Paxillin Phosphorylation and Association with Lck and Pyk2 in Anti-CD3- or Anti-CD45-stimulated T Cells*

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Antibodies to either CD3 or CD45 have been shown to induce dramatic changes in cell morphology, increased tyrosine phosphorylation of cellular proteins, and the association of a subset of these proteins with the tyrosine kinase Lck. The current study was initiated to determine the identity of the tyrosine-phosphorylated 70–80 kDa protein that becomes Lck-associated after stimulation with anti-CD45 or anti-CD3. We demonstrate that the cytoskeletal protein paxillin becomes tyrosine-phosphorylated when cells are plated on immobilized antibodies specific for CD45 or CD3. Only tyrosine-phosphorylated paxillin is associated with Lck, suggesting that the association is through the SH2 domain of Lck. Consistent with this we demonstrate that the SH2 domain of Lck binds tyrosine-phosphorylated paxillin. In contrast, the association of paxillin with the FAK-related kinase Pyk2 was found to be constitutive and not altered by the phosphorylation of either protein. Finally, we establish that the phosphorylation of paxillin is dependent on the expression of Lck. Taken together, these results demonstrate that paxillin is physically associated with kinases from two different families in T cells and suggest that paxillin may function as an adaptor protein linking cellular signals with cytoskeletal changes during T cell activation.

T cells have been shown to undergo dramatic changes in cell morphology, coincident with cytoskeletal rearrangements, upon activation (1, 2). A number of studies over the past few years have indicated that protein tyrosine phosphorylation and the formation of focal adhesions, the sites of contact between integrins on the cell surface and extracellular matrix, are tightly linked (3, 4). It is plausible that some of the phosphorylation events that are observed upon T cell activation regulate cytoskeletal reorganization, leading to the observed changes in cell morphology.

CD45 is a protein-tyrosine phosphatase expressed on all cells of hematopoietic origin (5). We have previously demonstrated that antibodies to CD45 induce rapid and dramatic changes in T cell morphology (6). Coincident with these changes in morphology is an increase in the tyrosine phosphorylation of proteins at approximately 70 kDa and 120 kDa and an association of these proteins with the Src-related protein-tyrosine kinase Lck (6). Phosphorylation of these proteins also occurs upon stimulation through the T cell receptor (6). Furthermore, phosphorylation of these proteins is enhanced when both anti-CD45 and anti-CD3 are coinmobilized, suggesting that engagement of CD45 or CD3 stimulates the phosphorylation of an overlapping set of proteins (6). The identification of these proteins might therefore provide insight into the regulation of T cell activation.

Phosphorylation of a Src-related kinase at its negative regulatory site results in the intramolecular interaction of the phosphorylated residue with the SH2 domain of the same molecule, rendering it inactive (7–9). An implication of this model is that dephosphorylation of Tyr-505 would not only allow the kinase to become activated but would also allow for the interaction of other phosphorylated proteins with the newly available SH2 domain of Lck, which may have important biological consequences. Consistent with this notion, recent studies have suggested that the SH2 domain of Lck is required for T cell activation (10, 11).

Paxillin is a cytoskeletal protein that localizes to sites of adhesion to extracellular matrix (12). It becomes tyrosine-phosphorylated upon integrin engagement and associates with focal adhesions (12), possibly as a consequence of its direct association with focal adhesion kinase (FAK)1 (13, 14). As a cytoskeletal protein that targets to focal adhesions, paxillin is thought to be intimately involved in cell spreading. Very little is known about the role, if any, of paxillin in T cell activation. Because T cells undergo dramatic changes in cellular morphology upon T cell activation we sought to examine the possible link of Lck with the cytoskeleton. In the current study we establish that paxillin becomes phosphorylated upon T cell action and associates with Lck and may therefore function as an adaptor protein linking tyrosine phosphorylation events with the cytoskeleton by way of its association with Lck.

EXPERIMENTAL PROCEDURES

Cell Lines—The murine H-2b-specific CTL clones 11 and AB.1 have been described previously (15). Cells were stimulated weekly with irradiated C57BL/6J spleen cells in media supplemented with interleukin-2, and experiments were performed 4–6 days after stimulation. All of the experiments that are shown were done with AB.1 and confirmed with clone 11. Jurkat and J.CaM1.6 were obtained from the ATCC.

Antibodies and Reagents—The monoclonal antibodies 145-2C11 (2C11, anti-mouse CD3) and OKT3 (anti-human CD3) were obtained through the ATCC. 15-2 (anti-CD45) was obtained from Dr. I. Trowbridge, and PY-72 (anti-phosphotyrosine) was obtained from Dr. B. Setton, both of whom are at The Salk Institute (La Jolla, CA).

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1 The abbreviations used are: FAK, focal adhesion kinase; CTL, cytotoxic T lymphocyte; GST, glutathione S-transferase; MHC, major histocompatibility complex; PAGE, polyacrylamide gel electrophoresis; SH2, Src homology 2; BSA, bovine serum albumin.
Hybridomas were grown in Protein-Free Hybridoma Medium-II (Life Technologies, Inc.). The monoclonal antibodies were purified by ammonium sulfate precipitation and, if necessary, by protein A or protein G chromatography. The monoclonal antibody specific for paxillin was obtained from Transduction Laboratories (Lexington, KY). Antiserum to the carboxyl terminus of Lck was generated in our laboratory using a bovine serum albumin-coupled peptide based on amino acids 476–509 of human Lck (6). Many of the experiments have also been done with anti-Lck (carboxyl terminus) antiserum obtained from Upstate Biotechnologies Inc. (Lake Placid, NY). Anti-Pyk2 antisera was generated in our laboratory as described previously (16). Anti-GST was obtained from Sigma. Horseradish peroxidase-coupled goat anti-mouse antibody and goat anti-hamster IgG were purchased from Jackson Immunologicals (Mississauga, ON) and protein A from Pierce. The GST fusion proteins used in this study have also been described previously (17).

**Protein Immunization**—60 mm Petri dishes were incubated with 5 ml of phosphate-buffered saline containing 15 μg/ml of the indicated antibody (2C11 or I3/2) overnight at 4 °C. Plates were then washed twice with phosphate-buffered saline, blocked with 2% bovine serum albumin in phosphate-buffered saline at 37 °C for 30 min, washed twice with phosphate-buffered saline, and then used immediately for assay.

**Immunoprecipitation, Polyacrylamide Gel Electrophoresis, and Immunoblotting**—Cells (7.5 × 10⁶ per plate) were incubated for 20 min at 37 °C and were lysed directly on the plates by adding 1.5× lysis buffer to give a final concentration of 1% Nonidet P-40 in 20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM sodium vanadate. Postnuclear lysates were incubated with the indicated antibody for 30 min on ice after which protein A-Sepharose (Pharmacia, Ste-Anne-de-Bellevue, QC) was added prior to a 90-min incubation with rotation. If a monoclonal antibody was used, rabbit anti-mouse antisera was added to facilitate immunoprecipitation. Immunoprecipitates were washed 4 times with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS) before SDS-PAGE on 7.5% gels. Immunoblotting was performed by either anti-phosphotyrosine or anti-paxillin followed by rabbit anti-mouse coupled to horseradish peroxidase. Blots were developed by chemiluminescence (NEN Life Science Products) as described in the product bulletin.

**RESULTS**

**Immunoblots, Polyclonal Gel Electrophoresis, and Immunoblotting**—Cells (7.5 × 10⁶ per plate) were incubated for 20 min at 37 °C and were lysed directly on the plates by adding 1.5× lysis buffer to give a final concentration of 1% Nonidet P-40 in 20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM sodium vanadate. Postnuclear lysates were incubated with the indicated antibody for 30 min on ice after which protein A-Sepharose (Pharmacia, Ste-Anne-de-Bellevue, QC) was added prior to a 90-min incubation with rotation. If a monoclonal antibody was used, rabbit anti-mouse antisera was added to facilitate immunoprecipitation. Immunoprecipitates were washed 4 times with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS) before SDS-PAGE on 7.5% gels. Immunoblotting was performed by either anti-phosphotyrosine or anti-paxillin followed by rabbit anti-mouse coupled to horseradish peroxidase. Blots were developed by chemiluminescence (NEN Life Science Products) as described in the product bulletin.

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CD3-stimulated cells. This phosphorylation is coincident with a significant shift in the migration of paxillin as can be seen in the anti-paxillin blot in Fig. 2B, which has been confirmed with anti-phosphotyrosine immunoblotting (data not shown). A large proportion of the paxillin, about 60–70% in most experiments, becomes phosphorylated upon anti-CD3 stimulation (Fig. 2B).

To directly determine if paxillin is present in Lck immunoprecipitates they were probed with anti-paxillin antibodies. Paxillin can be easily detected in Lck immunoprecipitates from cells plated on the indicated antibody or on BSA as a control. B, anti-paxillin blot of postnuclear lysates from cells stimulated as in A. The open arrow indicates the position of non-tyrosine-phosphorylated paxillin and the closed arrow the position of tyrosine-phosphorylated paxillin.

**FIG. 1. Antibodies to CD45 or CD3 induce the tyrosine phosphorylation and association of an 80-kDa protein with Lck.** Anti-phosphotyrosine blots of lysates or anti-Lck immunoprecipitates from AB.1 CTL cells plated on BSA as a control or on immobilized anti-CD45 (I3/2) or anti-CD3 (2C11). Antiserum to ZAP-70 was used as a negative control for the associations. The closed arrowhead indicates the 80-kDa protein that associates with Lck after either anti-CD45 or anti-CD3 stimulation, and the open arrowhead indicates the position of Lck.

**FIG. 2. Paxillin becomes tyrosine-phosphorylated in response to immobilized anti-CD3 or anti-CD45.** A, anti-phosphotyrosine blot of paxillin immunoprecipitated from cells plated on the indicated antibody or on BSA as a control. B, anti-paxillin blot of postnuclear lysates from cells stimulated as in A. The open arrow indicates the position of non-tyrosine-phosphorylated paxillin and the closed arrow the position of tyrosine-phosphorylated paxillin.
Paxillin Associates with the SH2 Domain of Lck—That the association between Lck and paxillin requires the tyrosine phosphorylation of paxillin implies that the interaction may be mediated through the SH2 domain of Lck. We are able to compete for binding of essentially all of the Lck-associated proteins with phenyl phosphate (data not shown), which is consistent with what has been shown previously with Lck immunoprecipitates prepared from activated T cells (18). Anti-phosphotyrosine immunoblotting of paxillin immunoprecipitates prepared from phenyl phosphate eluates of Lck immunoprecipitates reveal that tyrosine-phosphorylated paxillin is bound to Lck; however no tyrosine-phosphorylated paxillin is observed in the control rabbit anti-mouse Ig immunoprecipitate from 2C11-stimulated cells, and very little is detected in paxillin immunoprecipitates from unstimulated cells (Fig. 4). These results further confirm that paxillin associates with Lck in CTL clones and suggest that these proteins associate in a phosphotyrosine-dependent manner and possibly a SH2-dependent manner.

To directly address the question of whether paxillin associates with the SH2 domain of Lck, we generated GST fusion proteins containing either the SH2 or the SH3 domain of Lck. The GST fusion proteins were mixed with lysates from AB.1 stimulated with either anti-CD45 or anti-CD3, and the complexes were captured with glutathione beads. An 80-kDa tyrosine-phosphorylated protein can associate with the Lck-SH2 domain GST fusion protein after stimulation of cells with 2C11 and to a low level after plating of cells on immobilized anti-CD45 (Fig. 5). This phosphoprotein does not associate with our SH3 domain preparations or with GST alone (Fig. 5). When this same blot is probed with anti-paxillin (Fig. 5), it is clear that paxillin specifically associates with the SH2 domain of Lck. Although not apparent on this blot, only the more slowly migrating phosphorylated form of paxillin is associating with the fusion protein (data not shown).

The argument could still be made that paxillin does not interact with the SH2 domain of Lck directly but rather forms a complex with a phosphoprotein that itself interacts with Lck. To assess whether paxillin binds directly to the SH2 domain of Lck, a Far Western blot of paxillin immunoprecipitates using GST-SH2-Lck as a probe was completed. The data presented in Fig. 6 show that the SH2 domain of Lck binds to phosphorylated paxillin. Interestingly, the GST-SH2-Lck probe fails to interact with immunoprecipitates from unstimulated cells, which contain only nonphosphorylated paxillin (Fig. 6). Since anti-CD45 does not induce extensive phosphorylation of paxillin, we did not include it in these studies. These experiments confirm that there can be a direct interaction between the SH2 domain of Lck and tyrosine-phosphorylated paxillin.

Paxillin Is Constitutively Associated with Pyk2 in CTL—It has been demonstrated that paxillin can directly associate with FAK through an interaction that is phosphotyrosine independent (13, 14). Recently paxillin has also been shown to constitutively associate with the FAK-related kinase Pyk2, also known as related adhesion focal tyrosine kinase (RAFTK), in T cells with only a modest increase in the level of association after T cell activation (19). Since both FAK and Pyk2 are expressed in...
Lck is required, either directly or indirectly, for paxillin phosphorylation in the J.CaM1.6 cells (Fig. 9). Taken together these results demonstrate that paxillin does not become tyrosine-phosphorylated in the J.CaM1.6 cells. However, examination of paxillin phosphorylation reveals that PP1 completely inhibited its tyrosine phosphorylation after stimulation (Fig. 7). These results indicate that paxillin is constitutively associated with Pyk2 but not with FAK in these T cell clones.

Lck Is Required for the Tyrosine Phosphorylation of Paxillin

Since paxillin is physically associated with at least two different tyrosine kinases in T cells, it is of interest to determine if either of these kinases might be responsible for its phosphorylation. To address this question we made use of the Src-related tyrosine kinase inhibitor PP1 (20). When cells are treated with this drug prior to anti-CD3 stimulation, as previously observed (20), no increased tyrosine phosphorylation is detected (Fig. 8A). In this experiment cells were stimulated with cross-linked anti-CD3 in solution since we have observed that PP1 prevents cell spreading thereby limiting the ability of cells to contact immobilized antibody (data not shown). When the phosphorylation status of paxillin was examined, we found that PP1 completely inhibited its tyrosine phosphorylation (Fig. 8B), suggesting that a Src-related kinase is required upstream of paxillin phosphorylation. However, this inhibition could be either direct or indirect because PP1 also inhibits the induction of Pyk2 phosphorylation, although PP1 does not directly inhibit Pyk2 kinase activity as measured by autophosphorylation (data not shown).

To more directly assess the role of Lck in the induction of tyrosine phosphorylation of paxillin we examined paxillin phosphorylation in the Lck-deficient variant of Jurkat called J.CaM1.6. We have previously demonstrated that Lck is not required for the induction of FAK or Pyk2 phosphorylation (17). However, examination of paxillin phosphorylation reveals that it does not become tyrosine-phosphorylated in the J.CaM1.6 cells (Fig. 9). Taken together these results demonstrate that Lck is required, either directly or indirectly, for paxillin phosphorylation.

DISCUSSION

We have provided the first demonstration that antibodies to CD45 or CD3 stimulate the phosphorylation of paxillin leading to its association with the SH2 domain of Lck. Paxillin contains a number of potential tyrosine phosphorylation sites, five of which conform to SH2-binding motifs (21), and it binds to the SH2 domain of Src upon tyrosine phosphorylation (22). Since Lck is the predominant Src-related kinase found in T cells it could be predicted that it might bind to paxillin via its SH2 domain. Paxillin also contains a proline-rich domain that may be important for linking it to other signaling molecules or cytoskeletal components (21). Because paxillin is a major component of the cytoskeleton and can bind to multiple signaling molecules it may function as an adaptor molecule linking intracellular signals with morphologic changes known to accompany T cell activation. The phosphorylation of paxillin may therefore be important for regulating its adaptor function during T cell activation.

In addition to the association with Lck, we have also found that paxillin constitutively associates with Pyk2 in T cells, as previously shown (19). We could detect little or no association between FAK and paxillin. These results show that paxillin is able to bind to at least two kinases from different tyrosine kinase families in T cells; however it is not clear which kinase, if either, is directly responsible for phosphorylating paxillin. Both FAK and Pyk2 have been shown to phosphorylate paxillin.
It is therefore possible that one or both of these kinases is responsible for paxillin phosphorylation in CTL clones. It is also possible that Lck might phosphorylate paxillin since paxillin has also been shown to be a substrate for Src (22). Consistent with this we have demonstrated that the Src kinase inhibitor PP1 is able to inhibit phosphorylation of paxillin. Although we cannot formally exclude a role for FAK or Pyk2 in phosphorylating paxillin, we reason that paxillin is unlikely to be a direct substrate of these kinases since both FAK and Pyk2 become phosphorylated (17) in the Lck-deficient cells while paxillin does not become phosphorylated (Fig. 9). It is also conceivable that both Pyk2 and Lck phosphorylate paxillin at different sites. Studies are currently underway to distinguish these possibilities.

Adhesion through a number of different integrin receptors is known to play an important role in T cell activation. The signals that are required to induce the cytoskeletal changes required for increased adhesion remain largely unknown. Our previous results show that cell spreading, which is coincident with increased adhesion, correlates with increased tyrosine phosphorylation (6). Which is cause and which is effect remains to be determined. Interestingly, only those antibodies or ligands that induce cell spreading induce tyrosine phosphorylation of paxillin. So far this is limited, in our hands, to purified class I MHC molecules (data not shown) and antibodies to the T cell receptor complex or CD45. We have shown that antibodies to β3 integrins, which are expressed on these T cells, stimulate phosphorylation of FAK and Pyk2 (16); however no significant cell spreading or paxillin phosphorylation is stimulated by these antibodies. Furthermore, antibodies to LFA-1 or class I MHC induce limited phosphorylation of FAK and Pyk2; however little or no cell spreading and paxillin phosphorylation is observed with these antibodies (data not shown). Taken together these results suggest that cell spreading correlates with paxillin phosphorylation. Paxillin phosphorylation does not appear to require adhesion since phosphorylation of paxillin occurs, albeit very transiently, upon stimulation of CTL with soluble cross-linked antibody to CD3 (Fig. 8 and data not shown). We believe, therefore, that paxillin phosphorylation leads to cell spreading and not vice versa.

That CD45 plays a role in regulating the phosphorylation of paxillin suggests that it might be involved in the regulation of adhesion. It has been clearly established that antibodies to CD45 can induce homotypic adhesion in various cell types (24–26). We have observed that CD45-deficient cell lines do not appear to be as adhesive as their CD45-expressing counterparts. Recently Roach et al. (27) have shown that macrophages from CD45-deficient mice are unable to maintain integrin-mediated adhesion. They further demonstrated that this disregulation of adhesion correlates with the hyperphosphorylation of Src-related kinases (27). It is possible that CD45 might regulate adhesion in a global manner by dephosphorylating Src-related kinases which in turn function to regulate the phosphorylation of paxillin. Interestingly, a CD45-related transmembrane tyrosine phosphatase, LAR, has been found to localize to focal adhesions, where FAK and paxillin are located, and may induce their disassembly (28). Taken together these studies support the provocative idea that CD45 regulates adhesion by regulating FAK, Pyk2, and paxillin phosphorylation, perhaps via Lck or other Src-related kinases.

In summary, the phosphorylation of paxillin, and likely its function, are regulated through the T cell receptor and can be modulated through CD45 engagement. It appears that paxillin might act as a type of adaptor protein that is able to bind to tyrosine kinases and cytoskeletal proteins and may therefore be important for integrating growth signals and cellular adhesion, both of which are essential for mitogenic signals in T cells.

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