Identification of the Sites of Interaction between Lymphocyte Phosphatase-associated Phosphoprotein (LPAP) and CD45*

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Human lymphocyte phosphatase-associated phosphoprotein (LPAP) is a phosphoprotein of unknown function that noncovalently associates with CD45 in lymphocytes. In CD45-deficient human T cells, LPAP protein is synthesized at normal levels but is more rapidly degraded than in wild-type cells. Expression of CD45 cDNA rescues LPAP protein expression. This strongly suggests that LPAP is protected from degradation through its interaction with CD45. We have mapped the sites of interaction between LPAP and CD45 employing chimeric CD45 molecules and LPAP deletion mutants. Our data demonstrate that the interaction between LPAP and CD45 is mediated via the transmembrane regions of both molecules. In addition, the intracytoplasmic amino acids adjacent to the transmembrane region of LPAP may influence its binding to CD45.

Stimulation of the T cell antigen receptor (TCR) with anti-T cell activity of protein-tyrosine kinases, including the Src kinases p56

CD45 are sufficient to activate TCR signaling, the extracellular and/or transmembrane domains could optimize these processes, possibly through interaction with other proteins.

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Recent staining of the T cell antigen receptor (TCR) with anti-TCR antibodies leads to rapid activation of protein-tyrosine kinases, including the Src kinases p56

CD45 is also present in CD45-deficient Jurkat variants (15). In addition, transfection of J45.01 cells with a cDNA encoding a chimeric A2/A2/CD45 molecule (containing the extracellular and transmembrane domains of the human HLA-A2 molecule fused at the eighth cytoplasmic amino acid to the CD45 phosphatase domain) also restores TCR-mediated signal transduction (16). However, the efficiency of signaling in these cells is reduced when compared to the parental J urkat line. These data suggest that, although the phosphatase domains of CD45 are sufficient to activate TCR signaling, the extracellular and/or transmembrane domains could optimize these processes, possibly through interaction with other proteins.

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Recently, the mouse and human cDNAs coding for a novel 30–32-kDa phosphoprotein that noncovalently associates with CD45 have been cloned (21–26). The human protein has been termed LPAP (lymphocyte phosphatase-associated phosphoprotein) (25), whereas the mouse molecule was named CD45-AP (CD45-associated protein) (26). We will use the name LPAP in this paper.

LPAP is predominantly expressed in lymphocytes and contains a potential transmembrane domain and a short, 10-amino acid-long extracellular domain (25). Importantly, expression of CD45 appears to correlate with the expression of CD45 in human T lymphocytes, since the CD45-negative Jurkat T cell line 45.01 expresses LPAP mRNA but barely detectable levels of LPAP protein (25). Analysis of J 45.01 has demonstrated that, although similar amounts of LPAP protein are synthesized, the half life time of LPAP in J 45.01 is 10-fold less than in the wild-type cells (25). Thus, it appears that LPAP is degraded in the absence of CD45. Re-expression of wild-type CD45 but not the above A2/A2/CD45 chimera rescues LPAP expression in J 45.01. Moreover, when the A2/A2/CD45 chimera is transfected into wild type J urkat cells, no association is seen with LPAP, although LPAP protein is present in these cells (25). Collectively, these data suggest that CD45 stabilizes LPAP protein expression, possibly through interaction via the extracellular and/or transmembrane domains.

To understand further the composition of the CD45-associated protein complex and its role in TCR signaling, we have mapped the sites of interaction between CD45 and LPAP using CD45 chimeras and LPAP deletion mutants. Our data suggest that the transmembrane regions of both molecules are required for their association. Interestingly, it appears that membrane proximal amino acids in the cytoplasmic domain of LPAP must also be present for complete association.

MATERIALS AND METHODS

Site-directed Mutagenesis—Mutations of the LPAP gene (the full-length LPAP cDNA construct (25) cloned in the EcoRI site of pBlue

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site-directed mutagenesis kit from Clontech according to the manufacturer's protocol. Plasmid DNA from identified mutant bacterial colonies was sequenced. Since LPAP represents a transmembrane molecule, the leader sequence was deleted from all constructs. Therefore, mutant A (mutA) corresponds to the mature LPAP protein except that the initiator methionine residue is incorporated into the construct. Fig. 1A summarizes all deletion mutants that were used in this study. The following oligonucleotides were used for site-directed mutagenesis: selection oligo (converts the unique Asp700 site of the Bluescript I vector into an Eco47III site), CATCATTGGGAGCGTTCTTGGGGGC; mutA, GGCGTTGCGGACAAATCTGGTGACGCGCGGCGG; mutB, GGCGTGGCAGGACATTGCTGGCGAGCGCGG; mutC1, CGGGCTGGGACCAATGGGCACTGCGGCTC; mutC2, GTGGGGAACAGCTGGC; mutC4, CGGCTGGGACCAATGGGCACTGCGGCTC; mutD1, CCTAGCACTGCGGACCAATGTCGGGTGGCAGCGCGG; mutD2, GGGCCATGGGACCAATGTCGGGTGGCAGCGCGG; mutE3, CTACCAGGGGCGGCGCGCGCGCGCGCGCG; mutF1, T7GAAGCCAGAGACAGTGGCAGGAGCCGG; mutG1, CTGGGGAACAGCTGGC; mutH1, GGGCCATGGGACCAATGTCGGGTGGCAGCGCGG; mutT1, GGGCCATGGGACCAATGTCGGGTGGCAGCGCGG; mutY2, GGGCCATGGGACCAATGTCGGGTGGCAGCGCGG; mutZ1, GGGCCATGGGACCAATGTCGGGTGGCAGCGCGG; mutZ2, GGGCCATGGGACCAATGTCGGGTGGCAGCGCGG.

In Vitro Transcription/Translation—[3H]Leucine-labeled LPAP protein (mutA) was added to Nonidet P-40 lysates of J urkat cells, which were then immunoprecipitated with antibodies directed against the proteins listed below the individual lanes.

antibody or 9.4 (anti-CD45) monoclonal antibody bound to 30 μl of Staphylococcus aureus protein A (Sigma) pre-armed with 1 μg of rabbit anti-mouse IgG. Immunoprecipitates were washed four times in high salt lysis buffer (Nonidet P-40 lysis buffer with 0.35 M NaCl) and one time in lysis buffer and run on 10% reducing SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose, and LPAP was detected as described previously (25).

RESULTS AND DISCUSSION

LPAP Associates with CD45 via Its Transmembrane Region—We have previously shown that wild-type CD45, but not an A2A2CD45 chimeric containing the cytoplasmic domain of CD45 fused to A2 extracellular and transmembrane regions, associates with LPAP in vivo (25). These data suggested that the transmembrane or extracellular region of these proteins could mediate the CD45-LPAP interaction. We attempted to map the sites within the LPAP molecule responsible for its association with CD45 employing deletion LPAP mutants. Mutant mutants were expressed in vitro in rabbit reticulocyte lysates and subsequently investigated for their ability to bind wild-type CD45 in lysates of Jurkat cells. This approach was chosen because we expected that cells transfected with LPAP constructs bearing deletions in the transmembrane domain would likely not produce proteins that can be expressed on the cell surface. The deletion constructs used for in vitro translation are summarized in Fig. 1A. The data shown in Fig. 1B demonstrate the specificity of our assay. In vitro translated LPAP (mutA) associates with CD45 but not with HLA-class I, CD49d, CD2, or CD3.

We next analyzed mutB, which lacks the extracellular and transmembrane domains of LPAP, for its ability to bind CD45. Fig. 2A demonstrates that while mutA readily associates with CD45, mutB does not. These data indicate that the association of LPAP with CD45 involves the extracellular and/or transmembrane regions of LPAP.

We investigated the role of the extracellular domain of LPAP further using mutC1 and C2, which both have a five-amino acid deletion in the extracellular domain (see Fig. 1A). Fig. 2A shows that both mutC1 and mutC2 bind to CD45, demonstrating that deletions in the extracellular domain of LPAP do not diminish the capacity of the protein to interact with CD45.

In contrast, all deletions within the transmembrane segment of LPAP (mutC3-C7) result in a loss of binding to CD45 (Fig. 2A, lanes 1–5). MutC3 and mutC4 (lanes 1 and 5) have a greatly diminished capacity to associate with CD45, while mutC5, mutC6, and mutC7 have completely lost their ability to bind CD45. Although there appear to be quantitative differences in the contribution of individual stretches of amino acids, our data demonstrate that the intact transmembrane region is required to mediate optimal association between the two molecules.

LPAP Cytoplasmic Sequences Are Also Required for Association with CD45.
ation with CD45—Deletions in the cytoplasmic domain of LPAP (mutD1 and mutD2) were designed to investigate whether the LPAP-CD45 association is exclusively mediated via the transmembrane region. The 5 cytoplasmic amino acids directly adjacent to the transmembrane domain are deleted in mutD1, while mutD2 contains a deletion of 30 cytoplasmic amino acids closer to the COOH terminus. As shown in Fig. 2B, lanes A and E, mutD2 associates with CD45 similarly to mutA, demonstrating that this region of the cytoplasmic domain is not required for the association of LPAP and CD45. Interestingly, the deletion in mutD2 appears to induce conformational changes in the molecule that mask the amino terminus because mutD2 is not recognized by antisera directed against the first 10 amino acids of LPAP (not shown).

Sequence analysis of mutD2 verified that this construct does not contain mutations in the amino-terminal epitope region that would explain loss of accessible epitopes. Thus, the deletion in the COOH-terminal portion of LPAP apparently alters the conformation of the protein, resulting in non-reactivity with the amino-terminal antisera. However, the mutation within mutD2 does not appear to dramatically alter the conformation of the transmembrane region since the molecule retains the ability to bind CD45. It is important to note at this point that we have most recently obtained a mutant Jurkat cell line (clone 14) that expresses a LPAP variant that carries a single point mutation in the cytoplasmic domain, resulting in a new stop codon 36 amino acids upstream of the original COOH terminus. Interestingly, the mutant LPAP protein expressed by clone 14 is, similarly to mutD2, not recognized by our amino-terminal antisera but still associates with CD45 in vivo.²

In contrast to mutD2, the protein encoded by mutD1 does not bind to CD45 (Fig. 2B, lane C), suggesting that the cytoplasmic amino acids closest to the transmembrane region also contribute to the association with CD45.

Three mutants (mutE1, mutE2, and mutE3), each containing contiguous deletions of five amino acids starting with those directly adjacent to the deletion in mutD1, were designed to address the question of whether or not additional amino acids within the cytoplasmic domain of LPAP are required to mediate association with CD45. Fig. 2B indicates that mutE1 and mutE2 are strongly impaired in their ability to bind to CD45, while mutE3 associates similarly to wild-type LPAP (mutA). Deletions further downstream of mutE3 do not influence the association between LPAP and CD45 (not shown). These results along with the lack of binding of mutD1 suggest that the 15 cytoplasmic amino acids adjacent to the transmembrane region play a role in CD45 association.

The Transmembrane Region of CD45 Is Necessary and Sufficient for Association with LPAP—We hypothesized that the transmembrane region of CD45, including adjacent cytoplasmic amino acids, was likely the site of interaction with LPAP since this was the region of LPAP that mediated its association with CD45. To confirm this assumption, we created a chimera, A2CD45 (A2 extracellular, CD45 transmembrane and 8 NH₃-terminal cytoplasmic amino acids followed by a stop codon, Fig.

² E. Bruyns, L. R. Hendricks-Taylor, S. Meuer, G. A. Koretzky, and B. Schraven, unpublished observation.
The molecular interaction between the TCR and CD3 is stabilized through charged amino acids within the transmembrane domains of both molecules. Mutation of only one of these charged amino acids leads to a loss of interaction (34–37). In contrast, all deletions in the transmembrane domain of LPAP result in an almost complete loss of binding to CD45, suggesting that their association depends upon the entire sequence of amino acids present in the transmembrane region of LPAP. This pattern of binding activity, together with the high content of hydrophobic amino acids and the absence of charged amino acids in the transmembrane regions of LPAP and CD45, leads us to conclude that the association is mediated by hydrophobic interactions. Similar interactions have been postulated for the interaction of the bovine papillomavirus E5 oncoprotein with the 16-kDa membrane pore-forming protein and the platelet-derived growth factor receptor (38, 39).

It is not clear whether the 15 LPAP cytoplasmic amino acids that contribute to the LPAP-CD45 interaction represent a portion of the binding site or whether their deletion results in conformational changes of the transmembrane region that abrogate its ability to interact with CD45. However, a requirement for transmembrane as well as flanking cytoplasmic amino acids has been observed previously for the interaction of MHC class I molecules with the chaperone protein p88 (40).

Our previous data as well as the transfection data shown here demonstrate that LPAP associates with the transmembrane domain of CD45 and that the LPAP protein is stabilized through its binding to CD45. It is tempting to speculate that in the absence of CD45, particular sequences within the transmembrane region of LPAP become unmasked and thus become accessible to a protease that subsequently induces degradation of the protein.

We have also presented data that suggest that deletions in the COOH-terminal region of LPAP alter the conformation of the extracellular amino acids, resulting in non-reactivity with amino-terminal antisera (mutD2, done 14). In addition, we have reported previously that resting but not activated human T lymphocytes express a hyperphosphorylated form of LPAP (pp29) that is also not reactive with the amino-terminal antisera (25). Together, these results suggest that receptor-mediated phosphorylation/dephosphorylation events affecting the COOH-terminal portion of LPAP may indirectly alter the conformation of the extracellular domain. These events could represent a mechanism to regulate the ability of LPAP to bind an external ligand via its intracellular phosphorylation pattern.

The biological function of LPAP and its role in lymphocyte activation are unknown. We have shown previously that CD45 and p56Lck associate with LPAP (21, 22). Furthermore, the A2A2CD45 chimera that does not associate with LPAP also does not associate with Lck. This lack of CD45-Lck association may explain the decreased efficiency of TCR signaling seen with A2A2CD45 in transfected J 45.01 (CD45-deficient) when compared to wild-type J urkat (16). We are currently testing this hypothesis by establishing J 45.01 stable transfectants expressing A2CD45 and A2CD45CD45 to assay LPAP and Lck binding and TCR signaling efficiency.

CD45 associates with LPAP in the absence of Lck in J Cam.1 cells (25). In addition, we have demonstrated that CD45 and LPAP associate in the absence of Lck in COS cells. Collectively, these data lead us to speculate that LPAP may serve as a bridge to facilitate the interaction of CD45 with Lck. LPAP thus could be involved in the regulation of the CD45-Lck complex by controlling the interaction between both molecules. Conversely, the conformation and function of LPAP (e.g., its ability to interact with other proteins) could be regulated by the enzymatic activities of CD45 and p56Lck.
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