotting with anti-CD45, Fyn, Lck, ZAP-70, or Csk antibodies. B, the two forms of GST-tagged recombinant CD45-AP: F (full-length; amino acid positions 1–185 including the transmembrane segment (TM) of amino acid positions 21–41) and C (amino acid positions 47–185). C, binding of recombinant CD45-AP to CD45 and protein-tyrosine kinases. The cell lysates described in A were incubated with either the F or the C form of CD45-AP bound to glutathione-Sepharose 4B beads. Material bound to the beads was analyzed by Western blotting with the aforementioned antibodies. D, percentages of Lck and ZAP-70 capable of binding to CD45-AP. Cell lysates were prepared from unstimulated WT or N1 cells (15 × 10⁶) and were subjected repeatedly to fresh aliquots of recombinant CD45-AP-conjugated beads. Material bound to each aliquot of the beads (lanes 1–9) was analyzed by Western blotting with either anti-Lck or anti-ZAP-70 antibody. The total amount of Lck and ZAP-70 bound to recombinant CD45-AP was estimated by densitometric analysis of the immunoblot. Percentages were obtained by comparison to the total cellular amounts of Lck and ZAP-70 detected in lysates of 0.1 × 10⁶ and 0.05 × 10⁶ cells, respectively, determined by densitometric analysis (shown in the left panels).
Western blotting with the aforementioned antibodies. As shown in Fig. 1C, binding of CD45 to recombinant CD45-AP remained unchanged with stimulation. As expected from the fact that CD45 and CD45-AP interact through their respective transmembrane segments (23), no CD45 bound to the C form of CD45-AP. Lck and ZAP-70 specifically bound to recombinant CD45-AP, and specificity of the binding was demonstrated by the fact that Pyn, Csk (Fig. 1C), and other proteins such as Grb2 and protein-tyrosine phosphatase-1C (not shown) did not bind to CD45-AP under the same conditions. Interestingly, binding of Lck and ZAP-70 to CD45-AP did not require the presence of CD45 since it occurred in CD45-negative variant cells as well. Furthermore, the binding of Lck and ZAP-70 to CD45-AP decreased for both the wild-type lymphocytes and their CD45-negative variants when cells were stimulated by TCR ligation although the total amounts of these protein-tyrosine kinases in the cell lysates remained unchanged (see Fig. 1A). The greater decrease of bound Lck in WT cells compared with that seen in N1 was not a consistent observation as N1 cells exhibited greater decreases in some experiments. Lck and ZAP-70 did not associate with the C form of CD45-AP indicating that the transmembrane and/or the first five amino acid residues of the cytoplasmic domain play critical roles for their interactions. In addition, the binding of Lck and ZAP-70 to CD45-AP occurred independently of the TCR coreceptor, CD4, since the cell lines used did not express CD4 on their cell surfaces (27).

The percentage of endogenous Lck and ZAP-70 that is capable of binding to recombinant CD45-AP was then determined by using cell lysates obtained from unstimulated CD45-positive YAC-1 cells (WT) and CD45-negative N1 cells. The cell lysates were repeatedly subjected to fresh aliquots of recombinant CD45-AP-conjugated beads, and the material bound to each aliquot of beads was analyzed by Western blotting with either anti-Lck or anti-ZAP-70 antibody (Fig. 1D). Approximately 2.1% of total Lck and 0.95% of total ZAP-70 present in WT cells bound to recombinant CD45-AP. On the other hand, approximately 0.84% of total Lck and 0.66% of total ZAP-70 present in N1 cells bound to CD45-AP.

Lck and ZAP-70 Coimmunoprecipitate with CD45 after TCR-mediated Stimulation Independently of CD45—Next, we analyzed the effect of anti-CD3 ε stimulation on associations between endogenous CD45-AP and Lck or ZAP-70 in wild-type CD45-positive YAC-1 cells (WT) and their CD45-negative variant cells (N1). Anti-CD45-AP immunoprecipitates were prepared at various time points after stimulation, and material coimmunoprecipitated with CD45-AP or with control beads was analyzed by Western blotting with antibodies against Lck or ZAP-70 (Fig. 2). Lck and ZAP-70 coimmunoprecipitated with CD45-AP after stimulation both in WT and N1 cells. In agreement with the results of recombinant CD45-AP binding analysis (Fig. 1C), association of Lck or ZAP-70 with endogenous CD45-AP did not require the presence of CD45 since it occurred in CD45-negative variant cells as well. Larger amounts of Lck and ZAP-70 coimmunoprecipitated with CD45-AP in N1 than in WT cells despite the greatly diminished presence of CD45-AP in N1 compared with WT cells (20).

Interestingly, the binding of Lck and ZAP-70 to recombinant CD45-AP decreased with stimulation (Fig. 1C), whereas association of endogenous CD45-AP with these protein-tyrosine kinases increased (Fig. 2). This indicates that after 60 min stimulation, the protein-tyrosine kinase populations available for binding to CD45-AP have already bound to endogenous CD45-AP and are not available for binding to recombinant CD45-AP.

Specific Binding of CD45-AP to Lck and ZAP-70 Can Be Demonstrated with the Respective Recombinant Proteins—We have further investigated the association of CD45-AP with Lck and ZAP-70 by binding analysis employing recombinant forms of each protein. As shown in Fig. 3A, GST-tagged recombinant Lck protein bound to histidine-tagged recombinant CD45-AP that was conjugated to the nickel-nitriolutartriacetate beads, and the binding was prevented by adding increasing amounts of free GST-tagged CD45-AP as a competitor for binding. An excess amount of control GST protein did not compete for the binding. Likewise, as shown in Fig. 3B, histidine-tagged recombinant ZAP-70 protein bound to GST-tagged recombinant CD45-AP that was conjugated to the glutathione-Sepharose 4B beads, and the binding was prevented by adding increasing amounts of free histidine-tagged CD45-AP as a competitor for binding. An excess amount of control histidine-tag protein did not compete for the binding. The results demonstrate that both Lck and ZAP-70 recombinant proteins bind specifically to recombinant CD45-AP and that the interactions do not require other lymphocyte-specific proteins.

Lck and ZAP-70 Coimmunoprecipitate with CD45 after TCR-mediated Stimulation—Direct associations have been observed between CD45 and Lck (31), between CD45 and TCR ε chain (15), between TCR γ chain and ZAP-70 (3, 4), and between Lck and ZAP-70 (32). To determine the effect of stimulation on these associations, wild-type CD45-positive YAC-1 cells were stimulated by TCR cross-linking with anti-CD3 ε antibody, and anti-CD45 immunoprecipitates were prepared at various time points after stimulation. Material coimmunoprecipitated with CD45 was then analyzed by Western blotting with antibodies against CD45, CD45-AP, Pyn, Lck, or ZAP-70 (Fig. 4). In agreement with the results obtained by recombinant CD45-AP binding analysis (Fig. 1C), the amount of CD45-AP coimmunoprecipitating with CD45 did not change after stimulation. Fyn and Lck coimmunoprecipitated with CD45 after 60 min stimulation, whereas a small amount of ZAP-70 coimmunoprecipitated with CD45 without stimulation and the amount increased after a short period of stimulation. These results indicate that TCR-associated protein-tyrosine kinases bind to CD45 in response to
Interactions of CD45-AP with Lck and ZAP-70

approximately 4.2% of total Lck and 2.5% of total ZAP-70 coimmunoprecipitated with CD45 as previously reported (18). However, a subpopulation of free CD45-AP exists in CD45-positive cells, CD45 does not prevent the interaction of Lck with CD45 since Lck associates with Lck under certain circumstances (35–37). The cell lines used in the present study, YAC-1 and N1 (27), express neither CD4 nor CD8. Therefore, it is possible that the constitutively activated Lck in N1 cells is already bound to various endogenous molecules and thus is less capable of interacting with recombinant CD45-AP.

A Subpopulation of CD45-AP Is Not Bound to CD45—In a previous study, two-dimensional diagonal SDS-PAGE analysis of anti-CD45 immunoprecipitates obtained from YAC-1 cell lysates treated with a chemical cross-linker revealed that approximately 70% of CD45 exists as a complex with CD45-AP with a stoichiometry of 1 to 1 (18). To determine whether a population of free CD45-AP exists in CD45-positive cells, CD45 was depleted from lysates of CD45-positive YAC-1 cells by repeated immunoprecipitation with anti-CD45-conjugated beads, and the amount of CD45-AP in the lysates was determined (Fig. 5). After the exhaustive immunoprecipitation, CD45 was barely detectable in lysates by Western blotting as expected. On the other hand, approximately 24% of total CD45-AP was estimated by densitometric analysis of the immunoblot to be present in the lysate after CD45 depletion.

A similar estimate of the free form of CD45-AP was obtained by sucrose gradient ultracentrifugation as well (Fig. 6). YAC-1 cell lysate was fractionated by the ultracentrifugation, and each fraction was analyzed by Western blotting with anti-CD45 and CD45-AP-antibodies. A majority of CD45-AP cosedimented with CD45 as previously reported (18). However, a subpopulation of CD45-AP sedimented at a much slower rate with a distinct peak in fraction 18. The amount of CD45-AP present in fractions 16–20 was estimated to be 26% of the total.

DISCUSSION

The present study demonstrates that CD45-AP specifically interacts with Lck and ZAP-70 and that the interactions do not require CD45 (Figs. 1 and 2) or any other lymphocyte-specific proteins (Fig. 3). Binding of Lck and ZAP-70 to exogenously added recombinant CD45-AP decreases upon 60 min stimulation by TCR ligation (Fig. 1C), whereas the amounts of these protein-tyrosine kinases coimmunoprecipitating with endogenous cellular CD45-AP increase in both CD45-positive YAC-1 cells and the CD45-negative variant N1 (Fig. 2). The most plausible explanation for the reduced binding of recombinant CD45-AP to Lck and ZAP-70 after TCR-mediated stimulation is that the stimulation causes these protein-tyrosine kinases to associate with endogenous CD45-AP, hence rendering them inaccessible to recombinant CD45-AP. This is consistent with the fact that at a given time only a small percentage of the total cellular protein-tyrosine kinases is able to interact with CD45-AP (Fig. 1D). The protein-tyrosine kinase subpopulation that can interact with CD45 after stimulation is also small (Fig. 4B), and similar percentages of total Lck and ZAP-70 have been reported in association with the TCR (8, 33). Therefore, the percentages of protein-tyrosine kinase subpopulations engaged in TCR signaling at a given time are consistent with the percentages of the protein-tyrosine kinase subpopulations capable of associating with CD45-AP.

The absence of CD45 results in increased protein-tyrosine kinase activity of Lck in N1 cells compared with CD45-positive YAC-1 cells (34), probably due to the elevated level of tyrosine phosphorylation on the positive regulatory site (17). As shown in Fig. 1D, the percentage of endogenous Lck that is capable of binding to recombinant CD45-AP is lower in N1 cells than in YAC-1 cells. However, no corresponding increase in binding to endogenous CD45-AP is observed in N1 cells in the absence of stimulation (Fig. 2). It is therefore possible that the constitutively activated Lck in N1 cells is already bound to various endogenous molecules and thus is less capable of interacting with recombinant CD45-AP.

The mechanism that determines the availability of certain protein-tyrosine kinase subpopulations for binding to CD45-AP in cells is not known. Posttranslational modifications, interactions with other molecules, and cellular localization may be required for protein-tyrosine kinases to interact with CD45-AP. Likewise, it is possible that endogenous CD45-AP acquires the ability to interact with protein-tyrosine kinases only after certain stimulation-dependent events that result in modification of CD45-AP. Lck binds noncovalently to the cytoplasmic domain of the TCR coreceptors, CD4 or CD8 (5, 7). Upon TCR ligation, these coreceptors are thought to augment antigen-mediated signals by binding to major histocompatibility complex molecules of target cells and bringing Lck closer to the TCR. On the other hand, CD4 may also exert a negative signal by sequestering Lck from the TCR under certain circumstances (35–37). The cell lines used in the present study, YAC-1 and N1 (27), express neither CD4 nor CD8. Therefore, it is possible that the absence of CD4 and CD8 releases Lck from certain constraints and increases the amount of Lck available for interacting with CD45 and CD45-AP in these cell lines. On the other hand, it is clear that the presence of the coreceptors does not prevent the interaction of Lck with CD45 since Lck coimmunoprecipitates with CD45 in normal splenic T cells (24) as well as in a CD4-positive T cell line (38). It is of interest to examine whether transfection of CD4 to YAC-1 and N1 cells would alter the interactions of Lck with CD45 and CD45-AP.

We have previously demonstrated that CD45-AP protein levels are much lower in three out of four CD45-negative variant lymphoid cell lines, including N1 cells, compared with their CD45-positive wild-type parental lymphocytes (20). However, larger amounts of Lck and ZAP-70 coimmunoprecipitate with CD45-AP in N1 than in wild-type YAC-1 cells after 60 min

Fig. 3. Specific binding of CD45-AP to Lck and ZAP-70 using the respective recombinant proteins. A, histidine-tagged recombinant CD45-AP bound to glutathione-Sepharose 4B beads was incubated with a constant amount of histidine-tagged recombinant CD45-AP or an excess of control GST protein. The protein amount of control GST used corresponds approximately to the highest amount of GST-tagged CD45-AP. Lck bound to the beads was analyzed by Western blotting with anti-Lck antibody. B, GST-tagged recombinant CD45-AP bound to glutathione-Sepharose 4B beads was incubated with a constant amount of histidine-tagged recombinant ZAP-70 protein in the presence or absence of increasing amounts of histidine-tagged recombinant CD45-AP or an excess of histidine tag control protein. The amount of control protein used corresponds approximately to the highest amount of the histidine-tagged CD45-AP. ZAP-70 bound to the beads was analyzed by Western blotting with anti-ZAP-70 antibody.
stimulation (Fig. 2). Therefore, it is possible that CD45-AP/kinase dimers formed in N1 cells without CD45 are more efficiently immunoprecipitated with anti-CD45-AP antibody than CD45-AP/CD45/kinase trimers formed in wild-type cells. Alternatively, CD45 and CD45-AP may not bind cooperatively to the protein-tyrosine kinase to form ternary complexes, but instead the protein-tyrosine kinase may be transferred from the CD45-AP/kinase complex to CD45 to form a CD45/kinase complex in CD45-positive cells. In that case, fewer CD45-AP/kinase complexes would accumulate in the presence of CD45. Approximately 25% of total CD45-AP exists free of CD45 in CD45-positive T cells (Figs. 5 and 6). On the other hand, our earlier results indicate that approximately 30% of total CD45 is not bound to CD45-AP (18). These significant subpopulations of CD45-AP and CD45 may form binary complexes with protein-tyrosine kinases as a part of the CD45-mediated signaling process.

The C form of recombinant CD45-AP that encompasses the bulk of the cytoplasmic domain except for the five amino acid residues adjacent to the transmembrane segment failed to bind to Lck and ZAP-70 (Fig. 1C). Therefore, the transmembrane segment and/or the membrane-proximal region of the cytoplasmic domain is critical for the interactions. This region may be directly involved in the binding or, alternatively, the cytoplasmic domain may not be able to form a correct conformation without this region and thus fail to provide binding sites for the protein-tyrosine kinases.

Fyn does not bind to recombinant CD45-AP but coimmunoprecipitates with CD45 while Lck binds to recombinant CD45-AP and also coimmunoprecipitates with CD45 (Figs. 1 and 4). Thus CD45-AP appears to be involved in the interaction between CD45 and Lck but not in the interaction between CD45 and Fyn in YAC-1 cells. These findings are consistent with the hypothesis that CD45-AP may act as a "catalytic amplifier" of the protein-tyrosine kinase activity in the CD45-mediated signaling process in T cells.
with the fact that Fyn and Lck have distinct characteristics and roles in lymphocyte signal transduction. For example, Fyn has been reported to be much less efficiently regulated than Lck by CD45 in vivo (39–41), and overexpression of Lck but not Fyn leads to enhanced phosphorylation of ZAP-70 and Syk (42). Furthermore, Fyn and Lck are required to different degrees at different stages of T cell development (43–46). It is possible that CD45-AP plays a role in a subset of CD45-mediated signal transduction pathways by mediating interactions of CD45 with a selected group of potential substrates.

There is substantial evidence that Lck is a CD45 substrate in vivo (2, 11, 13, 39–41). In addition, it has been shown that the cytoplasmic domain of recombinant CD45 binds directly to recombinant Lck (31). However, data from CD45-AP-null mice created by gene targeting indicate that CD45-AP is required for an optimal CD45-Lck interaction and antigen receptor-mediated signaling in lymphocytes (24). T cells of CD45-AP-null mice exhibit markedly reduced proliferative and functional responses to TCR-mediated stimuli. Interestingly, the interaction between CD45 and Lck is significantly diminished in these CD45-AP-null T cells. Moreover, the amount of Lck associated with CD45 increases in wild-type cells after stimulation by TCR ligation, whereas it remains at constantly lower levels in CD45-AP-null T cells. Combined with the functional impairments exhibited by CD45-AP-null T cells, the results indicate that CD45-AP is required to coordinate the interaction of CD45 with Lck for appropriate signaling. Further support for this notion comes from experiments in which CD45 and Lck were coexpressed in fibroblasts (47). The failure of CD45 to stably dephosphorylate kinase-active Lck in these experiments may well be due to the absence of CD45-AP expression in fibroblasts. It would be interesting to determine whether cotransfection of a CD45-AP-expressing construct would restore the ability of CD45 to dephosphorylate Lck in this system.

It is thought that upon TCR-mediated stimulation, Src family kinases such as Lck phosphorylate tyrosine residues of a signaling motif, termed ITAM (immune receptor tyrosine-based activation motif), present in the TCR z chain and CD3 peptides (48). The tyrosine-phosphorylated z chain recruits ZAP-70 by binding through the SH2 domains (3, 4). The TCR-associated ZAP-70 then becomes tyrosine-phosphorylated and activated by Lck leading to phosphorylation of other cellular substrates and amplification of the signal (49, 50). Thus, Lck and ZAP-70 are thought to work in synergy to transduce TCR-mediated stimulatory signals. A direct interaction of Lck with ZAP-70 has been reported (32). However, the interaction of ZAP-70 with CD45-AP probably does not proceed via Lck since recombinant ZAP-70 protein directly binds to recombinant CD45-AP (Fig. 3). The specific interaction of ZAP-70 with CD45-AP and the communoprecipitation of ZAP-70 with CD45 (Fig. 4) demonstrated in the present study support the possibility that ZAP-70 is also a CD45 substrate. Phosphorylation on Tyr-492 and -493 of ZAP-70 leads to down- and up-regulation of its protein-tyrosine kinase activity, respectively, whereas phosphorylation of Tyr-292, the major autophosphorylation site, is thought to down-regulate ZAP-70 function without affecting the kinase activity (51, 52). It is possible that the CD45 protein-tyrosine phosphatase activates ZAP-70 by dephosphorylating one or both of its down-regulatory tyrosyl residues. As described above, responses to TCR-mediated stimuli are diminished in CD45-AP-knock-out mice. These data suggest that CD45-AP coordinates the interaction between ZAP-70 and Lck during TCR signal transduction.

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