CD45, a leukocyte-specific transmembrane protein tyrosine phosphatase, is required for critical signal transduction pathways in immune responses. To elucidate the molecular interactions of CD45 with other proteins involved in CD45-mediated signal transduction pathways, we have recently cloned a 30-kDa phosphorylated protein, CD45-AP, which specifically associates with CD45. Binding analysis employing several deleted or chimeric forms of CD45-AP and CD45 demonstrated that the potential transmembrane segment of CD45-AP bound to the transmembrane portion of CD45. CD45-AP was found in particulate fractions of lymphocytes along with CD45, indicating that it is likely to be a transmembrane protein. In addition, CD45-AP was resistant to proteolysis by tosylphenylalanyl chloromethyl ketone-treated trypsin applied to intact cells. This is consistent with the most likely membrane orientation of CD45-AP predicted from the amino acid sequence, that is, only a short amino-terminal segment of CD45-AP is extracellular. The cytoplasmic portion of CD45-AP specifically interacts with CD45. In the present study, we propose that CD45-AP interacts with CD45 at the plasma membrane and that the bulk of CD45-AP located in the cytoplasmic domain of CD45 mediates signal transduction pathways.

CD45 plays a critical role in signal transduction pathways essential for immune responses (1). The intracellular domain of CD45 exhibits protein tyrosine phosphatase activity (2) and differential usage of extracellular NH2-terminal exons results in several isoforms of CD45 which are variably expressed in a cell lineage- and developmental stage-specific manner (1, 3). It has been proposed that CD45 activates src family protein tyrosine kinases associated with the T cell receptor signaling complex, p56lck (4, 5) and p59fyn (6, 7), by dephosphorylating their down-regulatory tyrosyl residues. Thus, CD45 appears to be required at the onset of the T cell receptor-mediated signal transduction pathway. CD45 appears to play a similar role in B lymphocytes in combination with the src protein tyrosine kinases (8). Furthermore, CD45 is required for signal transduction pathways of monocytes (9), natural killer cells (10), and mast cells (11). Physical associations between CD45 and various membrane proteins involved in T cell activation, such as CD2 (12), CD4 (13, 14), CD8 (13, 14), Thy-1 (15), T cell receptor (14), CD26 (16), and LFA-1 (14), as well as components of the B cell antigen receptor complex (17), have been documented under certain circumstances. It is not clear how specifically these proteins interact with CD45 and what their binding sites are. A cytoskeletal protein, fodrin, has been reported to interact with the cytoplasmic portion of CD45 and stimulate its protein tyrosine phosphatase activity (18). In addition, CD45 has been reported to be one of surface glycoproteins to which CD22, the B lineage-specific cell surface glycoprotein and a sialic acid-binding lectin, binds (19).

We have recently reported the molecular cloning of a novel 30-kDa phosphorylated protein, CD45-AP, which is specifically associated with CD45 (20). CD45-AP shares no homology with previously known sequences and appears to be expressed specifically in leukocytes. The association of CD45-AP with CD45 has been observed in various types of lymphocytes (21), indicating that the association involves the isoformically invariant portion of CD45. No tyrosine phosphorylation of CD45-AP has been detected either in vivo or in vitro, and the predicted sequence contains no consensus tyrosine phosphorylation sites (20), indicating that it is not a substrate for protein tyrosine kinases. In addition, the predicted sequence of CD45-AP does not contain conserved sequences of GTP-binding proteins. Given the importance and complexity of CD45-mediated signal transduction, it is essential to determine how this novel protein, CD45-AP, interacts with CD45. In the present study, we characterize the interaction between CD45 and CD45-AP and summarize our findings in a model.

**EXPERIMENTAL PROCEDURES**

Rabbit Antiserum—Antiserum against CD45-AP were raised by immunizing rabbits with CD45-AP (20) isolated from YAC-1 cells, a murine T cell line (22), as well as with a recombinant glutathione S-transferase (GST)-CD45-AP fusion protein (described below). Rabbit antiserum to the cytoplasmic domain of CD45 (23) was a generous gift from Dr. H. Ostergaard. Rabbit antiserum which recognizes a common segment present in the extracellular portion of all CD45 isoforms (24) was a generous gift from Dr. J. Marth.

Cell Culture and Radiolabeling—YAC-1 cells were cultured as described before (20). For metabolic labeling with amino acids, cells were cultured overnight at 1.2 × 106 cells/ml with 2 μCi/ml of an L-14C-labeled amino acid mixture (52 mCi/mmol of carbon, Amersham Corp.) as described (20).

Construction of Deleted Forms of CD45-AP cDNA—CD45-AP cDNA was subcloned into pGEM-3Z plasmid (Promega) downstream from the T7 RNA polymerase promoter using the EcoRI site, and two deleted forms, “N” and “C”, of the cDNA were derived from it. The N form contains a termination codon at amino acid position 75 and the region 3′ to the new termination codon is eliminated. The C form starts from amino acid position 47, and the region 5′ to the new initiation codon is

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†To whom correspondence should be addressed: Dept. of Pathology, Roger Williams Medical Center-Brown University, 825 Chalkstone Ave., Providence, RI 02908. Tel.: 401-456-6557; Fax: 401-456-6569.

‡From the Department of Pathology, Roger Williams Medical Center-Brown University, Providence, Rhode Island 02908 and the Department of Microbiology & Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.

¶The abbreviations used are: GST, glutathione S-transferase; BRIJ 96, polyoxyethylene 10 oleyl ether; CD45-AP, CD45-associated protein; mAb, monoclonal antibody; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; TPCK, tosylphenylalanyl chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis.
eliminated. For construction of the pN1 form, the sequence from nucleotide positions 67 to 158 was amplified from the full-length CD45-AP cDNA by the polymerase chain reaction (PCR) and was cloned in pGEM-3Z. Therefore, the pN1 form contains a segment which corresponds to amino acid positions 1–24. Likewise, the pN2 form was constructed by amplifying the sequence from nucleotide positions 67 to 241 which encodes amino acid positions 1–52. A schematic drawing of each construct is shown in Fig. 1.

Binding Assay of in Vitro Translated CD45-AP—In vitro transcription and translation were carried out with the TNT™ T7-coupled reticulocyte lysate system lacking cysteine (Promega) with the addition of Tris-5' label™ (1076 Ci/mmol, ICN Biochemicals), CD45 immunocomplexes formed by incubating a YAC-3 cell lysate in 0.1 M Mops, 0.1 M MgCl₂, 0.1 M EGTA, 2.5 mM thiglycolic acid, and 1 mM PMSF, 2 μg/ml chymostatin, 2 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor were incubated with various cell lysates, CD45 purified from YAC-1 cells, or purified recombinant cytoplasmic CD45 at 37 °C for 1 h for binding. After extensive washing, material bound to the beads was analyzed by SDS-PAGE followed by autoradiography. Western blotting was carried out with a combination of rabbit anti-CD45 and horseradish peroxidase-conjugated Protein A or a combination of rat mAb and horseradish peroxidase-conjugated anti-rat 1g antibody using the ECL Western blotting system (Amersham Corp.).

Subcellular Fractionation of Lymphocytes and Localization of CD45 and CD45-AP—YAC-3 cells were washed and suspended at 4 × 10⁶ cells/ml in cold hypotonic buffer consisting of 25 mM HEPES-NaOH, pH 7.4, 5 mM KCl, 1 mM MgCl₂, 2.5 mM thiglycolic acid, and 1 mM PMSF and were disrupted with a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 200 × g for 10 min at 4 °C, and the 200 × g pellet (nuclear fraction) was resuspended in the hypotonic buffer supplemented with 0.8% BRIJ 96 and 150 mM NaCl. Insoluble material was removed by a brief centrifugation in a microcentrifuge and the final volume of the fraction was adjusted to be equal to the volume of the original cell suspension. The 200 × g supernatant was centrifuged at 100,000 × g for 1 h at 4 °C, and 0.8% BRIJ 96 and 150 mM NaCl were added to the 100,000 × g supernatant (cytoplasmic fraction). The 100,000 × g pellet (microsomal fraction) was resuspended in gradient buffer consisting of 25 mM HEPES-NaOH, pH 7.4, 5 mM KCl, 5 mM NaCl, 0.1 mM EDTA, 2.5 mM thiglycolic acid, and 1 mM PMSF and was overlaid on a discontinuous sucrose gradient of 20, 35, 40, and 50% sucrose in gradient buffer. After centrifugation at 200,000 × g for 2 h at 4 °C (40,000 rpm in an SW40 swinging bucket rotor), material at the interfaces was collected into three fractions, i.e. M1 (the pooled interfaces of 0% and 20%), M2 (the 35%–40% interface), and M3 (the 40%/50% interface) (35). Each fraction was diluted in gradient buffer without sucrose and was centrifuged at 100,000 × g for 40 min at 4 °C. Each pellet was then resuspended in the same manner as described above for the nuclear fraction. A portion of each fraction was subjected to SDS-PAGE for Western blotting with antisera against CD45 and CD45-AP and horseradish peroxidase-conjugated Protein A. Another portion of the same fraction was immunoprecipitated by anti-CD45 mAb (25), and the immunoprecipitates were analyzed by SDS-PAGE.

TPCK-Trypsin Treatment of Lysates and Cells—YAC-1 cell lysates (10 × 10⁶ cells/ml lysis buffer) prepared in Hank’s balanced salt solution containing 0.8% BRIJ 96, 25 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, and 2.5 mM thiglycolic acid or intact cells (10 × 10⁶ cells/ml) suspended in Hank’s balanced salt solution containing 25 mM HEPES-NaOH, pH 7.4, and 1 mM EDTA were treated without or with TPCK-treated trypsin (10 μg/ml, specific activity 12,200 units/mg of protein) for 1 h. After the incubation, PMSF and trypsin inhibitor were added to the lysate samples to final concentrations of 2 μg/ml each, respectively. The cell samples were washed once in the presence of 0.1% fetal calf serum and 20 μg/ml TPCK-trypsin inhibitor (followed by 0.8% BRIJ 96 containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 2.5 mM thiglycolic acid, 1 mM PMSF, 2 μg/ml chymostatin, 2 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor were incubated with various cell lysates, CD45 purified from YAC-1 cells, or purified recombinant cytoplasmic CD45 at 37 °C for 1 h for binding. After extensive washing, material bound to the beads was analyzed by SDS-PAGE followed by autoradiography.

RESULTS

Binding of Various Deleted Forms of in Vitro Translated CD45-AP to CD45—In a previous report, we have shown that in vitro translated CD45-AP bound specifically to CD45 (20). In the present study, the segment of CD45-AP that is responsible for the interaction with CD45 was determined by constructing deleted forms of CD45-AP cDNA and examining the ability of their in vitro translated products to bind to CD45. The in vitro translated full-length (F) or the deleted forms (N or C) of CD45-AP cDNA (Fig. 1) were either analyzed directly in SDS-PAGE or were examined for their ability to bind specifically to CD45 (Fig. 2). As expected, the in vitro translated full-length CD45-AP exhibited specific binding to the CD45 immunocomplex but not to the immunocomplexes of LFA-1 and the trans-

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[2] A. Takeda, unpublished results.
ferrin receptor. The in vitro translated products of the N and C forms migrated at 14 and 22 kDa, respectively. The N form exhibited specific binding to CD45 whereas the C form failed to bind to CD45. These results indicate that interaction between CD45-AP and CD45 requires only a small NH₂-terminal segment of CD45-AP, and the rest of CD45-AP, including most of the consensus phosphorylation sites, is not directly involved in the interaction.

Binding of Various Deleted Forms of Recombinant CD45-AP to CD45—In order to further define the segment of CD45-AP required for binding to CD45, two additional deleted forms of CD45-AP cDNA, designated pN1 and pN2, were prepared by PCR (Fig. 1). The full-length and the various deleted forms of CD45-AP cDNA were then ligated to pGEX-1xT expression vector downstream from GST, and recombinant GST fusion proteins were produced in E. coli. The recombinant proteins bound to equal amounts of glutathione-Sepharose 4B were incubated with lysates of YAC-1 cells metabolically labeled by ¹⁴C-labeled amino acid incorporation. Material bound to the beads was analyzed by SDS-PAGE (Fig. 3). As expected, a prominent band of 180 kDa which comigrates with CD45 immunoprecipitated from YAC-1 cells bound to the beads containing the GST fusion protein of full-length CD45-AP. The 180-kDa protein also bound to the GST fusion proteins of the N and pN2 forms but not to the GST fusion proteins of the C and pN1 forms. These results demonstrate that the potential transmembrane segment of CD45-AP is required for binding to CD45. Binding of Recombinant Cytoplasmic Portion of CD45 to CD45—First, a recombinant cytoplasmic CD45 which lacks the entire extracellular domain and the transmembrane segment was produced in E. coli with six histidine residues fused to its amino terminus and purified as described previously (29). GST fusion proteins of the full-length (F) or the deleted forms (N or C) of CD45-AP bound to glutathione Sepharose 4B beads were incubated with either CD45 isolated from YAC-1 cells or the purified recombinant cytoplasmic CD45 protein. Material bound to the recombinant CD45-AP beads was subjected to SDS-PAGE and analyzed by Western blotting with antiserum against the cytoplasmic portion of CD45 (23) (Fig. 4). CD45 isolated from YAC-1 cells (YAC-1 CD45) bound to the full-length and the N form of CD45-AP, but not to the C form, in agreement with the results described above (Fig. 4A). The recombinant cytoplasmic form of CD45 (Cyto-CD45), however, failed to exhibit any significant amount of binding to any of the recombinant CD45-AP (Fig. 4B). The minute amount of the cytoplasmic CD45 bound to the N and C forms probably reflects nonspecific binding, since a similar amount of binding was also detected with control glutathione beads that did not contain any protein.²

Binding of Recombinant Nocytoplasmic Portion of CD45 to CD45—In order to examine whether the nocytoplasmic portion of CD45 binds to CD45-AP, recombinant forms of CD45 were expressed in L cells. When an anti-CD45 immunoprecipitate was obtained from L106A6 cells which were transfected with the full-length CD45RABC and was analyzed by Western blotting, a 220-kDa protein was detected, as expected, by antiserum which recognizes a common segment in the extracellular domain of all CD45 isoforms (24) (Fig. 5A). A minor band of 200 kDa observed in this sample probably represents a degradation product of the full-length CD45RABC.³ When a similar analysis was carried out for L1285 cells transfected with the nocytoplasmic form of CD45 which consists of the entire extracellular domain of CD45RABC and the transmembrane segment, but lacks most of the cytoplasmic portion, 160-, 125-, and 95-kDa proteins were detected. The 160- and 125-kDa proteins appear to be derived from a single precursor protein,³ and they comigrate as a 115-kDa protein after N-glycanase treatment.² The 95-kDa protein may represent an incompletely translated product or a degradation product of the recombinant nocytoplasmic CD45.

For binding analysis, GST fusion proteins of the full-length

³ A. Maiti and P. Johnson, unpublished results.

![Fig. 1. Deleted forms of CD45-AP cDNA. Various deleted forms of CD45-AP were derived from the full-length (F) cDNA as described under "Experimental Procedures." The potential transmembrane segment is indicated with filled bars. Consensus phosphorylation sites are indicated with arrows. Downward arrows indicate phosphorylation sites favored by more than one enzyme.](image)

![Fig. 2. Binding of in vitro translated deleted forms of CD45-AP to CD45. Full-length (F) and deleted (N and C) forms of CD45-AP cDNA were tested for specific binding to CD45. The first lane of each panel is direct analysis of in vitro translated products by SDS-PAGE. The next three lanes are material bound to immunocomplexes of CD45, LFA-1, and transferrin receptor, respectively. The F and C forms were analyzed in an 8–15% acrylamide gradient and the N form in a 12–19.2% acrylamide gradient, under reducing conditions. The positions of molecular mass markers (expressed in kilodaltons) are shown.](image)

![Fig. 3. Binding of recombinant deleted forms of CD45-AP to CD45. Control glutathione-Sepharose 4B beads with GST alone (−) or beads bound to recombinant GST-fusion proteins of CD45-AP were incubated with lysates of YAC-1 cells metabolically labeled by ¹⁴C-labeled amino acid incorporation. Material bound to the beads was analyzed by SDS-PAGE (Fig. 3). As expected, a prominent band of 180 kDa which comigrates with CD45 immunoprecipitated from YAC-1 cells bound to the beads containing the GST fusion protein of full-length CD45-AP. The 180-kDa protein also bound to the GST fusion proteins of the N and pN2 forms but not to the GST fusion proteins of the C and pN1 forms. These results demonstrate that the potential transmembrane segment of CD45-AP is required for binding to CD45.](image)
CD45 and CD45-AP Interaction

Fig. 4. Binding of recombinant cytoplasmic CD45 to CD45-AP. Panel A, GST fusion proteins of CD45-AP bound to glutathione-Sepharose 4B beads and control beads with GST alone (—) were incubated with CD45 isolated from YAC-1 cells. F, N, and C refer to the full-length and deleted forms shown in Fig. 1. CD45 isolated from YAC-1 cells (YAC-1 CD45) and material bound to equal amounts of beads were subjected to SDS-PAGE in an 8–15% acrylamide gradient under reducing conditions and were analyzed by Western blotting with antiserum against the cytoplasmic portion of CD45. Panel B, GST fusion proteins of CD45-AP bound to glutathione-Sepharose 4B beads and control beads with GST alone (—) were incubated with purified recombinant cytoplasmic CD45. Recombinant cytoplasmic CD45 (Cyto-CD45) and material bound to equal amounts of beads were subjected to SDS-PAGE in an 8–15% acrylamide gradient under reducing conditions and were analyzed by Western blotting with antiserum against the cytoplasmic portion of CD45. The positions of molecular mass markers (expressed in kilodaltons) are shown.

Fig. 5. Binding of recombinant noncytoplasmic CD45 to CD45-AP. Panel A, immunoprecipitates were obtained from untransfected L cells (L40B7), L cells transfected with full-length CD45RABC (L106A6), L cells transfected with the noncytoplasmic portion of CD45RABC (L12B5), or YAC-1 cells using anti-CD45 mAb and were subjected to SDS-PAGE in an 8–15% acrylamide gradient under reducing conditions. They were then analyzed by Western blotting with antiserum against the extracellular domain of CD45. Panel B, full-length CD45-AP GST fusion protein (F) or its deleted forms (N and C, shown in Fig. 1) bound to glutathione-Sepharose 4B beads were incubated with lysate of L40B7, L106A6, L12B5, or YAC-1 cells. Material bound to equal amounts of beads was subjected to SDS-PAGE in an 8–15% acrylamide gradient under reducing conditions and was analyzed by Western blotting with antiserum against the extracellular portion of CD45. The positions of molecular mass markers (expressed in kilodaltons) are shown.

Fig. 6. Binding of the CD45 transmembrane domain to CD45-AP. Panel A, immunoprecipitates were obtained from untransfected L cells (L40B7) or L cells transfected with CD44.TM2 (CD44.TM2) using anti-CD44 mAb and were subjected to SDS-PAGE in an 8–15% acrylamide gradient under nonreducing conditions. They were then analyzed by Western blotting with the same mAb. Panel B, full-length CD45-AP GST fusion protein (F) or its deleted forms (N and C, shown in Fig. 1) bound to glutathione-Sepharose 4B beads were incubated with lysate of L40B7 or the CD44.TM2 transfectant. Material bound to equal amounts of beads was subjected to SDS-PAGE in an 8–15% acrylamide gradient under nonreducing conditions and was analyzed by Western blotting with anti-CD44 mAb. The positions of molecular mass markers (expressed in kilodaltons) are shown.

(F) or the deleted forms (N or C) of CD45-AP bound to glutathione-Sepharose 4B beads were incubated with a cell lysate of untransfected control L cells (L40B7), L106A6, L12B5, or YAC-1 cells. Material bound to the recombinant CD45-AP beads was subjected to SDS-PAGE and was analyzed by Western blotting with antiserum against the extracellular portion of CD45 (Fig. 5B). The full-length as well as the noncytoplasmic form of CD45RABC bound to the full-length and N forms of CD45-AP. This indicates that CD45-AP interacts with the noncytoplasmic portion of CD45.

Binding of the Transmembrane Segment of CD45 to CD45-AP—As described above, the potential transmembrane segment of CD45-AP is required for binding to CD45 (Figs. 2 and 3). Therefore, if CD45-AP is indeed a transmembrane protein, it is most likely that the transmembrane segment of CD45 is directly involved in the interaction with CD45-AP. In order to examine whether the transmembrane portion of CD45 alone is capable of binding to CD45-AP, we utilized an L cell transfectant expressing recombinant chimeric CD44 (CD44.TM2) in which the transmembrane segment of CD45 replaces that of CD44 (34). Immunoprecipitates were obtained from untransfected control L cells (L40B7) and the CD44.TM2 transfectant with an anti-CD44 mAb, KM201 (36), and were analyzed by Western blotting with the same antibody (Fig. 6A). L cells express endogenous CD44 (37), and this was detected as a broad 85-kDa band in the control L cells. A similar band in the transfectant represents both endogenous CD44 and the transfected chimera, since they cannot be discriminated by size.

For binding analysis, GST-fusion proteins of the full-length (F) or the deleted forms (N or C) of CD45-AP bound to glutathione-Sepharose 4B beads were incubated with a cell lysate of the control L cells or the L cells transfected with CD44.TM2. Material bound to the recombinant CD45-AP beads was subjected to SDS-PAGE and was analyzed by Western blotting with the anti-CD44 mAb (Fig. 6B). In the control L cells, endogenous CD44 did not bind to any form of CD45-AP. In the L cells transfected with CD44.TM2, on the other hand, CD44 bound to the full-length and N forms of CD45-AP but not to the C form. This indicates that the transmembrane portion of CD45 is sufficient for specific interaction with CD45-AP.

Subcellular Localization of CD45-AP—The CD45-AP cDNA predicts a stretch of hydrophobic amino acids near the NH₂ terminus that qualifies as a potential transmembrane segment (38). It was of great interest, therefore, to examine in which subcellular fraction CD45-AP is found and whether the CD45-AP localization pattern is similar to that of CD45. Subcellular fractions of YAC-1 cells derived from equal numbers of cells were subjected to SDS-PAGE and Western blotting with antiserum against CD45-AP (Fig. 7A), while another portion of the same fraction was immunoprecipitated by anti-CD44 mAb and the immunoprecipitates were analyzed by SDS-PAGE (Fig. 7B). CD45-AP was found in particular fractions and not at all in the cytoplasmic fraction, indicating that it is likely to be a membrane protein. CD45-AP was found largely in the microsomal fraction and to a lesser extent in the nuclear fraction which would contain nondisrupted cells and cytoskeletal components besides nuclei. When the microsomal fraction was further separated into three subfractions, CD45-AP was found in all three fractions. The two high density fractions, M2 and M3, contained an equal amount of CD45-AP and the low density fraction, M1, contained somewhat less. The distribution of CD45 among all subcellular fractions was quite similar to that
The positions of molecular mass markers (expressed in kilodaltons) are shown.

Blue. The positions of molecular mass markers (expressed in kilodaltons) were subjected to SDS-PAGE in an 8–15% acrylamide gradient under reducing conditions. The SDS-PAGE gel was then stained in Coomassie Blue. The positions of molecular mass markers (expressed in kilodaltons) are shown.

Membrane Orientation of CD45-AP—The results described above indicate that CD45-AP is likely to be a membrane protein and that it interacts with CD45 at the membrane. It is critical to determine the membrane orientation of CD45-AP in order to understand the role of CD45-AP in CD45-mediated signal transduction. YAC-1 cell lysates or intact cells were treated without or with TPCK-treated trypsin and a portion of each sample derived from equal numbers of cells was subjected to SDS-PAGE. The gel was then analyzed by Western blotting with antiserum against CD45-AP (Fig. 8A) or against a peptide segment present in the extracellular domain of CD45 (24) (Fig. 8B). TPCK-trypsin treatment of the lysates digested CD45-AP to a completely undetectable level and cleaved CD45 to a smaller fragment of about 120 kDa.

On the other hand, TPCK-trypsin treatment of intact cells did not alter the amount of CD45-AP detected at all, indicating that no arginine or lysine residue of CD45-AP is located extracellularly or is accessible to TPCK-treated trypsin. The 28-kDa band seen both in the presence and absence of TPCK-treated trypsin (Fig. 8A) was detected in control samples lacking cells as well1 and represented a component present in the fetal calf serum used for washing cells at the end of the incubation. In contrast to CD45-AP, the amount of CD45 detected was significantly reduced after TPCK-trypsin treatment of intact cells due to degradation of the extracellular domain. The 120-kDa fragment is not seen with TPCK-trypsin treatment of intact cells indicating that it probably results from digestion of the cytoplasmic domain of CD45.

In order to confirm that the proteolytic conditions employed above for intact cells were appropriate for degrading other transmembrane proteins as well, a similar experiment was carried out using cells that were radiolabeled by incorporation of 14C-labeled amino acid mixture. The labeled intact cells were treated without or with TPCK-treated trypsin as described above, and immunoprecipitates of LFA-1 (27) or the transferrin receptor (28) were analyzed by SDS-PAGE (Fig. 8C). The TPCK-trypsin treatment dramatically reduced the amounts of LFA-1 (the 180-kDa α chain and the 95-kDa β chain) and the mature form of the transferrin receptors (95 kDa) but not the nascent intracellular form (90 kDa) demonstrating that extracellular proteins were degraded under the proteolytic conditions employed.

**DISCUSSION**

Binding analysis employing several different deleted forms of CD45-AP demonstrated that the potential transmembrane segment of CD45-AP is required for binding to CD45 and that the rest of CD45-AP, including most of the consensus phosphorylation sites, is not directly involved in the interaction (Figs. 2 and 3). Interestingly, binding analysis employing recombinant cytoplasmic or noncytoplasmic forms of CD45, and various recombinant forms of CD45-AP showed that the noncytoplasmic portion of CD45 bound to CD45-AP (Figs. 4 and 5). Furthermore, binding analysis employing a recombinant chimeric transmembrane protein which consists of the extracellular and intracellular domains of CD44 and the transmembrane segment of CD45 demonstrated that the transmembrane segment of CD45 is sufficient for specific interaction with CD45-AP (Fig. 6). These data indicate that CD45 and CD45-AP interact at the plasma membrane through their respective transmembrane domains.

Consistent with this notion are the following observations: (i) CD45-AP can associate with various isotypes of CD45 (21), indicating that the interaction involves the isotypically invariant portion of CD45, (ii) the association between CD45-AP and CD45 is disrupted by some nonionic detergents (20), suggesting that the interaction between the two proteins involves hydrophobic segments, and (iii) CD45-AP is found in particulate fractions along with CD45 by subcellular localization studies (Fig. 7), indicating that CD45-AP is likely to be a membrane protein. The recombinant noncytoplasmic and transmembrane forms of CD45 used for binding studies were produced in L cell fibroblasts and the recombinant forms of CD45-AP were purified from E. coli. Therefore, these binding studies establish that the physical association between CD45 and CD45-AP does not require the presence of other leukocyte-specific proteins, such as p56Lck and p59Fyn(T).

When the CD45-AP cDNA was in vitro transcribed and translated with the rabbit reticulocyte lysate system in the absence of microsomal fraction, the product migrated as 30 kDa in SDS-PAGE (Fig. 2). Moreover, the in vitro translated product comigrated with the mature form of CD45-AP obtained from YAC-1 cells (20). In the absence of microsomal fraction, NH2-terminal signal peptides of nascent proteins cannot be cleaved (39). Therefore, it seems unlikely that the predicted sequence of the CD45-AP cDNA contains an NH2-terminal signal peptide for protein translocation because, in that case, the mature form of CD45-AP would be smaller than the in vitro translated product. Instead, CD45-AP is likely to be a single-pass membrane protein with an internal signal peptide that remains as a membrane spanning segment. All three charged amino acid residues in the NH2-terminal side of the potential transmembrane segment of CD45-AP are acidic amino acids. In contrast, the 42-residue peptide segment immediately on the carboxyl side of the potential transmembrane segment contains nine charged residues which are all basic. This charge distribution of the amino acids in the vicinity of the potential signal transmembrane segment of CD45-AP would most likely cause translocation of the amino-terminal end into the endoplasmic reticulum lumen (40, 41), as seen with the β-adrenergic receptor (42) and glycoporphin C (43). As a result, only a short segment at the NH2 terminus of CD45-AP would be located extracellularly, and the bulk of the protein would be intracellular. This is consistent with the results obtained by treating cell lysates and intact cells with TPCK-treated trypsin (Fig. 8A). CD45-AP was completely resistant to proteolysis by TPCK-treated trypsin applied to intact cells but was susceptible when the enzyme was applied to cell lysates. CD45-AP contains 10 arginine and one lysine residues, the TPCK-treated trypsin target sites, and all of them are located in the carboxyl-terminal side of the potential transmembrane segment (20). A proposed model of the physical orientation of CD45-AP is depicted in Fig. 9.
Recently, a cDNA clone of a CD45-AP human homologue has been reported (44). The predicted amino acid sequence of the human homologue has a high percentage of sequence identity with murine CD45-AP. Interestingly, the human homologue differs from murine CD45-AP in two important aspects. (i) The human homologue appears to have an NH₂-terminal signal peptidethatiscleavedtoproducethematureform,and(ii)the human homologue has a high percentage of sequence identity with murine CD45-AP if the human homologue plays a role as a cation, and (ii) why is the tyrosine phosphorylation lacking in murine CD45-AP that also predicts a protein of very high homology with CD45 and other molecules involved in CD45-mediated signal transduction pathways. Proteins that interact with the intracellular portion of CD45-AP may be a substrate or a regulator of CD45 protein tyrosine phosphatase, and such proteins are currently under investigation. Distinct differences in the specific protein tyrosine phosphatase activity of CD45 have been detected among various populations of CD45 separated by sucrose gradient ultracentrifugation with a nondisruptive detergent (21). It is possible that CD45-AP affects the protein tyrosine phosphatase activity of CD45 directly or indirectly since a population of CD45 without associated CD45-AP appeared to have a higher specific activity. This possibility can now be more closely examined by using various forms of recombinant CD45-AP.

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