# Definition of Amino Acids Sufficient for Plasma Membrane Association of CD45 and CD45-Associated Protein†

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ABSTRACT: The transmembrane protein tyrosine phosphatase CD45 functions to activate Src-family member kinase activity in T lymphocytes. The inability to activate $p56^{lck}$ in CD45-deficient cells results in a higher threshold of signaling through the T cell receptor. The lymphoid-specific CD45-associated protein, CD45AP, interacts with CD45 through transmembrane interactions. Cells lines and mice deficient in CD45 express CD45AP mRNA, yet the protein is poorly expressed, indicating that CD45 is required for efficient expression of CD45AP. Pulse−chase analysis indicates that CD45 associates with CD45AP within minutes of biosynthesis. Cell surface labeling and coimmunoprecipitation demonstrate that CD45AP associates with surface-expressed CD45. Therefore, CD45AP is localized to the plasma membrane. To further characterize this interaction, chimeric proteins containing mutations in CD45 transmembrane regions were expressed, and their ability to associate with CD45AP was determined. Alanine-scan mutations of the CD45 transmembrane region demonstrate that no single amino acid is essential for the interaction with CD45AP. However, the expression of chimeric transmembrane regions indicates that a minimum of three and a maximum of eight amino acids in this region are sufficient to allow interaction with CD45AP.

CD45 is a transmembrane protein tyrosine phosphatase (PTPase)[1] expressed by all leukocytes. In T cells, where the function of CD45 has been best defined, CD45 is required for efficient signaling through the T cell antigen receptor (TcR) (Trowbridge & Thomas, 1994). Cell lines deficient in CD45 expression require increased levels of antigen or anti-T cell receptor antibody to induce proliferation and cytokine production (Pingel & Thomas, 1989; Koretzky et al., 1990; Weaver et al., 1991). Mice ablated in CD45 expression by homologous recombination have a profound block in T cell development (Kishihara et al., 1993). Signal transduction through the TcR is mediated through the concerted actions of Src-family kinases and the ZAP-70 tyrosine kinase (Weiss, 1993). In T cells, CD45 activates two members of the Src-family tyrosine kinases, $p56^{lck}$ and $p59^{fyn}$, by dephosphorylating the carboxy-terminal negative tyrosine phosphorylation site (Ostergaard et al., 1989; Cahir McFarland et al., 1993; Sieh et al., 1993). Therefore, the requirement for CD45 in TcR signaling reflects the ability of CD45 to regulate Src-family tyrosine kinases. Interestingly, resting CD45-deficient cells exhibit an 8−10-fold decrease in $p56^{lck}$ tyrosine kinase activity when compared to $p56^{lck}$ activity from resting CD45-expressing cells (Pingel et al., 1994). Hence, CD45 activates $p56^{lck}$ prior to antigen stimulation.

The ability of CD45 to activate $p56^{lck}$ prior to antigenic stimulation suggests that the interaction of CD45 with $p56^{lck}$ is regulated. In support of this idea, a minimal effect on kinase activity is observed when CD45 is coexpressed with $p56^{lck}$ in nonlymphoid cells, suggesting that there are lymphoid-specific factors required for CD45 to regulate $p56^{lck}$ activity. Immune complex kinase reactions of CD45 immunoprecipitates reveal $p56^{lck}$ kinase and another phosphorylated protein of 32 000 $M_r$, named CD45-associated protein (CD45AP) (Schraven et al., 1991, 1992, 1994; Takeda et al., 1994). It has been postulated that CD45AP may link $p56^{lck}$ with CD45. cDNAs for CD45AP have been isolated, and the coding region predicts a protein with a short extracellular domain of 7 amino acids, a transmembrane region, and a cytoplasmic domain of 150 amino acids. The cytoplasmic domain of CD45AP contains a membrane proximal domain with significant similarities to WW domains, a protein module that binds proline-enriched sequences (Cahir McFarland & Thomas, 1995; Sudol, 1996). Interestingly, CD45AP is a lymphoid-specific protein.

Coexpression studies have demonstrated that CD45 and CD45AP directly interact, and this interaction has been mapped to their respective transmembrane regions (Cahir McFarland & Thomas, 1995; Kitamura et al., 1995; Bruyns et al., 1996). Significantly, when coexpressed in HeLa cells, $p56^{lck}$ can only be detected in CD45AP and/or CD45 immunoprecipitates by kinase reaction, but not by immunoblot analysis, suggesting that the interaction is weak. Thus, it is possible that CD45AP links CD45 to other cytoplasmic proteins but not necessarily to $p56^{lck}$. In this regard, it is interesting to note that CD45 has been implicated in the regulation of integrin-mediated adhesion in both lymphoid and nonlymphoid cells (Wagner et al., 1993;

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[1] Abbreviations: PTPase, protein tyrosine phosphatase; TcR, T cell antigen receptor.

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Lorenz et al., 1993; Bernard et al., 1994; Coombe et al., 1994). Since CD45AP expression is limited to lymphocytes, it is possible that CD45AP links CD45 to the regulation of antigen receptors versus the regulation of integrin-mediated adhesion. While the interaction of CD45AP and $p56^{lck}$ may not be direct, it may, nonetheless, be important in linking CD45 to the regulation of $p56^{lck}$.

To further characterize the interaction of CD45 with CD45AP, we have analyzed the temporal and physical requirements for association. Our studies indicate that CD45 associates with CD45AP in the endoplasmic reticulum and that CD45 is required for plasma membrane association of CD45AP. While the transmembrane region of CD45 is required for interaction with CD45AP, no single amino acid is essential. A minimum of three and a maximum of eight amino acids in the context of an exogenous transmembrane region are sufficient for the interaction to occur.

## EXPERIMENTAL PROCEDURES

*Antibodies.* Anti-CD8 monoclonal antibody, 53-6.72, was obtained from the ATCC (TIB 105) (Rockville, MD) or purchased from Pharmigen (San Diego, CA). Anti-CD45 monoclonal antibodies, I3/2.3, 30F11, and M1/9 (TIB) were obtained from Dr. Ian Trowbridge (Salk Institute, San Diego, CA), Dr. Jeff Ledbetter (Bristal Meyers-Squibb), and the ATCC, respectively. Anti-c-*myc* monoclonal antibody, 9E10, was obtained from the ATCC (CRL 1729) (Evan et al., 1985) and recognizes a specific epitope contained within the sequence SMEQKLISEEDLNN. Anti-$p56^{lck}$ monoclonal antibody 3A5 was obtain from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-$p56^{lck}$ antiserum was made to the peptide EVRDPLVYEGSLPPASPLQDN. Rabbit anti-mouse CD45 antisera was generated to recombinant CD45 cytoplasmic domain. Rabbit anti-mouse CD45AP antiserum was made to the peptide AEALLSDLHAFSGSAAWDDSA.

*Cells.* The L3 T cell clone was obtained from Dr. Andy Glasebrook (Eli Lilly, Indianapolis, IN) and is alloreactive to H2-$L^d$ (Glasebrook & Fitch, 1980). The derivation of the CD45-deficient L3M-93 T cell clone has been previously described (Weaver et al., 1991). The L3.H hybridoma was generated by fusing the L3 T cell clone with a $\alpha^-\beta^-$ BW5147 thymoma line using poly(ethylene glycol). A $CD45^-$ variant of the fusion partner, $\alpha^-\beta^-$ BW5147, was generated by mutagenesis with 7.5 mg/mL *N*-nitrosoguanidine (Sigma, St. Louis, MO), and $CD45^-$ cells were selected by treatment with anti-CD45 mAb and complement. The fusion partner was deficient for both CD45 protein and mRNA (data not shown). The $\alpha^-\beta^-$, $CD45^-$ BW5147 cell line was fused with the $CD45^-$ L3M93 T cell clone to generate L3M93H. This hybridoma was deficient in both CD45 mRNA and protein.

*cDNA Reagents.* CD8α cDNA was a gift from Dr. Hiromitsu Nakauchi (RIKEN, Tsukuba-City, Japan). A cDNA for CD44 isolated from CEM-1 T cells was provided by Barton Haynes (Duke University, Durham, NC). CD45 and CD45AP cDNAs have been previously described (Cahir McFarland & Thomas, 1995).

*Pulse−Chase Analysis.* L3H cells, $1.5 \times 10^8$, were incubated in DMEM without cysteine and methionine for 1 h, media were removed, and 1.2 mCi of [$^{35}$S]-translabel in 5 mL of media added (Amersham, Arlington Heights, IL) for 5 min, washed once in cold PBS, and resuspended in complete media for the indicated times. Cells, $2.5 \times 10^7$, were pelleted and lysed in 2 mL of lysis buffer (0.9% Brij 58, 0.1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $Na_3VO_4$, 10 mM NaF, 2 mM PMSF, 10 mg/mL leupeptin, and 50 mg/mL aprotinin). Lysates were equally divided for immunoprecipitations for CD45AP and CD45. Immunoprecipitation was as described below. After the immunoprecipitates were washed twice with lysis buffer, endoglycosidase H digestion was performed as described by manufacturer (New England Biolabs, Beverly, MA). Reactions were incubated overnight at 37 °C, sample buffer was added, and the proteins were resolved on a 7% SDS−polyacrylamide gel.

*Surface Biotinylation.* L3H cells, $6.5 \times 10^7$, were washed twice with PBS at 4 °C and resuspended in 1 mL of ice-cold PBS. Sulfo-NHS-biotin (Pierce, Rockford, IL), 500 mg in 50 mL of PBS, was added. The cells were incubated for 5 min, washed once in PBS containing 20 mM glycine, pH 8, separated on a Ficol Hypaque gradient, and washed twice with PBS−glycine before being resuspended in lysis buffer. Proteins were immunoprecipitated and resolved by SDS−PAGE, and immunoblot analysis was performed using strepavidin-conjugated horseradish peroxidase or alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN). Proteins were visualized by enhanced chemiluminesence (Amersham).

*Construction of Chimeric cDNAs.* A panel of chimeric cDNAs were generated by using PCR products derived by site overlap extension. Each PCR product contained unique restriction endonuclease sites for directional ligation behind the T7 promoter in the Bluescript vector (Stratagene, San Diego CA). CD8/8* and CD8/45* represent proteins that terminate carboxy terminal to the transmembrane region and have been previously described (Cahir McFarland & Thomas, 1995). Briefly, restriction sites were introduced by site overlap extension 5′ and 3′ to the region encoding the CD45 transmembrane domain. The 3′ oligonucleotide introduced a stop codon after the 14th amino acid of the cytoplasmic domain. The region encoding the extracellular domain of CD8 was generated by PCR to contain the appropriate restriction sites and was used to replace the region encoding the extracellular domain of CD45. This construct was termed CD8/CD45*. The cDNA encoding the CD8 transmembrane region and eight amino acids of the cytoplasmic domain was generated by PCR and used to replace the region encoding the CD45 transmembrane region. This construct was termed CD8/CD8*. The construct, TM1, which encodes the CD8 extracellular domain, the 22 amino acid CD45 transmembrane region, and 8 amino acids of the intracellular domain of CD8, was created by overlapping 4 oligonucleotides spanning the transmembrane region, single-stranded regions repaired with Klenow, and the fragment used to replace the transmembrane region of CD8/CD8*. In the process, restriction sites were added over the length of the transmembrane region to facilitate oligonucleotide mutagenesis. For the subsequent alanine scan, insert and frame-shift mutants were constructed by using oligonucleotide replacement in TM1. The CD44 chimeric proteins were constructed by subcloning oligonucleotides in a similar manner. Oligonucleotides that correspond to the transmembrane domain of CD44 were generated and subcloned into the unique restriction enzyme sites flanking the region encoding the CD45 transmembrane

domain within TM1. Constructs were verified by sequence analysis.

*Expression of Chimeric Proteins.* HeLa cells were plated at $5 \times 10^5$ cells/well in 6 well plates at 24 h prior to transfection. Cells were washed twice with DMEM containing penicillin, streptomycin, and 2 mM L-glutamine. Recombinant vaccinia virus expressing the T7 RNA polymerase (Fuerst et al., 1986) was added at a multiplicity of infection of 10:1 for 45 min at 37 °C in 1 mL of DMEM. A maximum amount of 5 mg of DNA was resuspended in a volume of 500 mL of DMEM and vigorously mixed, and 15 mL of Lipofectase (GIBCO BRL, Grand Island, NY) was added. The DNA mixture was added dropwise to the cells. After incubation for 12−14 h, the cells were resuspended in lysis buffer. Alternately, cells were removed from the plate by treating with PBS containing 50 mM EDTA. A portion of the cells was used for flow cytometry analysis to verify expression of the CD8 chimeras, and the remainder was resuspended in lysis buffer.

*Immunoprecipitation.* One milliliter of lysate was precleared with 10 mL of a 10% solution of Pansorbin (CalBiochem, La Jolla, CA). Appropriate antibodies, 1 mL, were added with 30 mL of a 50% slurry of either protein G− or protein A−Sepharose. Lysates were rotated at 4 °C for 2 h; immunoprecipitates was collected, washed once with lysis buffer, and then boiled in 20 mL of SDS−PAGE sample buffer containing 2-mercaptoethanol.

*Immunoblot Analysis.* Immunoprecipitates were resolved on either a 10% or a 12% SDS−polyacrylamide gel, transferred to nitrocellulose, and blocked in PBS containing 0.05% Tween-20 (PBS-T), 2% bovine serum albumin, and 1% non-fat dry milk. Antibodies were used at a 1:500 dilution in PBS-T and incubated with the membranes for 1 h. Following the primary antibody, the membranes were washed 3 times with PBS-T for 5 min and developed with either horseradish peroxidase conjugated protein A or anti-mouse IgG for 30 min, and then washed 3 times with PBS-T. Proteins were visualized using the Enhanced Chemiluminescence Kit (Amersham).

## RESULTS

*CD45 Is Required for CD45AP Expression.* It has been previously demonstrated that the expression of CD45 is required for expression of CD45AP in a CD45-deficient variant of the thymoma, Jurkat (Schraven et al., 1994). While CD45AP mRNA is present, no protein can be detected in the absence of CD45 expression. To confirm and extend this finding, we examined both the CD45-deficient T cell clone, L3M-93, and thymocytes from mice deficient in CD45 expression. Whereas CD45AP mRNA could be detected in L3M-93, the protein was undetectable by immunoblot analysis (Figure 1A). Using thymocytes from CD45-deficient mice (Kishihara et al., 1993), a small amount of CD45AP could be detected by immunoprecipitation. Thus, CD45AP is expressed in CD45-deficient thymocytes, but at greatly reduced levels (Figure 1B). Importantly, mice deficient in CD45 due to gene targeting are also deficient in CD45AP. These data are consistent with the greatly reduced expression of CD45AP in CD45-deficient cell lines. The small amount of CD45AP present in the immunoprecipitate could conceivably be associated with another protein.

*Endoplasmic Reticulum Association of CD45 and CD45AP.* To examine the subcellular compartment in which the

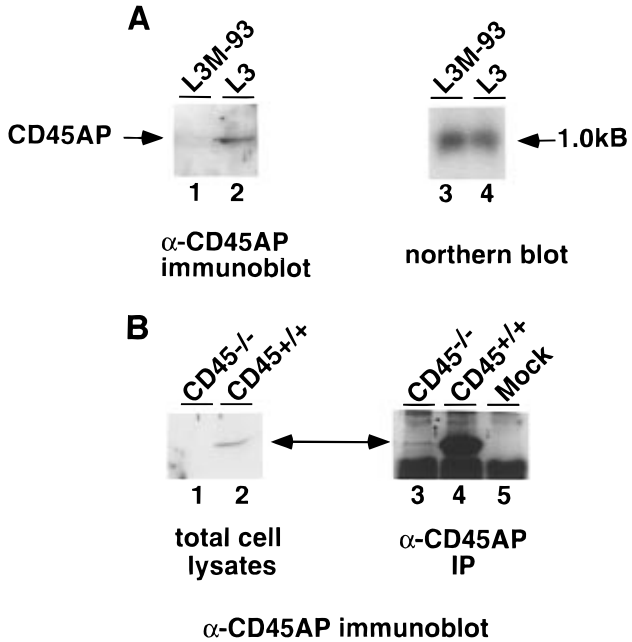



FIGURE 1: CD45AP expression is dependent on CD45 expression. (A) Immunoblot and Northern blot analyses of CD45AP expression. Total cell lysates, $3 \times 10^5$ cell equiv/lane, were separated on an 8% SDS−polyacrylamide gel. Proteins were transferred to membranes and probed with anti-CD45AP antisera. Lane 1, CD45-deficient L3M93H T cell hybridoma; lane 2, L3.H T cell hybridoma. Total RNA, 5 mg/lane, was separated on a formaldehyde−agarose gel, transferred to membrane, and hybridized with radiolabled CD45AP cDNA probe. Lane 3, L3M93H; lane 4, L3.H. (B) Immunoblot analysis of CD45AP expression in CD45-deficient thymocytes. Total cell lysates, $3 \times 10^5$ cell equiv/lane, from either CD45-deficient, lane 1, or wild-type thymocytes, lane 2; or CD45AP immunoprecipitates, $4 \times 10^7$ thymocytes/lane, from CD45-deficient, lane 3, wild type, lane 4, or antibody alone, lane 5, were immunoblotted for CD45AP. Immunoprecipitation with preimmune serum gave similar results to that shown in lane 5 (data not shown).

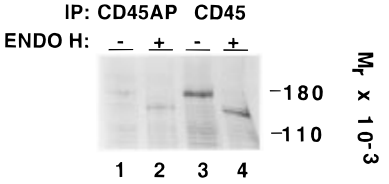



FIGURE 2: CD45AP associates with endoglycosidase H-sensitive CD45. Cells, $2.5 \times 10^7$, were metabolically labeled for 5 min with [$^{35}$S]methionine/cysteine, washed, and chased with complete media containing nonradioactive methionine for 10 min. Cells were immunoprecipitated for CD45AP, lanes 1 and 2, or CD45, lanes 3 and 4. Immunoprecipitates were digested with, lanes 2 and 4, or without endoglycosidase H, lanes 1 and 3, for 16 h.

interaction between CD45 and CD45AP first occurs, pulse−chase analysis was performed. CD45AP and CD45 were immunoprecipitated after metabolic labeling of a cytotoxic T cell hybridoma, L3.H, for 5 min, followed by incubation in the absence of radioactivity for various lengths of time. CD45AP coimmunoprecipitates with CD45 at all time points measured, including a 60 min cold chase (data not shown). Therefore, CD45 associates with CD45AP early in biosynthesis, suggesting an association in the endoplasmic reticulum. After a chase for 10 min in the absence of radioactivity, the CD45 that coimmunoprecipitates with CD45AP is endoglycosidase H-sensitive (Figure 2), indicating that CD45AP interacts with CD45 in a pre-Golgi compartment.

*Association of CD45 and CD45AP at the Plasma Membrane.* To determine whether CD45 is associated with

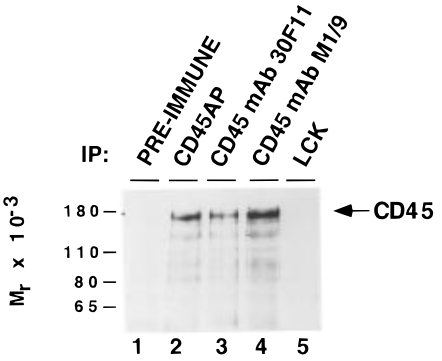
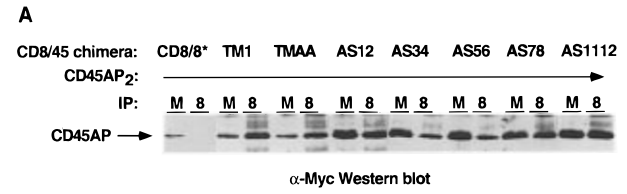



FIGURE 3: CD45AP associates with CD45 at the plasma membrane. L3.H T cell hybridoma cells were surface-biotinylated and lysed, and immunoprecipitations were performed on $1 \times 10^7$ cell equiv/lane with either preimmune sera, lane 1; anti-CD45AP sera, lane 2; anti-CD45 mAb, 30F11, lane 3; anti-CD45 mAb, M1/9, lane 4; or anti-$p56^{lck}$ mAb, lane 5.

CD45AP at the plasma membrane, L3.H cells were biotinylated, and either CD45AP or CD45 was immunoprecipitated. Probing the immunoprecipitate with streptavadin demonstrated that the protein that migrated coincident with CD45 was coimmunoprecipitated with CD45AP (Figure 3, lane 2). Furthermore, this protein was biotinylated, indicating that CD45AP is a plasma membrane protein. Significantly, $p56^{lck}$ was not biotinylated, demonstrating that only cell surface proteins had been labeled (Figure 3, lane 5).

*No Single CD45 Transmembrane Amino Acid Is Necessary for Association with CD45AP*. We and others have demonstrated that CD45 associates with CD45AP through the transmembrane regions. Our previous analysis demonstrated that a 39 amino acid region encompassing the CD45 transmembrane region was both necessary and sufficient to interact with CD45AP. To determine if residues that flanked the transmembrane domain were important for the interaction with CD45, a chimeric protein, TM1, consisting of the CD8 extracellular domain, the 22 amino acid CD45 transmembrane domain, and 8 amino acids of the CD8 cytoplasmic domain was coexpressed with CD45AP that had been appended with an epitope-tag derived from c-*myc*. The expression level of CD8/CD45 chimeric proteins was assessed by flow cytometry and varied between 2- and 3-fold between different transfections (data not shown). As assessed by immunoprecipitation and immunoblot analysis of the epitope-tagged protein, the level of CD45AP expression varied between 2- and 5-fold between various transfections (Figures 4−7). It should be noted, though, that vaccinia-driven expression results in vast overexpression of the protein (Fuerst et al., 1987) and, therefore, the expressed protein is in excess. While expression levels varied between experiments, the data shown are representative of three or more independent experiments. Immunoprecipitation with anti-CD8 monoclonal antibody demonstrated the association of TM1 with CD45AP, indicating that the 22 amino acid CD45 transmembrane domain is sufficient for the interaction with CD45AP (Figure 4A). To further define amino acids critical for CD45/CD45AP association, we performed an alanine scan of the CD45 transmembrane region. Blocks of 4−5 amino acids within TM1 were mutated to alanine, and the chimeric proteins were expressed with CD45AP in HeLa cells (Figure 4B). Immunoprecipitation with anti-CD8 monoclonal antibody demonstrated that all mutations associated with CD45AP (Figure 4A). Therefore, no single




B

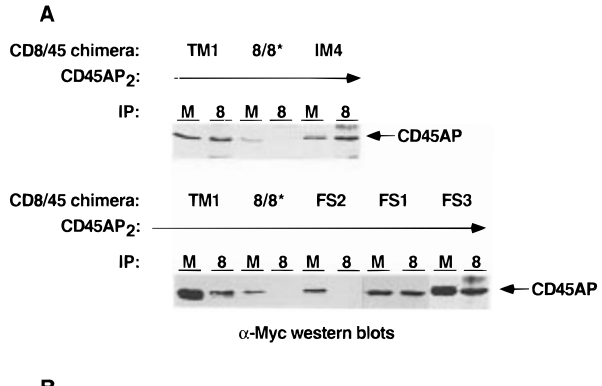
| | | BINDING |
|---|---|---|
| CD8/8/* | TSLDFACD IYIWAPLAGICVAPLLSLIITLI | CYRRHTID - |
| CD8/45/* | TSNFNAK ALIIFLVFLIIVTSIALLVVLY | KIYDLRKKRSSNID + |
| CD45 TM1 | TSLDFACD ALIIFLVFLIIVTSIALLVVLY | CYRRHTID + |
| TMAA | TSLDFACD ALIIFLVFLIIV**AA**IALLVVLY | CYRRHTID + |
| AS 1,2 | TSLDFACD A**AAAA**LVFLIIVTSIALLVVLY | CYRRHTID + |
| AS 3,4 | TSLDFACD ALII**AAAAA**IIVTSIALLVVLY | CYRRHTID + |
| AS 5,6 | TSLDFACD ALIIFLVF**AAAAA**SIALLVVLY | CYRRHTID + |
| AS 7,8 | TSLDFACD ALIIFLVFLIV**AAAL**LLVVLY | CYRRHTID + |
| AS 11,12 | TSLDFACD ALIIFLVFLIIVTSIALL**AAAA** | CYRRHTID + |

FIGURE 4: No single amino acid within the CD45 transmembrane region is essential for binding to CD45AP. (A) Alanine scan mutations of the CD45 transmembrane region were expressed with CD45AP that had been appended with an epitope-tag derived from c-myc in HeLa cells. All the chimeras use the CD8 extracellular domain. $CD45AP_2$ is the full-length CD45AP containing an epitope-tag derived from c-myc at the carboxy terminus and has been previously described (Cahir McFarland & Thomas, 1995). Cells were lysed, and immunoprecipitation was performed with either anti-c-myc mAb, 9E10 (M), or anti-CD8 mAb (8). Immunoprecipitation with anti-c-myc serves as a control for protein expression. Anti-CD8 immunoprecipitation determines the association between the chimeric proteins and CD45AP. CD45AP was visualized by immunoblot analysis developed with anti-c-myc mAb using enhanced chemiluminescence. Exposure times were less than 30 s. The data shown are representative of three or more independent experiments. The band representing CD45AP is indicated. (B) Sequences of the chimeric protein transmembrane region and summary of association with CD45AP.

amino acid is essential for the association of CD45 with CD45AP.

*Identification of the CD45 Transmembrane Region Sufficient for Association with CD45AP*. To examine whether multiple contacts within the transmembrane region are required for CD45 to associate with CD45AP, two isoleucine residues were inserted in the middle of the CD45 transmembrane region. It is likely that this mutation, IM4, will alter the relative orientation of amino acids within the transmembrane region (Figure 5B). If the transmembrane region passes through the membrane as an α helix, the insertion of two amino acids within the transmembrane region should shift half of the face of the α helix relative to the other half. The chimeric protein IM4 retained the ability to associate with CD45AP (Figure 5A). Thus, it is likely that the position of the entire transmembrane amino acids relative to each other is not critical for CD45 to associate with CD45AP.

To determine whether the amino-terminal portion of the CD45 transmembrane region is important for the association with CD45AP, frameshift and deletion mutants were examined. Two frameshift mutations that alter the sequence of the last five residues, FS1 and FS3, were generated (Figure 5B). Neither mutation altered the ability to interact with CD45AP, demonstrating that substitution of the carboxy-terminal residues is permitted (Figure 5A). However, a frameshift mutation, FS2, that introduces two glutamine residues within the CD45 transmembrane region failed to associate with CD45AP (Figure 5A). This indicates that the CD45−CD45AP interaction cannot tolerate polar residues in the CD45 transmembrane region. Importantly, as determined by flow cytometric analysis, all of the CD45 transmembrane mutations were expressed at the plasma membrane (data not shown).

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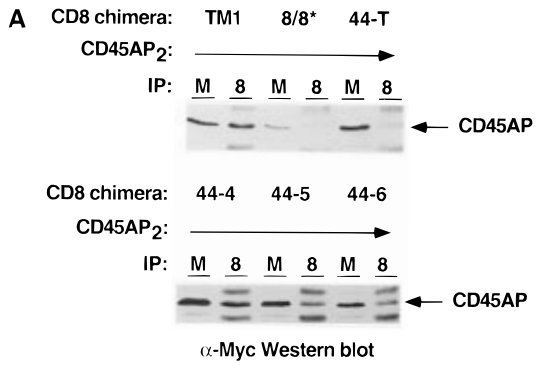
| | | BINDING |
|---|---|---|
| TM1 | ALIIFLVFLIIVTSIA---LLVVLY CYRRHTID | + |
| IM4 | ALIIFLVFLIIVIITSIA-LLVVLY CYRRHTID | + |
| FS1 | ALIIFLVFLIIVTSIA---LCIVIV DTLSIKN | + |
| FS2 | ALIIFLVFLIIVTSIAQLQLCIVIV DTLSIKN | - |
| FS3 | AAAAALVFLIIVTSIACWLFCIVIV DTLSIKN | + |

FIGURE 5: Conservative amino acid substitution within the CD45 transmembrane region allows for association with CD45AP. (A) (Top panel) Insertion of two isoleucine residues does not affect the interaction with CD45AP. The chimeric proteins TM1, 8/8*, or IM4 were expressed with $CD45AP_2$ as described in the Figure 4 legend. Association was determined by coimmunoprecipitation using either anti-c-myc mAb (M) or anti-CD8 mAb (8) and immunoblot analysis with anti-c-myc mAb using enhanced chemiluminescence. Exposure times were less than 30 s. The data shown are representative of three or more independent experiments. (Bottom panel) Frameshift mutations that preserve the hydrophobic nature of the carboxy terminus of the CD45 transmembrane domain interact with CD45AP. Frameshift mutations, FS1, FS2, and FS3 and chimeric proteins, TM1 and 8/8*, were expressed in HeLa cells with $CD45AP_2$, and immunoprecipitation and immunoblot analysis were performed as above. The band representing CD45AP is indicated. (B) Sequences of the chimeric protein transmembrane regions and summary of association with CD45AP. IM4 has two isoleucine residues (boldface underline) inserted into the transmembrane region of CD45. The frameshift mutants are altered after the 16th amino acid, alanine. FS1 and FS2 are deletions of 10 and 4 base pairs, respectively, in the transmembrane region of CD45. FS3 is a deletion of 1 base pair derived from AS12 (described in Figure 4).

To define potential contact amino acids between CD45 and CD45AP, chimeric transmembrane regions were generated utilizing the transmembrane segments of CD44 and CD45 (Figure 6B). The CD44 transmembrane domain does not associate with CD45AP [data not shown and Xiao et al. (1995)]. Successive replacement of the CD45 transmembrane region with residues from CD44 indicates that the first six amino acids of the CD45 transmembrane regions are sufficient to generate the interactions with CD45AP (Figure 6A, compare 44-6 to 44-T). 44-6 and 44-T differ at only three residues in the transmembrane domain: position 1, Ala to Trp; position 5, Phe to Leu; and position 6, Leu to Ala. Since five amino acids are conserved between CD45 and CD44 in this region, the data indicate that the association between CD45 and CD45AP requires a minimum of three amino acids and a maximum of eight amino acids. However, simultaneous mutation of residues 2−5 (LIIF), or 5−9 (FLVFL), to alanines did not diminish binding (Figure 4). Therefore, this region is not essential, but it is sufficient for the interaction with CD45AP. While there may be other sites of contact between CD45 and CD45AP, the contact does not require the conservation of the entire transmembrane sequence.

Since position 1 of the CD44 transmembrane domain is a large bulky hydrophobic residue, tryptophan, it was possible

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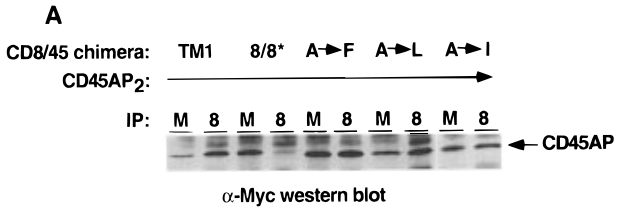
| | 123456789012345678901 2 | BINDING |
|---|---|---|
| TM1 | ALIIFLVFLIIVTSIALLVVLY | + |
| 44-4 | ALIIFLVFLIIVTSIAVCIAVN | + |
| 44-5 | ALIIFLVFLIIALILAVCIAVN | + |
| 44-6 | ALIIFLSLLALALILAVCIAVN | + |
| 44-T | WLIILASLLALALILAVCIAVN | - |

FIGURE 6: The six most amino-terminal amino acids of the CD45 transmembrane region are sufficient to induce the interaction with CD45AP. (A) Coexpression of chimeric proteins containing part of the CD45 and CD44 transmembrane regions associate with CD45AP. All the chimeras use the extracellular domain of CD8. Chimeric proteins TM1, 44-4, 44-5, 44-6, and 44-T were expressed in HeLa cells with $CD45AP_2$. Cells were lysed, and immunoprecipitation was performed with either anti-c-myc mAb (M) or anti-CD8 mAb (8). Immunoblots were developed with anti-c-myc mAb, 9E10, using enhanced chemiluminescence. Exposure times were less than 30 s. The data shown are representative of three or more independent experiments. CD45AP is indicated. (B) Sequences of the chimeric protein transmembrane region and summary of association with CD45AP. CD44 transmembrane sequences are underlined. Sequence differences between 44-6 and 44-T are underlined in boldface.




B

| | | BINDING |
|---|---|---|
| TM1 | ALIIFLVFLIIVTSIALLVVLY | + |
| A→F | FLIIFLVFLIIVTSIALLVVLY | + |
| A→L | LLIIFLVFLIIVTSIALLVVLY | + |
| A→I | ILIIFLVFLIIVTSIALLVVLY | + |

FIGURE 7: Alanine at position 1 of the CD45 transmembrane domain is not required for association with CD45AP. (A) Mutation at position 1 to bulky hydrophobic amino acids does not prevent association with CD45AP. All the chimeras use the CD8 extracellular domain. Chimeric proteins, TM1, 8/8*, A→F, A→L, and A→I were expressed in HeLa cells with $CD45AP_2$. Cells were lysed, and immunoprecipitation was performed with either anti-c-myc mAb (M) or anti-CD8 mAb. Immunoblots were developed with anti-c-myc mAb, 9E10, using enhanced chemiluminescence. Exposure times were less than 30 s. The data shown are representative of three or more independent experiments. CD45AP is indicated. (B) Sequences of the chimeric protein transmembrane region and summary of association with CD45AP.

that the presence of this residue prevented the association with CD45AP. To address this issue, alanine at position 1 of the CD45TM was mutated to phenylalanine, leucine, or isoleucine (Figure 7B). These mutants retained the ability to interact with CD45AP (Figure 7A). Therefore, this residue may contribute to the specificity of the interaction with

CD45AP as demonstrated by the ability of 44-6 to interact; however, it is not essential.

## DISCUSSION

CD45 is required for stable expression of CD45AP. This suggests that the function of CD45AP is intimately linked to that of CD45. The biosynthesis of CD45 is complex in that multiple isoforms are expressed in single cells, due to the use of alternatively spliced exons, and extensive post-translational modification occurs in the form of both N- and O-linked glycosylation. A potential function for CD45AP is to serve as a chaperone. However, the observation that CD45AP exists at the cell surface would argue against a strict chaperone function. Alternatively, CD45AP may function to regulate signal transduction through CD45. In our hands, however, the direct association between CD45AP and $p56^{lck}$ is weak. Thus, other proteins besides CD45AP may be required to regulate CD45 and $p56^{lck}$ interactions.

Two types of transmembrane complexes assemble via transmembrane interactions; those that function as chaperones or those that function as signaling complexes at the cell surface. For example, calnexin, a molecular chaperone, retains MHC class I molecules in the endoplasmic reticulum (ER) through a transmembrane interaction (Margolese et al., 1993; Rajagopalan & Brenner, 1994; Jackson et al., 1994). However, after assembly of MHC class I molecules with $\beta_2$-microglobulin and peptides, class I and calnexin are never found together at the cell surface. In contrast to calnexin/MHC interactions, the multisubunit antigen receptors assemble via transmembrane interactions in the ER and transport together to the plasma membrane. The absence of a single subunit prevents the transport of the remaining receptor. Therefore, transmembrane interactions can direct whether the complexes are either retained within the ER or allowed to be further processed and trafficked to the plasma membrane (Sussman et al., 1988; Suzuki et al., 1991; Rutledge et al., 1992; Reth et al., 1991). Here we have presented evidence that CD45 and CD45AP associate within the ER. It is likely that in cells deficient in CD45 expression, CD45AP is retained in the ER and degraded, resulting in reduced expression.

CD45 functions to regulate Src-family member kinase activity in leukocytes. For lymphocytes, CD45 is required to activate Src-family members that function in antigen receptor signal transduction by dephosphorylating the negative regulatory tyrosine phosphorylation site (Mustelin et al., 1989; Cahir McFarland et al., 1993; Shiroo et al., 1992; Sieh et al., 1993). CD45 functions in macrophages to regulate Src-family members involved in integrin-mediated adhesion by decreasing kinase activity by dephosphorylation of the tyrosine phosphorylation site that potentiates activity within the kinase domain. CD45 also appears to regulate integrin-mediated adhesion in lymphocytes (Wagner et al., 1993; Lorenz et al., 1993; Bernard et al., 1994). CD45AP is uniquely expressed by lymphoid cells. Thus, it is possible that CD45AP serves to distinguish the ability of CD45 to regulate antigen receptors versus the ability to regulate integrin-mediated adhesion.

CD45 serves to increase $p56^{lck}$ kinase activity prior to antigen-mediated signal transduction. This suggests that the phosphatase and kinase activity may be compartmentalized to sequester enzymatic activity prior to the reception of extracellular signals. A potential function for CD45AP is that it is involved in such a compartmentalization process. Indeed, CD45 interacts with the cytoskeleton, and while sites within CD45 have been identified that potentially interact with the cytoskeletal component fodrin, CD45AP may also function in this regard. CD45AP contains a putative WW domain. WW domains are protein modules that interact with other proteins through a proline-base motif (Sudol, 1996). WW domains have been demonstrated to function in linking transmembrane receptors to substrates and to the cytoskeleton. Dystroglycan interacts with dystrophin via a proline-rich region and WW domain. The amiloride-sensitive $Na^+$ channel binds to the WW domain of the cytoplasmic protein Nedd4 (Schild et al., 1996; Staub et al., 1996). Mutation of the proline-rich region of the $Na^+$ channel results in the failure to interact with Nedd4 and gives rise to Liddles syndrome. Yap, a $p60^{yes}$, associated protein, binds to $p60^{yes}$ via a WW domain (Sudol et al., 1995). Thus, it is likely that the putative WW domain in CD45AP will link CD45 other proteins.

Coexpression of CD45 with CD45AP in HeLa cells demonstrates the interaction does not require other lymphocyte-specific proteins. CD45 interacts with CD45AP via the 22 amino acid transmembrane domain. To define residues within the CD45 transmembrane domain that mediate the interaction with CD45AP, mutants were constructed that substitute four or five residues with alanine. However, none of the mutants lost the ability to associate with CD45AP, suggesting that no single amino acid is essential in mediating the interaction. Chimeric proteins that replace the CD45 transmembrane domain with residues from the CD44 transmembrane domain indicate CD44 does not interact with CD45AP, but the addition of three residues from the CD45 transmembrane domain results in a protein that will bind CD45AP. Therefore, a potential minimum of three residues, alanine at transmembrane position 1, phenylalanine at position 5, and leucine at position 6, are sufficient to mediate the interaction of a CD45/44 chimera with CD45AP. However, it is possible that additional residues may contribute to the interaction between CD45 and CD45AP. Five amino acids are conserved between the transmembrane domain of CD45 and CD44, and these may also contribute to the interaction. Thus, potentially a maximum of eight residues may be required. Importantly, all eight residues are conserved in shark, chicken, mouse, rat, and human CD45 (Okumura et al., 1996) and five of the eight residues are on the same face of a predicted α helix. Furthermore, other residues may be important, but conservative substitutions may be allowed.

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