CD45 is a transmembrane protein tyrosine phosphatase playing an essential role during T-cell activation. This function relates to the ability of CD45 to regulate p56\(^{\text{Lck}}\), a cytoplasmic protein tyrosine kinase necessary for T-cell antigen receptor (TCR) signaling. Previous studies have demonstrated that CD45 is constitutively associated in T-lymphocytes with a transmembrane molecule termed CD45-AP (or lymphocyte phosphatase-associated phosphoprotein). Even though the exact role of this polypeptide is unclear, recent analyses of mice lacking CD45-AP have indicated that its expression is also required for optimal T-cell activation. Herein, we wished to understand better the function of CD45-AP. The results of our studies showed that in T-cells, CD45-AP is part of a multimolecular complex that includes not only CD45, but also TCR, the CD4 and CD8 coreceptors, and p56\(^{\text{Lck}}\). The association of CD45-AP with TCR, CD4, and CD8 seemed to occur via the shared ability of these molecules to bind CD45. However, binding of CD45-AP to p56\(^{\text{Lck}}\) could take place in the absence of other lymphoid-specific components, suggesting that it can be direct. Structure-function analyses demonstrated that such an interaction was mediated by an acidic segment in the cytoplasmic domain of CD45-AP and by the kinase domain of p56\(^{\text{Lck}}\). Interestingly, the ability of CD45-AP to interact with Lck in the absence of other lymphoid-specific molecules was proportional to the degree of catalytic activation of p56\(^{\text{Lck}}\). Together, these findings suggest that CD45-AP is an adaptor molecule involved in orchestrating interactions among components of the antigen receptor signaling machinery. Moreover, they raise the possibility that one of the functions of CD45-AP is to recognize activated Lck molecules and bring them into the vicinity of CD45.

Activation of T-lymphocytes by antigen is initiated by protein tyrosine phosphorylation (1–4). Although the T-cell antigen receptor (TCR)\(^1\) and the associated CD3 and \(\zeta\) subunits are devoid of intrinsic protein tyrosine kinase activity, they can recruit two classes of cytoplasmic protein tyrosine kinases to mediate this response, the Src family and the Syk/Zap-70 family. The Src-related enzymes Lck and Fyn\(^{\text{T}}\) initiate TCR-mediated signals by phosphorylating a signaling motif in the cytoplasmic domain of CD3 and \(\zeta\) termed ITAM (for immunoreceptor tyrosine-based activation motif). Following this phosphorylation, the Syk/Zap-70 family kinases are activated through binding of their tandem Src homology 2 (SH2) domains to doubly phosphorylated ITAMs. Together with Src family kinases, Zap-70 and Syk are responsible for subsequent tyrosine phosphorylation of several signal transduction molecules including phosphoprotein C-\(\gamma\)-, Cbl, Vav, Slp-76, and Lat.

CD45 is a 180–220-kDa transmembrane protein tyrosine phosphatase expressed on all nucleated hemopoietic cells (5, 6). In T-cells it constitutes ~10% of all cell surface glycoproteins. Previous studies have shown that CD45 is necessary for T-cell activation because of its ability to promote constitutive dephosphorylation of the inhibitory carboxyl-terminal tyrosine of p56\(^{\text{Lck}}\); tyrosine 505 (7–9). This dephosphorylation is necessary for Lck to become activated and phosphorylate the TCR complex upon antigen stimulation. There is also an indication that CD45 is able to dephosphorylate other substrates, including the positive regulatory site of Lck (tyrosine 394) and the \(\zeta\) subunit of TCR (10–12). Although the precise mechanism by which CD45 specifically targets these substrates is not established, it is noteworthy that small amounts of p56\(^{\text{Lck}}\), but not Fyn\(^{\text{T}}\), can be found to coimmunoprecipitate with CD45 in mild detergent lysates of T-cells (13–15). Furthermore, CD45 has been shown to colocalize on the cell with several other molecules, including the TCR complex, CD4, CD2, and Thy-1 (16–21). Thus, it is possible that the specificity of CD45 toward its substrates is dictated in part by its relative distribution on the cell surface.

Previous studies have demonstrated that the majority of CD45 molecules in T-cells is associated with a 30-kDa polypeptide termed CD45-associated protein (CD45-AP) or lymphocyte phosphatase-associated phosphoprotein (hereafter named CD45-AP) (22–24). Through molecular cloning, CD45-AP was shown to be a novel transmembrane protein with a short extracellular domain of ~9 amino acids, a single transmembrane segment, and a cytoplasmic region of ~145 residues (see Fig. 8A) (23, 24). Although CD45-AP has no clear homology with any other known polypeptide, one group pointed out that it may possess a WW domain in its intracytoplasmic region (25). CD45-AP is expressed on T-cells, B-cells, and some myeloid...
cells and is absent in non-hematological cell types (23, 26, 27). Structure-function analyses and experiments with recombinant proteins have revealed that the interaction between CD45 and CD45-AP is direct and that it occurs via their respective transmembrane segments (25, 28, 29). CD45-AP was also reported to associate directly with Lck in transfected HeLa cells (25); however, the affinity of this association was apparently low because it could be detected only through in vitro kinase reactions and not by Western blotting.

The importance of CD45-AP in normal cellular physiology was addressed recently by the creation of CD45-AP-deficient mice through homologous recombination in embryonic stem cells (30). Although T-cell development was not noticeably affected in these mice, thymocytes and splenic T-cells exhibited a decrease in proliferation in response to TCR stimulation. CD45-AP-deficient T-cells were also less efficient at mediating effector functions such as cytolysis. Although a precise biochemical explanation for these defects was not established, it is noteworthy that CD45-AP-deficient T-cells also demonstrated a reduction in CD45 expression and in the amount of p56⚲k that can be coimmunoprecipitated with CD45.

In this paper, we wish to explore further the role of CD45-AP in T-lymphocytes. The results of our experiments showed that CD45-AP is part of a complex that contains not only CD45 but also the antigen receptor, the CD4 and CD8 coreceptors, and p56⚲k. They also demonstrated clearly that an interaction between CD45-AP and Lck can occur in the absence of other lymphoid-specific molecules and that this association is proportional to the degree of enzymatic activation of p56⚲k.

MATERIALS AND METHODS

Cells—Thymocytes were extracted from 5–6-week-old Balb/c mice (Bi-141) on an antigen-specific mouse T-cell line (31). It expresses TCR, CD4, and CD8 but lacks CD4 and CD8. Because Bi-141 contains very low amounts of endogenous p56⚲k, a variant transfected with a wild-type lck cDNA was used for some of the experiments (32). Monoclonal cell lines expressing either Tac- or Tac- were generated by transfection. YAC-1 and the CD45-negative variant YAC-N1 were described previously (33) and were kindly provided by Dr. Jonathan Ashwell, National Institutes of Health. LSTRA is a lymphoid cell line expressing CD45 but lacking TCR, CD4, and CD8. It expresses markedly elevated levels of Lck as a result of retroviral promoter insertion (34, 35). All constructs were verified by sequencing to ensure that no unwanted changes had been introduced (data not shown). For transfection in COS-1 cells, the various cDNAs were cloned in the multiple cloning site of pXM139, which contains the SV40 origin of replication. Transfections in COS-1 cells were performed as described elsewhere (36), using the DEAE-dextran method and a fixed total amount of DNA (8 µg).

Antibodies—A polyclonal antiserum directed against CD45-AP was produced in rabbits using a fusion protein encompassing the cytoplasmic domain of CD45-AP as an immunogen. Rabbit antisera directed against Lck, Fyn, Csk, Chk, Zap-70, Syk, Cbl, Yav, and Shc were described previously (36, 42–48). Monoclonal antibodies (MAbs) reacting with CD45 (M1,89,18,7), TCR (F23.1 and H57), CD3 (145–2C11) for immunoprecipitation; HMT-3 for immunoblotting), (H146), Thy-1 (G7), CD4 (GK1,5), CD8 (2,43, TAC (7G7), and Mye (9E10) were reported elsewhere.

Immunoprecipitations and Immunoblots—After washing in phosphate-buffered saline, cells were lysed in Brij-97-containing buffer (50 mM Tris, pH 8.0, 1% Brij-97, 2 mM EDTA) supplemented with 10 µg/ml each of the protease inhibitors leupeptin, aprotinin, N-tosyl-l-phenylalanine chloromethyl ketone, N-p-tosyl-l-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride, as well as the phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (1 mM). For COS-1 cells, cells were lysed in TNE buffer (50 mM Tris, pH 8.0, 1% Nonidet-P40, 2 mM EDTA) supplemented with the same inhibitors. Polypeptides were recovered by immunoprecipitation from equivalent amounts of total cellular proteins using the indicated antibodies, pre-coupled to protein A or protein G-Sepharose (Amersham Pharmacia Biotech). In some cases, immune complexes were collected with Staphylococcus aureus protein A (Pansorbin; Calbiochem), coupled, if indicated, to rabbit anti-mouse immunoglobulin IgG. Immunoprecipitates were washed three times with lysis buffer containing 1 mM sodium orthovana- date. Proteins were then eluted in sample buffer, boiled, electrophoresed in 8% (Lck), 10% (CD45-AP and e) or 12% (e) SDS-polyacrylamide gels, and transferred onto Immobilon membranes (Millipore) for immunoblotting. Immunoblots were performed according to a protocol described previously (42). After incubation with 125I-protein A (Amersham Pharmacia Biotech) or 125I-goat anti-mouse IgG (ICN), immunoreactive products were detected by autoradiography and quantitated with a PhosphorImager (BAS 2000; Fuji).

RESULTS

Comuniprecipitation of CD45-AP with the Antigen Receptor Complex in T-lymphocytes—To gain more understanding of the function of CD45-AP in T-lymphocytes, its ability to interact with molecules other than CD45 was evaluated. Normal ex vivo mouse thymocytes were lysed in Brij-97-containing buffer, and various polypeptides were immunoprecipitated from post-nuclear cell lysates using the indicated antibodies. After separation in 10% SDS-polyacrylamide gels, the presence of CD45-AP in these immunoprecipitates was determined by immunoblotting with a rabbit antisera directed against the cytoplasmic domain of CD45-AP (Fig. 1A). As reported earlier (23, 24), large amounts of CD45-AP were found to coimmunoprecipitate with CD45 (lane 3). Unexpectedly, though, we observed that appreciable quantities of CD45-AP were also present in immunoprecipitates of Lck (lanes 4 and 5), CD4 (lane 8), CD8 (lane 9), and TCR-associated CD3 (lane 10). In contrast, no CD45-AP was detected in FynT immunoprecipitates (lanes 6 and 7) or in immunoprecipitates obtained with normal rabbit serum (lane 11). It is notable that CD45-AP migrated as three distinct species in these gels, in agreement with earlier reports (23). These variations in the apparent molecular mass of CD45-AP are apparently caused by serine phosphorylation (23).

To address the specificity of these associations, the ability to CD45-AP to coimmunoprecipitate with a series of other signal transduction molecules expressed in thymocytes was assessed (Fig. 1B). Contrary to p56⚲k (lane 1) and the TCR-associated ζ chain (lane 10), no CD45-AP was associated with the protein tyrosine kinases Csk (lane 3) and Chk (lane 4), the adaptor molecules Cbl (lane 7) and She (lane 9), and the guanine nucleotide exchange factor Vav (lane 8). However, a small quantity was detected in Zap-70 (lane 5) and Syk (lane 6) immunoprecipitates. Because Syk/Zap-70 family kinases are known to be constitutively associated with TCR in thymocytes (49), it is possible that these interactions were mediated via the TCR.

We also wanted to test whether the components of the TCR complex could be found in immunoprecipitates of CD45-AP (Fig. 2). Therefore, thymocyte lysates were immunoprecipitated with antibodies directed against either CD45-AP or CD45, and the presence of the ζ subunit of TCR in these immunoprecipitates was revealed by immunoblotting with anti-ζ MAb H146. This experiment demonstrated that significant

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amounts of $\zeta$ were immunoprecipitated both with CD45-AP (lane 1) and with CD45 (lane 2) but not when normal rabbit serum (lane 4) or an irrelevant MAb antibody (anti-Tac MAb 7G7; data not shown) was used as immunoprecipitating reagent. As expected, large quantities of $\zeta$ were also detectable in anti-CD3 immunoprecipitates (lane 3). In combination, these data revealed that CD45-AP could be coimmunoprecipitated not only with CD45 but also with several elements of the antigen receptor complex in thymocytes, including TCR, CD3, CD4, and CD8, as well as with p56$\text{Lck}$. The Interaction between CD45-AP and the TCR Complex Requires the Presence of CD45—To understand the mechanism(s) of these interactions, we first investigated whether they required the presence of CD4 and CD8 by performing similar analyses with the CD4-negative, CD8-negative mouse T-cell line BI-141 (Fig. 3). As was the case in thymocytes (Fig. 1), significant amounts of CD45-AP were present in immunoprecipitates of CD45 (Fig. 3, lane 2), CD3 (lane 6), TCR (lanes 7 and 8), and $\zeta$ (lane 9) in BI-141 cells. Smaller quantities were also detected in anti-Lck immunoprecipitates (lane 3). However, there was no CD45-AP associated with FynT (lane 4) or the glycosylphosphatidylinositol-linked molecule Thy-1 (lane 10), even though both molecules are expressed abundantly in BI-141 cells (data not shown). Moreover, no CD45-AP was recovered when an antibody against CD4 (lane 5) or normal rabbit serum (lane 11) was used for immunoprecipitation. Thus, the interaction of CD45-AP with TCR (and Lck) could take place in the absence of CD4 and CD8.

Next, we wished to determine whether the CD45-AP-TCR interaction was direct. Because CD45-AP contains only a few extracellular residues and its transmembrane region is already involved in binding to CD45, we were especially interested in testing the possibility that its intracytoplasmic region may bind the intracellular sequences of CD3 and/or $\zeta$. To this end, subclones of BI-141 bearing chimeras in which the cytoplasmic sequences of either $\zeta$ or the $\epsilon$ subunit of CD3 were fused to the extracellular and transmembrane regions of Tac were studied (Fig. 4). As in control BI-141 cells (Fig. 4A, lanes 2 and 3), substantial amounts of CD45-AP were immunoprecipitated...
with CD45 (lanes 7 and 12) and CD3 (lanes 8 and 13) in these derivatives. In contrast, though, none was associated with Tac-ε (lane 9) or Tac-ζ (lane 14), despite the fact that both chimeras were expressed at the cell surface (data not shown) and were present in amounts comparable or superior to those of the endogenous TCR-associated chains (Fig. 4; data not shown). Similar results were obtained when individual chains of CD3ζ were transfected with CD45-AP in COS-1 cells (data not shown). Hence, these results implied that the interaction between CD45-AP and TCR was not mediated by the cytoplasmic domain of either ε or ζ. Unfortunately, similar experiments could not be performed with chimeras encompassing the cytoplasmic domain of the δ or γ subunit of CD3 because fusions were internalized rapidly and not expressed stably at the cell surface (data not shown).

Studies of splicing isoforms of CD45 have indicated that CD45 and the TCR complex can interact by way of their extracellular domains (20, 50). On this basis, the possibility that the CD45-AP-TCR interaction was mediated by CD45 was ascertained by studying T-cells lacking CD45 expression (Fig. 5). CD45-positive YAC-1 T-cells, as well as a variant lacking CD45 (YAC-N1), were lysed and processed for immunoprecipitation as outlined above. In keeping with our earlier observations (Figs. 1 and 3), CD45-AP was present in immunoprecipitates of CD45-AP and TCR complex in CD45-positive and CD45-negative variants of YAC-1 T-cells (data not shown). The migrations of prestained molecular weight markers are indicated on the right; those of the heavy chain of immunoglobulin (IgH) and CD45-AP are shown on the left. Exposure was for 8.5 h. Panel B, anti-ε immunoblot. The presence of CD3 ε was detected by immunoblotting with an antibody directed against the cytoplasmic domain of ε (MAb HMT-3). The migrations of prestained molecular weight markers are indicated on the right; those of Tac-ε and ε are shown on the left. Exposure was for 16 h.

To examine further the idea that CD45-AP may interact directly with p56Lck, we first studied their capacity to be co-

![Image](https://via.placeholder.com/150)

**Fig. 4. Association of CD45-AP with CD3ε and ζ chimeras in BI-141 T-cells.** The ability of Tac-ε and Tac-ζ to associate with CD45-AP in BI-141 T-cells was ascertained as described for Fig. 1. 60 μg of cellular protein was used for immunoprecipitation of CD45 and CD45-AP; 300 μg was used for all other immunoprecipitations. Panel A, anti-CD45-AP immunoblot. The positions of molecular mass markers are indicated on the right; those of the heavy chain of immunoglobulin (IgH) and CD45-AP are shown on the left. Exposure was for 8.5 h. Panel B, anti-ε immunoblot. The presence of CD3 ε was detected by immunoblotting with an antibody directed against the cytoplasmic domain of ε (MAb HMT-3). The migrations of prestained molecular weight markers are indicated on the right; those of Tac-ε and ε are shown on the left. Exposure was for 16 h.

**Fig. 5. Role of CD45 in the CD45-AP-TCR interaction.** The ability of CD45-AP to coimmunoprecipitate with TCR in CD45-positive and CD45-negative variants of YAC-1 T-cells was examined as outlined in the legend of Fig. 1. 1 mg of protein was used in each immunoprecipitation. The migrations of prestained molecular weight markers are indicated on the right; those of the heavy chain of Ig (IgH) and CD45-AP are shown on the left. NRS, normal rabbit serum. Exposure was for 21 h.

**Association of CD45-AP with LckCan Occur Independently of CD45, CD4, CD8, and TCR**—Previously it was reported that small quantities of Lck could be observed in immunoprecipitates of the CD45 phosphatase obtained from mild detergent lysates of T-cells (13, 14). Although the basis for this association was not determined, it was shown to happen in the absence of CD4 and CD8 expression (15). Further experiments with recombinant proteins suggested that this interaction was possibly direct, as a result of an association between the cytoplasmic portion of CD45 and the unique and SH2 domains of p56Lck (52). Nevertheless, recent analyses of CD45-AP-deficient T-cells suggested that the association between CD45 and Lck may be facilitated by CD45-AP, especially in TCR-stimulated cells (30). Further credence for this view was provided by the observation that CD45-AP and Lck can associate weakly in HeLa cells, in the absence of CD45 (25).

To examine further the idea that CD45-AP may interact directly with p56Lck, we first studied their capacity to be co-

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munoprecipitated in LSTRA, a lymphoid cell line lacking not only CD4 and CD8, but also TCR, while expressing markedly elevated levels of Lck (Fig. 6A) (34, 35). This analysis showed that significant quantities of CD45-AP existed in anti-p56
lck
immunoprecipitates (lane 3) in LSTRA cells. As expected, CD45-AP was also associated with CD45 (lane 2) in these cells, but it was absent in immunoprecipitates obtained with preimmune serum (lane 4). Therefore, CD45-AP and Lck could interact in lymphoid cells in the absence of TCR, CD4, and CD8 (this figure and Fig. 3).

Second, the capacity of CD45-AP to associate with Lck was characterized in COS-1 cells, a non-lymphoid cell line lacking CD4, CD8, TCR, and CD45 (Fig. 6B). Cells were transiently transfected by the DEAE-dextran method with a mouse cd45-ap cDNA, in the absence or presence of a wild-type mouse lck cDNA. After 60 h, cells were lysed in Nonidet P-40-containing buffer, and the ability of CD45-AP to associate with Lck was determined as indicated under “Materials and Methods.” 250 μg of cellular proteins was used in each immunoprecipitation. The migrations of prestained molecular weight markers are shown on the right; those of CD45-AP and Lck are indicated on the left. Exposures were for 16 h.

![Image](image-url)

**Fig. 6.** Association of CD45-AP with Lck can occur independently of other lymphoid-specific components. Panel A, association of CD45-AP with p56
lck
in LSTRA cells. 100 μg of cellular proteins was used for immunoprecipitation in lanes 1 and 2; 500 μg was used in the other lanes. The positions of prestained molecular mass markers are indicated on the right; those of the immunoglobulin heavy chain (Ig(H)) and CD45-AP are shown on the left. Exposure was for 7 h. Panel B, association of CD45-AP with p56
lck in COS-1 cells. COS-1 cells were transiently transfected with the indicated cDNAs. After lysis in Nonidet P-40-containing buffer, the ability of CD45-AP to associate with Lck was determined as indicated under “Materials and Methods.” 250 μg of cellular proteins was used in each immunoprecipitation. The migrations of prestained molecular weight markers are shown on the right; those of CD45-AP and Lck are indicated on the left. Exposures were for 16 h.

Cules was revealed by immunoblotting of CD45-AP immunoprecipitates with an anti-Lck serum (Fig. 7B, top panel). This assay demonstrated that, compared with wild-type Lck (lane 9), Lck polypeptides carrying a deletion of the SH3 domain (ΔSH3 Lck; lane 11) or SH2 region (ΔSH2 Lck; lane 12), or a mutation of the inhibitory carboxyl-terminal tyrosine, tyrosine 505 (Y505F Lck; lane 14) exhibited a ~5–6-fold greater efficiency at binding CD45-AP. Interestingly, these three mutations have all been shown to cause constitutive activation of p56
lck
(41, 53–55). In contrast, variants lacking the unique domain (ΔU Lck; lane 10) or carrying a mutation of the site of positive regulation, tyrosine 394 (Y394F; lane 13) had binding capacities similar to that of wild-type p56
lck
(lane 9). No significant immunoprecipitation of any of these polypeptides occurred in the absence of CD45-AP (lanes 3–8).

To examine further the possibility that CD45-AP recognized activated Lck molecules preferentially, the impact of supplementary mutations on the capacity of Y505F Lck to associate with CD45-AP was assessed (Fig. 7C). This experiment revealed that derivatives of Y505F Lck carrying a mutation of the site of myristoylation (G2A/Y505F Lck; lane 3), ATP binding (K273R/Y505F Lck; lane 5), or positive regulation (Y394F/Y505F Lck; lane 6) had a reduced capacity to bind to CD45-AP compared with Y505F Lck (lane 2). Their binding efficiency was comparable to that of wild-type Lck (lane 1). In contrast, a mutant lacking the unique domain (ΔU/Y505F Lck; lane 4) behaved like Y505F Lck (lane 2). Once again, the ability of CD45-AP to bind Lck correlated perfectly well with enzymatic activation of p56
lck
(37, 41). Because the unique SH3 and SH2 domains of Lck were dispensable for the association with CD45-AP (Fig. 7B), these data suggested that the binding was mediated by the Lck catalytic domain. To test this idea more directly, we evaluated the capacity of an Lck variant lacking the kinase domain to bind CD45-AP. As expected, this mutant (ΔK Lck; Fig. 7C, lane 7) was incapable of interacting with CD45-AP.

Finally, we determined which region in CD45-AP was involved in binding Lck (Fig. 8). For this purpose, various carboxyl-terminal truncations of CD45-AP were created by polymerase chain reaction (Fig. 8A). The first mutant (∆C1 CD45-AP) lacked 38 amino acids at the carboxyl terminus of the protein. The second (∆C2 CD45-AP) was missing 98 residues, including
a region rich in aspartic acid and glutamic acid (“acidic” region). Finally, the third carried a truncation of 128 amino acids at the carboxyl terminus, including part of the potential WW domain. For adequate detection of these polypeptides, they were also engineered to possess a Myc-derived epitope at their carboxyl terminus.

When tested in the COS-1 system (Fig. 8B), ΔC1 CD45-AP (top panel, lane 4) was able to associate with Y505F Lck in a manner analogous to wild-type CD45-AP (lane 3). Although the association of this variant with Lck may appear greater than that of wild-type CD45-AP, it should be noted that the mutant protein accumulated in greater amounts in COS-1 cells (bottom panel). Contrary to ΔC1 CD45-AP, however, the ΔC2 CD45-AP (lane 5) and ΔC3 CD45-AP (lane 6) mutants were incapable of associating with p56<sup>Lck</sup> even though they were also expressed adequately in COS-1 cells (bottom panel). It is worth pointing out that, unlike wild-type CD45-AP, all truncated proteins migrated as a single species in these gels. This finding suggested that the sites of phosphorylation responsible for the variations in the electrophoretic mobility of CD45-AP (23) were positioned within the last 38 residues of the protein. Taken together, these results indicated that the CD45-AP-Lck association was mediated by an acidic-rich region in the cytoplasmic domain of CD45-AP and by the catalytic domain of p56<sup>Lck</sup>.

**DISCUSSION**

In this paper, we have examined the ability of CD45-AP to interact with various constituents of the antigen receptor signaling machinery in T-lymphocytes. We found that in mouse thymocytes, CD45-AP coimmunoprecipitated not only with CD45 but also with the antigen receptor complex (which incorporates TCR, CD3,ζ, CD4, and CD8 in these cells) and the protein tyrosine kinase p56<sup>Lck</sup>. These interactions were specific, as CD45-AP was not associated with Thy-1, FynT, Csk, Chk, Cbl, Vav, and She. Further studies showed that the association between CD45-AP and TCR occurred independently of the
Association of CD45-AP with TCR Signaling Machinery

CD45-AP was discovered on the basis of its capacity to associate with CD45, via its transmembrane domain (25, 28, 29, 51). In addition to confirming this association, our results revealed that CD45-AP could be coimmunoprecipitated with the antigen receptor complex in Brij-97 lysates of T-cells. Although the precise basis for this interaction needs to be established, several observations suggest that it was mediated by CD45. First, we observed that the cytoplasmic domain of at least two of the components of the TCR complex, CD3e and ζ, was unable to engage in an association with CD45-AP, suggesting that the extracellular and/or transmembrane segments of the TCR complex were responsible for the interaction. Because CD45-AP contains little extracellular sequences, and its transmembrane domain is already bound to CD45, it thus appears unlikely that CD45-AP made direct contact with the TCR complex. Second, we noted that the CD45-AP-TCR interaction was absent in cells lacking CD45. Even though the levels of CD45-AP were reduced in CD45-negative YAC-1 cells, the inability of the remaining CD45-AP (20% of control) to coimmunoprecipitate with TCR would be consistent with the notion that CD45 was bridging the interaction between these two molecules. This idea is also in keeping with the previous observations that CD45 can interact with the TCR complex by way of its extracellular domain (29, 50), whereas it associates with CD45-AP via its transmembrane domain (25, 28, 29, 51).

These findings indicate that CD45-AP is part of a multimolecular complex that includes TCR as well as the CD4 and CD8 coreceptors in addition to CD45 (Fig. 9). Obviously, the function of CD45-AP in this complex remains to be established. Nevertheless, it is provocative to note that the absence of CD45-AP in mice was accompanied by a reduction in CD45 expression at the cell surface (30). Thus, CD45-AP may be a “chaperone” for one or more of these molecules. In agreement with this notion, it has been reported that CD45-AP associates with CD4 and CD8 shortly after its synthesis (51). Although an analysis of the levels of TCR, CD4, and CD8 in CD45-AP-deficient mice has yet to be reported, CD45-AP may also provide, albeit indirectly, a similar function for TCR, CD4, and CD8. It is also conceivable that CD45-AP participates in organizing the membrane microdomains containing the components of the antigen receptor signaling machinery. Such an organization may be important for optimal generation of TCR-triggered signals and may explain the diminished antigen receptor-induced responses in CD45-AP-deficient mice (30). Clearly, future studies are warranted to test these various possibilities.

The mechanisms underlying the association between CD45-AP and Lck in T-cells are apparently complex. In part, this interaction is probably indirect, as a consequence of the capacity of p56\(^{Lck}\) to associate with CD4 and CD8 through its unique region (42, 56) and possibly with CD45 via its unique and SH2 domains (52). Nonetheless, our transient expression experiments in COS-1 cells, and those in HeLa cells reported by others (25), showed that an association between CD45-AP and Lck could take place in the absence of other lymphoid-specific components. Notably, however, the association of CD45-AP with wild-type or inactive Lck molecules was rather weak in these systems. It was much more efficient with activated Lck variants such as Y505F Lck, ΔSH3 Lck, and ΔSH2 Lck. Therefore, the capacity of CD45-AP to bind Lck in the absence of other lymphoid molecules seems to be dictated by the degree of enzymatic activation of Lck. Presumably, CD45-AP recognizes the “active” conformation of the Lck kinase domain. Along these lines, it is worth noting that this association was mediated by an acidic-rich region in the cytoplasmic domain of CD45-AP. Interestingly, an acidic domain in the β chain of the interleukin-2 receptor has also been shown to bind the kinase domain of p56\(^{Lck}\) (57), raising the possibility that the catalytic region of p56\(^{Lck}\) may recognize certain acidic motifs.

In light of these results, we propose the following scheme for the interaction between CD45-AP and p56\(^{Lck}\) (Fig. 9). The ability of CD4, CD8, and, in all likelihood, CD45 to bind Lck brings the kinase in the vicinity of CD45-AP in unstimulated T-cells. Once activated by engagement of TCR, CD4, or CD8, Lck molecules become more susceptible to bind CD45-AP. This binding may help deliver activated Lck polypeptides to CD45. Given the capacity of CD45 to dephosphorylate the positive regulatory site of Lck (tyrosine 394) and cause its inactivation (11, 12), this could be part of a negative feedback mechanism aimed at limiting TCR signaling. Such a concept was also suggested by the earlier findings that TCR stimulation resulted in an enhancement of the extent of association of CD45 with Lck and that such an increase was absent in T-cells lacking CD45-AP (30). Alternatively, CD45-AP may bring activated p56\(^{Lck}\) into closer proximity to TCR, thereby facilitating ITAM phosphorylation and amplifying TCR signaling. Additional analyses of T-cells from CD45-AP-deficient mice should help distinguish between these two opposing scenarios. Finally, it is plausible that CD45-AP has a direct impact on the kinase activity and/or substrate specificity of Lck via steric hindrance or conformational modification. However, this notion seems less likely because we found that CD45-AP expression alone...
had little effect on the activity of Y505F Lck in transfected fibroblasts or COS-1 cells.  

In summary, the results presented in this manuscript show that CD45-AP is part of a multimolecular complex that includes the TCR complex, CD4, CD8, CD45, and p56lck. These associations are mediated at least in part through the ability of CD45-AP to associate with CD45 via its transmembrane domain and with activated Lck molecules via its cytoplasmic region. Even though additional studies are necessary to determine the precise role of CD45-AP in this complex, our data are consistent with the notion that it is an adaptor or chaperone-like molecule coordinating interactions among components of the antigen receptor signaling machinery.

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