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# THE ASSOCIATION BETWEEN CD45 AND *LCK* DOES NOT REQUIRE CD4 OR CD8 AND IS INDEPENDENT OF T CELL RECEPTOR STIMULATION

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CD45, the major transmembrane tyrosine phosphatase of lymphoid cells, is required for optimal signaling via a number of receptors. A model for how CD45 regulates signaling is that it controls phosphorylation of the COOH-terminal tyrosine of *src* family kinases. We have shown that CD45 physically associates with *lck*, one *src* kinase. Others have shown that CD45 also interacts with the CD4 and CD8 surface antigens expressed on many T cells. In this report we examine further the relationship between CD45 and *lck* in a CD4+ T cell line and in peripheral T cells. We show now that CD45 associates with *lck* independently of both CD4 and CD8. We show also the time course of an association between CD45 and a form of *lck* that migrates at an apparent higher molecular mass. Finally, we demonstrate that the interaction between CD45, *lck*, and a previously reported 32-34 kD protein is stable after stimulation of T cells. 

CD45 is a family of transmembrane tyrosine phosphatases expressed at high levels on all nucleated hematopoietic cells (1). Although details differ in the various systems, experiments from a number of laboratories utilizing mutant cells deficient in CD45 expression have demonstrated that CD45 is required for optimal signal transduction via the T cell antigen receptor (TCR) (2-5) and CD2 (6) on T lymphocytes, surface immunoglobulin on B lymphocytes (7), and CD2 on natural

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The abbreviations used are: TCR, T cell antigen receptor; PTK, protein tyrosine kinase; mAb, monoclonal antibody, MOPC, mineral oil plasmacytoma; PAGE, polyacrylamide gel electrophoresis; PBMC, peripheral mononuclear cells.

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killer cells (8). Proof for CD45's role in regulating several of these signaling events has been provided by reconstitution of CD45 expression with concomitant rescue of signaling (3,7,9-12).

One model for how CD45 regulates signaling relies on the observation that members of the *src* family of protein tyrosine kinases (PTK) appear to be important in the most proximal signaling events seen after receptor ligation (13,14). Each member of this PTK family possesses a COOH-terminal tyrosine which, when phosphorylated, down regulates enzymatic activity (15). One postulated role for CD45 in the regulation of signaling is to dephosphorylate this COOH-terminal tyrosine, enabling the PTK to become activated after the appropriate receptor is bound. Experimental evidence supporting this model includes 1) CD45 is able to dephosphorylate the COOH-terminal tyrosine of *lck*, one *src* family member, *in vitro* (16,17); 2) *lck* and *fyn* (another *src* PTK) appear to be hyperphosphorylated on their COOH-terminal tyrosines in a number of CD45-deficient cells (18-20); and 3) CD45 has been shown to physically associate with *lck* (21-23) and *fyn* (24) in T cells and *lyn*, a third member of the *src* family, in B cells (25).

In this report we examine further the relationship between CD45 and *lck* in human T cells. *Lck* has been shown to be essential for the most proximal signaling events seen after TCR ligation in a number of T cell lines (26,27). It has been demonstrated also that a significant portion of the *lck* present in T cells is bound to the CD4 and/or CD8 surface antigens (28). The relationship between CD4 and *lck* has been shown to be physiologically relevant in experiments which demonstrate that this association is required for an optimal T cell response to antigen (29). In contrast, however, although *lck* expression is required for normal thymocyte development (30), a recent study demonstrated that the association between *lck* and CD4 and/or CD8 is not important for thymocyte maturation (31). We therefore investigated whether the *lck* associated with CD45 represents the same pool which binds to CD4 in the Jurkat (CD4+/CD8-) T cell line. We were particularly interested in this question because others have shown that under some conditions CD45 interacts with CD4 and CD8 (32-34). Therefore, it was formally possible that the association between CD45 and *lck* demonstrated by our laboratories was indirect, being due to an interaction between CD45 and CD4 and/or CD8. In this report we show that there remains a physical association between CD45 and *lck* after preclearing all CD4 from Jurkat cell lysates. Additionally, we show that the association between CD45 and an as yet unidentified 32-34kD phosphoprotein (pp32-34) remains intact after CD4 is cleared. In this report we also extend these observations to normal peripheral blood T cells demonstrating that CD45 associates with *lck* and pp32-34 in the absence of CD4, CD8, CD2, and the TCR complex. We show also that the association between CD45 and *lck* is preserved after stimulation of the TCR. Others have reported previously that such stimulation modifies *lck*, presumably by a serine phosphorylation event, resulting in a shift in the apparent molecular mass of *lck* when analyzed by single dimension gel electrophoresis (35). In this report we show that the higher molecular mass form of *lck* transiently associates with CD45 after TCR stimulation.

## Materials and Methods

*Cell line.* The E6-1 clone of Jurkat was grown in RPMI 1640 supplemented with 10% fetal calf serum, glutamine, and penicillin and streptomycin. Stimulations were carried out with C305 (1:2500 dilution of ascites), a clonotypic antibody directed against the TCR (36).

*Peripheral mononuclear cells (PBMC).* PBMC were isolated from heparinized whole blood of healthy donors by Ficoll-Hypaque density centrifugation (Pharmacia, Uppsala, Sweden). Subsequently, resting human T lymphocytes were prepared by means of E-rosette formation as described (37). Negative selection for CD4-positive T cells was performed as follows: 2 x $10^8$ T-lymphocytes were incubated on ice for 30 minutes with CD8 mAb (AICD8.1, IgG1). The cells were washed three times, resuspended in 30 ml culture medium, layered upon plastic petri dishes (Greiner, Nurtingen, FRG) which were precoated with rabbit anti-mouse Ig (Dako, Hamburg, FRG). Following a 2 hour incubation at 4°C , nonadherent cells were collected. To induce modulation of surface antigens 12 x $10^6$, $CD4^+$ T lymphocytes were incubated overnight at 37°C with CD2 mAb (ICRFCD2.3.1, IgM), CD3 mAb (2AD2A2, IgM) or CD4 mAb (AICD4.2, IgM) at a density of 2 x $10^6$ cells/ml.

*Flow cytometry* was performed using culture supernatants of the following hybridomas: NS-1 (negative control); ICRFCD2.1.1A (anti-CD2 [38]); OKT-3 (anti-CD3, American Type Culture Collection, Rockville, MD); AICD4.1 (anti-CD4); AICD8.1 (anti-CD8 [39]); and AICD45.2 (anti-CD45 [38]). The secondary antibody was a FITC-conjugated goat anti-mouse IgG (Dako, Hamburg, FRG).

*Immunoprecipitations* were carried out as described previously (23). Briefly, cells were solubilized in lysis buffer (1% digitonin, 140mm NaCl, 10mm Tris, pH 7.4, .02% NaN3) containing a cocktail of protease (1mm phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 25 µg/ml pepstatin A, 5 µg/ml aprotinin) and phosphatase (.4mM Na3VO4, 10mM Na4P2O7, 10mM NaFl) inhibitors at $10^7$ cells/100µl. Postnuclear supernatants were precleared twice over staph A, subjected to ultracentrifugation, then sequential immunoprecipitations with a control monoclonal antibody (MOPC 195, Cappel), then anti-CD45 (9.4 [ATCC]) or anti-CD4 (Leu 3a [ATCC]). Immunoprecipitates were washed four times with lysis buffer containing 500mm NaCl, then once with lysis buffer. Samples were subjected to *in vitro* kinase assays, or SDS-PAGE.

*In vitro kinase assays* were performed as described previously (23). Briefly, immunoprecipitates were washed once in water, then once in lysis buffer with .5M LiCl. Samples were then incubated in kinase buffer (10mM MnCl2, 20mM Tris, pH 7.4, 10 µCi [$\gamma$-$^{32}$P] ATP) for 10 minutes at room temperature, washed twice then subjected to SDS-PAGE.

*Western blotting* was performed after transferring proteins to .2-µm nitrocellulose and blocking in TBST (150mM NaCl, 10mM Tris, pH 8, .05% polyoxyethylenesorbitan monolaurate [Tween 20]) with 5% bovine serum albumin. *Lck* was visualized using antiserum 337 (generously supplied by T. Potter, Denver, CO) and development with alkaline phosphatase conjugated goat anti-rabbit antibody (BioRad, Richmond, CA). CD45 was visualized using monoclonal antibody 9.4 (ATCC) and development with alkaline phosphatase.

*Biosynthetic labelling and phosphoamino acid analysis* was performed as described (23). Briefly, Jurkat cells were loaded with [$^{32}$P] orthophosphate, washed, then stimulated with medium or C305. Cell lysates (prepared in the presence of phosphatase and protease inhibitors) were subjected to immunoprecipitation with control mAb or anti-CD45 then subjected to 10% SDS-PAGE. The band of interest was excised from the polyacrylamide gels, hydrated, then subjected to acid hydrolysis and phosphoamino acid analysis.

## Results and Discussion

We have demonstrated previously that CD45 physically associates with *lck* in peripheral blood lymphocytes and several human T cell leukemic lines (21-23). Evidence from other laboratories has shown that the association between *lck* and CD4 is important in eliciting an optimal response to antigen (29), however a more recent study indicates that the association between CD4 and *lck* is not important in the ability of *lck* to regulate thymocyte development (31). It was therefore of interest to investigate whether the *lck* which associates with CD45 is also complexed with CD4 in the CD4+ Jurkat cell line. Figure 1 shows the result of a representative experiment where this was tested. Lysates obtained from Jurkat cells (either resting or stimulated with anti-TCR) were subjected to four sequential immunoprecipitations with a control mAb or with anti-CD4. Following this, the lysates were subjected to immunoprecipitation with anti-CD45. An *in vitro* kinase assay was performed on an aliquot of each immunoprecipitate (top panel) allowing for

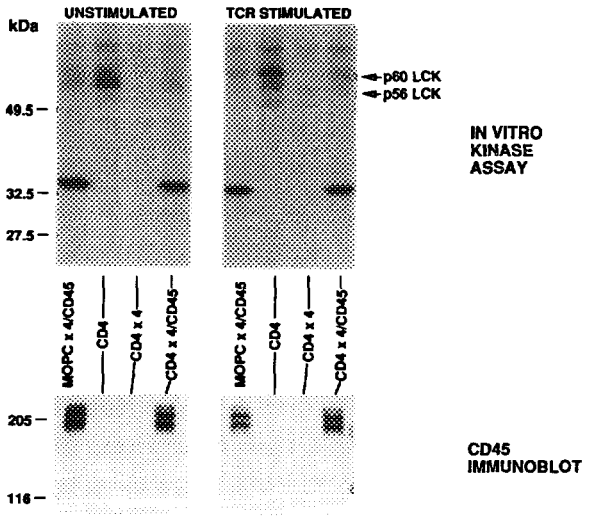


Figure 1. **CD45 associates with *lck* independently of CD4.** *Top panel, in vitro* kinase assays were performed on immunoprecipitates of lysates from Jurkat cells stimulated with medium alone (left) or C305 for 10 minutes (right). Lysates were subjected to 4 sequential immunoprecipitations with a control mAb (MOPC), then anti-CD45 (first lane, left and right); anti-CD4 (second lane, left and right); 4 sequential immunoprecipitations with anti-CD4 (third lane, left and right); or 4 sequential immunoprecipitations with anti-CD4 followed by anti-CD45 (fourth lane, left and right.) Arrows indicate the migration of p56 *lck* and p60 *lck*. An *in vitro* kinase assay was performed on each sample followed by 10% SDS-PAGE and autoradiography. 3 x $10^7$ cell equivalents were loaded per lane. *Bottom panel*, aliquots of each immunoprecipitate described above were subjected to 8% SDS-PAGE. Proteins were transferred to nitrocellulose for immunoblotting with anti-CD45 mAb. 7 x $10^6$ cell equivalents were loaded per lane.

the visualization of both *lck* and pp32-34. Another aliquot of each sample was subjected to SDS-PAGE, then immunoblotted for the presence of CD45 (bottom panel).

The autoradiograph of the *in vitro* kinase assays performed on CD45 immunoprecipitates obtained after 4 sequential preclearing steps employing a control mAb corroborate our previous findings. As shown, in lane 1, CD45 associates with *lck* (which becomes autophosphorylated under these conditions) and pp32-34 which becomes phosphorylated by *lck* during the *in vitro* kinase assay. This is seen when lysates are used from either resting (left) or TCR-stimulated (right) cells. The autoradiograph demonstrates that after TCR stimulation, the form of lck which associates with CD45 and pp32-34 migrates at an apparent molecular mass of 60kD, presumably due to serine phosphorylation of the kinase (35). The bottom panel of Figure 1 demonstrates that CD45 is present in the immunoprecipitates as expected.

Lanes 2 and 3 of Figure 1 show the results of in vitro kinase assays performed on the first and the fourth sequential CD4 immunoprecipitates. As shown, under our cell lysis conditions, pp32-34 is not seen in CD4 immunoprecipitates from either resting or stimulated Jurkat cells. As expected, *lck* is present in the first CD4 immunoprecipitate, however by the fourth sequential CD4 immunoprecipitate, *lck* is no longer detectable. These data indicate that our immunoprecipitation protocol removes all CD4-associated *lck* detectable using an *in vitro* kinase assay. However, even after depletion of all CD4 associated lck, there does not appear to be a decrease in the amount of

pp32-34 or lck which is coprecipitated by CD45 mAb (compare lanes 1 and 4 of both the left and right panels). These data indicate that CD45 can associate with *lck* independently of CD4.

To examine further the role of CD2, CD3, CD4 and CD8 in the association of CD45, *lck* and pp32-34, we prepared $CD4^+/CD8^-$ T lymphocytes by negative selection of resting human T cells. $CD4^+$ cells were further treated with mAb of the IgM isotype to induce individual modulation of the surface antigens CD2, CD3, or CD4. In a parallel experiment, $CD4^+$ T lymphocytes were incubated with a cocktail of CD2, CD3 and CD4 mAb to generate a T cell population which was $CD2^-$, $CD3^-$, $CD4^-$, $CD8^-$, and $CD45^+$. The FACS analysis of the different T lymphocyte populations is shown in Figure 2a and demonstrates that CD2, CD3 and CD4 were completely removed from the cell surface by this treatment. In contrast, the surface expression of CD45 remained unaffected. Figure 2b depicts the results of *in vitro* kinase assays performed on CD45 immunoprecipitates obtained from the different T cell populations. The immunoprecipitates were analyzed on 18% SDS-PAGE which allows separation of pp32-34 into two distinct bands. As indicated, almost equal amounts of *in vitro* phosphorylated $p56^{lck}$ (arrowhead) and pp32-34 (triangles) were coprecipitated by CD45 mAb from the T lymphocyte populations. These data confirm further that the association between CD45, $p56^{lck}$ and pp32-34 does not require surface expression of CD2, CD3, CD4 or CD8.

As noted above, the slower migration of *lck* after TCR stimulation (p60 *lck*) is thought to be due to serine phosphorylation mediated by protein kinase C following TCR ligation. We performed a number of experiments to address the time course of the binding of p60 *lck* to CD45. Figure 3 shows the result of a representative experiment where lysates were prepared from Jurkat cells stimulated through their TCR for up to 2 hours. At each time, lysates were subjected to immunoprecipitation with a control mAb (odd numbered lanes) or anti-CD45 (even numbered lanes). Each sample was then analyzed for the presence of CD45 (top panel), *in vitro* kinase activity (middle panel), and the presence of *lck* (bottom panel). As shown, stimulation of the TCR did not change the amount of CD45 recoverable by immunoprecipitation nor the appearance of pp32-34. However, stimulation of the TCR did result in the transient appearance of p60 *lck* with the CD45 immunoprecipitates. The time course reflects the appearance of p60 *lck* in whole cell lysates (not shown.) Additionally, a similar appearance of p60 *lck* in CD45 immunoprecipitates was seen in lysates obtained from cells stimulated with phorbol esters which directly activate protein kinase C and stimulate the appearance of p60 *lck* (21,22 and not shown).

We have reported previously that although pp32-34 is phosphorylated heavily on tyrosines in *in vitro* kinase assays, when cells are labelled *in vivo* with $^{32}P$ orthophosphate, the only detectable residues phosphorylated are serines in resting or CD2-stimulated cells (22,23). We addressed this issue further by performing phosphoamino acid analysis on the 32-34kD band isolated by SDS-PAGE after immunoprecipitation of CD45 from lysates of resting and TCR-stimulated Jurkat cells biosynthetically labelled with $^{32}P$ orthophosphate (Figure 4). As expected, we found evidence for phosphorylation of pp32-34 only on serine in lysates from unstimulated cells. Additionally, however, we could detect only serine phosphorylation of pp32-34 from cells stimulated with anti-TCR (Figure 4b) for 30 minutes, the time point where we consistently saw maximal amounts of p60 *lck* associating with CD45. In similar experiments we could detect only

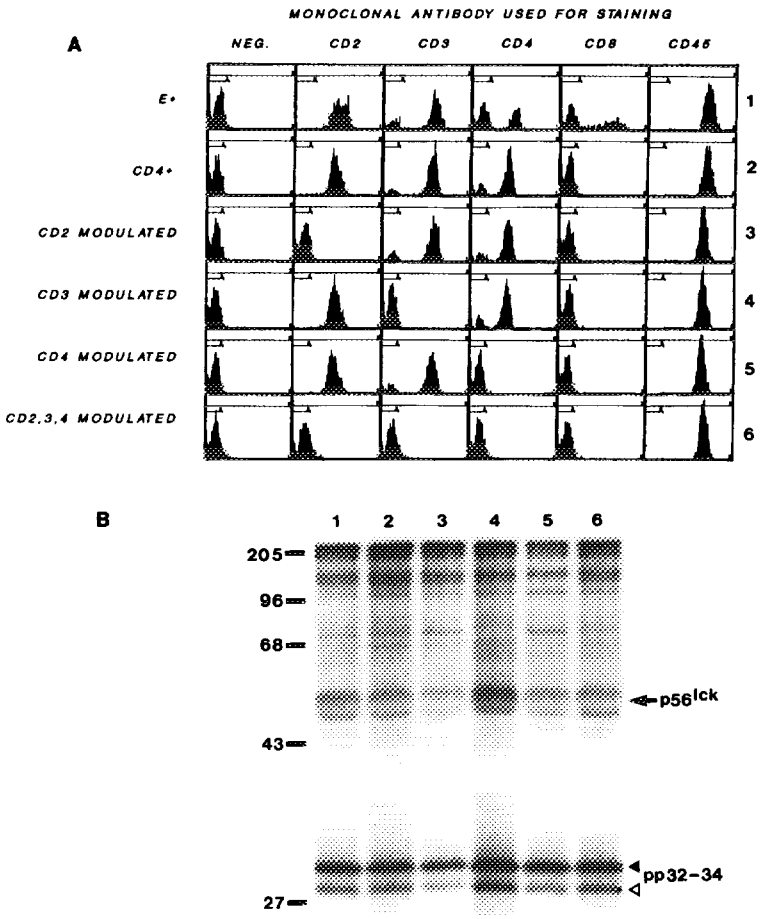


Figure 2. **Modulation of the CD2, CD3 and CD4 antigens does not alter the association of CD45, p56 *lck* and pp32-34 in CD4$^+$ T lymphocytes.** A: Expression of CD2, CD3, CD4, CD8 and CD45 on human T lymphocytes following modulation of individual surface antigens. Resting human CD4$^+$ T lymphocytes (panel 2) were prepared by means of negative selection from unseparated T cells (panel 1). CD4$^+$ cells were treated overnight with mAb of the IgM isotype directed at CD2 (panel 3), CD3 (panel 4), CD4 (panel 5) or, alternatively a cocktail of CD2, CD3 and CD4 mAb (panel 6). B: *In vitro* kinase assays of CD45 immunoprecipitates obtained from the T cell populations shown in Figure 2a. Lane 1: unseparated CD4$^+$/CD8$^+$ resting human T lymphocytes; lane 2: CD4$^+$/CD8$^-$ T lymphocytes; lane 3: CD2 modulated T lymphocytes; lane 4: CD3 modulated T lymphocytes; lane 5: CD4 modulated T lymphocytes; lane 6: CD2, CD3, CD4 modulated T lymphocytes. The position of *in vitro* labeled p56 *lck* is indicated by an arrowhead whereas the position of pp32-pp34 is depicted by triangles. Immunoprecipitates were analyzed by means of 18% SDS-PAGE to show the two individual bands of pp32 and pp34, respectively.

serine phosphorylation of pp32-34 after stimulation of the TCR for up to 2 hours (not shown). Of course, since our only method for detecting this protein is via immunoprecipitation with the CD45 tyrosine phosphatase, it is formally possible that pp32-34 is phosphorylated on tyrosines *in vivo*, then dephosphorylated by the association with CD45.

The data described in this report define further the parameters involved in the association between CD45 and *lck*. There currently is a great deal of interest in identifying protein-protein

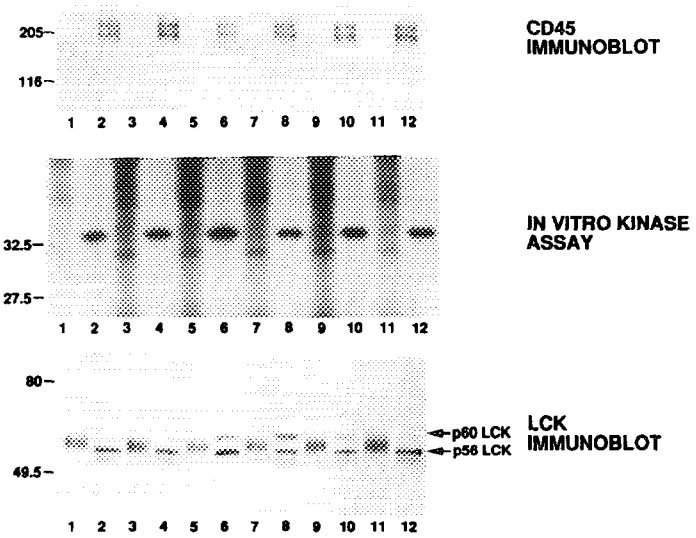


Figure 3. **Time course of the association of p60 *lck* and pp32-34 with CD45 after TCR stimulation.** Jurkat cells were left unstimulated (lanes 1 and 2) or stimulated with C305 for 1 minute (lanes 3 and 4), 5 minutes (lanes 5 and 6), 30 minutes (lanes 7 and 8), 60 minutes (lanes 9 and 10) or 120 minutes (lanes 11 and 12) then subjected to immunoprecipitation with a control mAb (odd numbered lanes) or anti-CD45 (even numbered lanes). *Top panel*, 7 x $10^6$ cell equivalents were loaded per lane then subjected to 8% SDS-PAGE and immunoblotted for the presence of CD45. *Middle panel*, 3 X $10^7$ cell equivalent samples were subjected to an *in vitro* kinase assay then subjected to 10% SDS-PAGE and autoradiography. *Bottom panel*, 3.5 x $10^7$ cell equivalents were loaded per lane then subjected to 10% SDS-PAGE followed by immunoblotting for *lck*. The migration of p56 *lck* and p60 *lck* are indicated by arrows.

interactions for a number of molecules felt to be important in modulating signal transduction. CD45 has been shown to physically associate with a number of antigens present on the surface of T cells and B cells including Thy1 (40), CD22 (41), CD2 (42,43), CD4 (32-34), and CD8

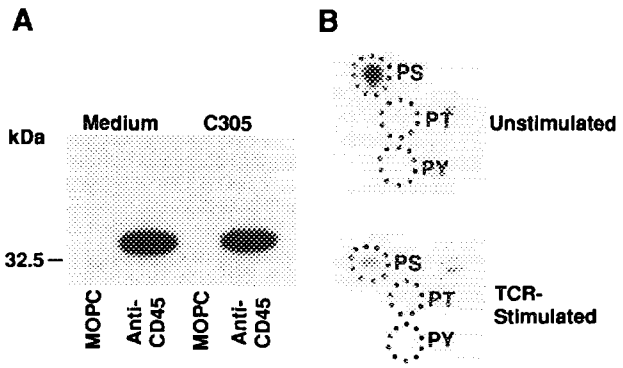


Figure 4. ***In vivo* phosphorylation on serines of pp32-34 in unstimulated and TCR-stimulated Jurkat cells.** A: Jurkat cells were loaded with [$^{32}$P] orthophosphate and stimulated with medium alone (lanes 1 and 2) or C305 for 30 minutes (lanes 3 and 4). Lysates prepared from the cells were subjected to immunoprecipitation with a control mAb (MOPC) or anti-CD45. Samples were subjected to 10% SDS-PAGE (7 x $10^7$ cell equivalents per lane) then autoradiography. B: The 32-34kD band visualized in the two "anti-CD45" lanes on the autoradiograph shown in panel A were excised, rehydrated, then subjected to phosphoamino acid analysis. The migration of phosphoserine (PS), phosphotyrosine (PY) and phosphothreonine (PT) standards is shown.

(32,34). It was formally possible, therefore, that the association between CD45 and *lck* noted by our laboratories could have been mediated via an interaction between CD45 and CD4 in the Jurkat T cell line and an interaction between CD45 and CD4 and/or CD8 in peripheral blood lymphocytes. The data presented in this report indicate that the association between these other surface antigens and CD45 is not required for CD45 to interact with *lck*. Our data demonstrate further that CD45 associates with the p60 form of *lck* in approximately the same proportion as p60 *lck* is found in the cell indicating that serine phosphorylation of *lck* does not affect its ability to interact with CD45.

It is not yet clear what is the relationship between CD45, pp32-34, and *lck*. We have shown that CD45 associates with pp32-34 in a Jurkat-derived cell that is deficient in *lck* expression (23) indicating that *lck* is not required for CD45 to interact with pp32-34. However we do not yet know if the converse, i.e. the association of pp32-34 with CD45, is required for CD45 to associate with *lck*. The identity of pp32-34 is not known. Although others have shown an association between CD45 (44) and CD4 (45) with proteins of approximately 30kD, these molecules have not been cloned and their relationship to pp32-34 remains unclear. It is intriguing to speculate that pp32-34 binds directly with CD45 and is responsible for the interaction between CD45 and *lck*, a potential *in vivo* substrate. Once the identity of pp32-34 is known, it will be possible to determine more precisely the relationship between this molecule, CD45, and *lck* and investigate the role each plays in the regulation of TCR signal transduction.

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