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Control of TCR-Mediated Activation of $\beta_1$ Integrins by the ZAP-70 Tyrosine Kinase Interdomain B Region and the Linker for Activation of T Cells Adapter Protein

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One of the earliest functional responses of T lymphocytes to extracellular signals that activate the Ag-specific CD3/TCR complex is a rapid, but reversible, increase in the functional activity of integrin adhesion receptors. Previous studies have implicated the tyrosine kinase $\zeta$-associated protein of 70 kDa (ZAP-70) and the lipid kinase phosphatidylinositol 3-kinase, in the activation of $\beta_1$ integrins by the CD3/TCR complex. In this report, we use human ZAP-70-deficient Jurkat T cells to demonstrate that the kinase activity of ZAP-70 is required for CD3/TCR-mediated increases in $\beta_1$ integrin-mediated adhesion and activation of phosphatidylinositol 3-kinase. A tyrosine to phenylalanine substitution at position 315 in the interdomain B of ZAP-70 inhibits these responses, whereas a similar substitution at position 292 enhances these downstream signals. These mutations in the ZAP-70 interdomain B region also specifically affect CD3/TCR-mediated tyrosine phosphorylation of residues 171 and 191 in the cytoplasmic domain of the linker for activation of T cells (LAT) adapter protein. CD3/TCR signaling to $\beta_1$ integrins is defective in LAT-deficient Jurkat T cells, and can be restored with expression of wild-type LAT. Mutant LAT constructs with tyrosine to phenylalanine substitutions at position 171 and/or position 191 do not restore CD3/TCR-mediated activation of $\beta_1$ integrins in LAT-deficient T cells. Thus, these studies demonstrate that the interdomain B region of ZAP-70 regulates $\beta_1$ integrin activation by the CD3/TCR via control of tyrosine phosphorylation of tyrosine residues 171 and 191 in the LAT cytoplasmic domain. The Journal of Immunology, 2004, 172: 5379–5387.

In the immune system, the integrin family of adhesion receptors mediates critical interactions of T cells with other cells and extracellular matrix (ECM) proteins (1). These adhesive interactions mediate T cell homing and trafficking into and through secondary lymphoid organs and peripheral tissue sites, as well as mediate the intercellular interactions between T cells and APCs that are essential for the activation and subsequent clonal expansion of T cells during encounter with a foreign pathogen (1–4). To facilitate changes in T cell movement in response to environmental signals, the functional activity of integrin receptors expressed on T cells is dynamically regulated by the activation state of the T cell (5, 6). Unstimulated T cells express relatively inactive integrin receptors that mediate low levels of adhesion to relevant ECM proteins and cellular counterreceptors. However, activation of the T cell by ligand engagement of the CD3/TCR complex results within minutes in a rapid increase in the functional activity of integrin receptors that does not require increased levels of integrin receptors on the cell surface (7–9). Integrin function can also be enhanced via treatment of cells with phorbol esters or with unique Abs or changes in the composition of extracellular divalent cations, which induce conformational changes in integrin receptors that enhance integrin affinity (9–12). Studies in T cells have also suggested that integrin activation induced by the CD3/TCR complex involves cytoskeletal-dependent changes in integrin membrane distribution (13–16).

Productive stimulation of T cells by APCs bearing the appropriate MHC-peptide complex requires activation by the CD3/TCR of the $\zeta$-associated protein of 70 kDa (ZAP-70) tyrosine kinase, a member of the Syk family of tyrosine kinases (17). Phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM), which is found in the cytoplasmic domains of the TCR $\zeta$-chain and CD3 subunits of the CD3/TCR complex, results in the association of ZAP-70 with CD3/TCR via the dual src homology 2 (SH2) domains in ZAP-70 (18). Subsequent phosphorylation of ZAP-70 by the src family tyrosine kinase Lck at key tyrosine residues, including tyrosine 493 in the kinase domain, leads to enhanced ZAP-70 tyrosine kinase activity (19). Additional tyrosine residues in the interdomain B region of ZAP-70 have both positive and negative regulatory effects on ZAP-70 function without altering ZAP-70 catalytic activity (20–23). Tyrosine 292 in the interdomain B region of ZAP-70 functions as a negative regulatory site, as mutation of this tyrosine to phenylalanine enhances ZAP-70 activity (24). In addition, tyrosine 292 interacts with the negative regulatory adapter protein p120$^{CA}$ (Cbl) (25), and Cbl has been proposed to mediate ubiquitination of TCR$\zeta$ following CD3/TCR stimulation (26). In contrast, mutation of tyrosine 315 in the interdomain B region to phenylalanine attenuates ZAP-70 function (21). Although phosphorylation of tyrosine 315 following CD3/
TCR stimulation has been proposed to potentially serve as an inducible binding site for the SH2 domain of Vav, a guanine-nucleotide exchange factor for Rho family GTPases (21, 27), studies in mice suggest that the inhibitory effects of mutating tyrosine 315 may not be mediated by loss of ZAP-70 interaction with Vav (28).

Activation of ZAP-70 results in phosphorylation of multiple tyrosine residues in the cytoplasmic domain of the transmembrane adapter protein linker for activation of T cells (LAT) (29, 30), which leads to the formation of a multiprotein complex containing LAT and a number of additional signaling proteins, including the growth factor receptor-bound protein (Grb)2, the Grb2-related adapter downstream of Shc protein (Gads), the SH2 domain-containing leukocyte protein (SLP)-76, the tyrosine kinase Itk, phospholipase C (PLC)-γ1, and the lipid kinase phosphatidylinositol 3-kinase (PI3-K) (31–35). This complex is particularly critical for tyrosine phosphorylation and activation of PLC-γ1, resulting in Ca²⁺ mobilization and activation of Ras signaling events that ultimately lead to transcriptional activation and actin reorganization (29, 30). The critical function of LAT in TCR signaling is illustrated by defects in T cell function in LAT-deficient T cell lines (36, 37) and the profound block in T cell development in LAT-deficient Jurkat T cell lines (36) by which ZAP-70 regulates CD3/TCR signaling to integrins remains undefined. Furthermore, the role of LAT in CD3/TCR signaling to integrins remains undefined. In this report, we use genetic variants of the Jurkat T cell line to define the mechanism by which ZAP-70 regulates CD3/TCR signaling to βι integrin functional activity. Furthermore, the role of LAT in CD3/TCR signaling to integrins remains undefined. Our results demonstrate that ZAP-70 tyrosine residues 315 and 292 mediate positive and negative regulatory effects on CD3/TCR-dependent activation of βι integrins, activation of PI3-K, and phosphorylation of two key tyrosine residues, amino acids 171 and 191, in the cytoplasmic domain of LAT. In addition, we find that activation of βι integrins by the CD3/TCR complex is dependent on LAT expression and on tyrosine residues 171 and 191 in the LAT cytoplasmic domain.

Materials and Methods

Abs and reagents

The CD3-specific mAb OKT3 and the activating β1 integrin-specific mAb TS2/16 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The CD3-specific mAb 38.1 was provided by Dr. S. Shaw (Bethesda, MD). The anti-mouse CD3 mAb 2C11 was purchased from BD Pharmingen (San Diego, CA). Goat anti-mouse IgG was purchased from ICN Biomedical Pharmaceuticals (Costa Mesa, CA). The phosphotyrosine-specific mAb 4G10 and the anti-LAT antiserum were purchased from Upstate Biotechnology (Lake Placid, NY). Abs that recognize tyrosine phosphorylated 171 and tyrosine phosphorylated 191 in the LAT cytoplasmic domain were purchased from Cell Signaling Technology (Beverly, MA). The anti-hemagglutinin (HA) Ab 3F10 was purchased from Roche (Indianapolis, IN) and the anti-HA mAb 16B12 was purchased from Covance (Berkeley, CA). The goat anti-mouse IgG HRP conjugate was purchased from Caltag Laboratories (Burlingame, CA). Anti-CD3 beads (Dynabeads M-450 CD3) were purchased from Dynal Biotech (Lake Success, NY). The phorbol ester PMA was purchased from LC Laboratories (Woburn, MA). Human fibronectin was purchased from Invitrogen (San Diego, CA) and mouse VCAM-1 and ICAM-1 were purchased from R&D Systems (Minneapolis, MN). U73122 and U73343 were purchased from Biomol (Plymouth Meeting, PA).

DNA constructs

The pIRE6-EGFP-HA-ZAP-70 and pIRE6-EGFP-HA-ZAP-70 (Y292F) bicistronic plasmid expression constructs encoding for HA-tagged forms of human ZAP-70 and containing internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP) have been previously described (44). Additional ZAP-70 mutations were generated with the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA) using both sense and antisense oligonucleotides encoding for the substitution of tyrosine at position 315 with the corresponding alanine (Y315F) and for the substitution of tyrosine at position 493 with phenylalanine (Y493F). The pIRE6-EGFP-HA-LAT and pIRE6-EGFP-HA-Y171/191F LAT bicistronic plasmid expression constructs encoding for HA-tagged forms of human LAT have been previously described (41). The Y171F and Y191F LAT mutations were generated as previously described using the pIRE6-EGFP-HA-LAT construct as a template for site-directed mutagenesis. All mutations were confirmed by sequencing at the University of Minnesota Microchemical Facilities (Minneapolis, MN).

Cell lines and cell culture

The ZAP-70-deficient Jurkat T cell line P116 was provided by Drs. R. Abraham and B. Irvin (Mayo Clinic, Rochester, MN) (45). The LAT-deficient Jurkat T cell line J.CaM2 (36) was provided by Dr. A. Weiss (University of California, San Francisco, CA). Wild-type Jurkat T cells (ATCC) and the anti-HA mAb 16B12 were maintained in RPMI 1640 medium supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA), t-glutamine, and penicillin/streptomycin. Stable transfectants of P116 cells and J.CaM2 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, t-glutamine, penicillin/streptomycin and 2 mg/ml G418.

Stable transfectants of P116 T cells expressing wild-type and mutant forms of ZAP-70 and J.CaM2 cells expressing wild-type and mutant forms of LAT were isolated as previously described (46). G418-resistant cells were analyzed for GFP expression and sorted to obtain populations of cells expressing homogeneous levels of ZAP-70 or LAT (data not shown).

Isolation of murine T cells

C57BL/6 mice, 7–8 wk of age, were purchased from the National Cancer Institute (Frederick, MD) and housed in specific pathogen-free facilities at the University of Minnesota. Lymph node T cells were purified by negative magnetic immunoselection as previously described (47) and were ≥98% CD3⁺. All experimental protocols involving the use of mice were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Transient transfections

Transient transfections with ZAP-70 constructs (25 μg per 10 × 10⁶ cells) were performed as previously described using a BTX square wave electroporator (BTX, San Diego, CA) set at 240 V with a pulse length of 25 ms (48). After electroporation, cells were incubated for 10 min at room temperature before resuspension at 1 × 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% FCS, t-glutamine and penicillin/streptomycin. Cells were harvested after 14–18 h for use in Western blotting or in adhesion assays.

Adhesion assays

The adhesion of transiently transfected P116 T cells to fibronectin was performed as previously described (41, 46). For PMA stimulation, T cells were added to wells containing 10 ng/ml PMA. For direct βι integrin activation, T cells were added to wells containing the activating βι integrin-specific mAb TS2/16 (1/20 final dilution). For CD3 stimulation, cells precoated with OKT3 mAb (anti-CD3) were added to wells containing 1 μg/ml goat anti-mouse IgG. Plates were then rapidly warmed to 37°C for 10 min and washed to remove nonadherent cells. An aliquot of each cell sample representing the same volume used in each well for the adhesion assay was prepared for flow cytometric analysis for verification of the cell numbers added per well. Adherent cells were removed with PBS/0.1% EDTA and collected. Cells from six replicate wells were pooled into a tube, pelleted and resuspended in 200 μl of PBS/5% FCS supplemented with 50 μl of PKH26 reference microbeads (Sigma-Aldrich, St. Louis, MO) and 25 μl of propidium iodide (Sigma-Aldrich). Each sample was then analyzed on a Becton Dickinson FACScan (BD Biosciences, San Jose, CA) as previously described (41, 46). For each sample, forward scatter/side scatter profiles were used to identify the transfected cells and the microbeads. FL2 events in the microbead gate were used to determine the total number of beads acquired in each sample. The total number of reference microbeads acquired was divided by the bead density to obtain the total volume of

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sample acquired. FL1 fluorescence reflects eGFP expression and FL1-positive events were gated into four subpopulations: eGFP-negative cells and three subpopulations with increasing eGFP expression. Within these subpopulations, the total number of T cells in each sample was then determined by the following equation: [T cells acquired] (milliliters of sample acquired) · (0.25 µl) · (initial numbers of T cells added to each well at the start of the adhesion assay) = (data not shown). The adhesion of the transiently transfected population of P116 T cells to fibronectin following various stimulation conditions was assessed as previously described by using flow cytometry to quantitate the adhesion of P116 T cells expressing varying levels of GFP (41, 46). In the results shown in Fig. 1, transiently transfected cells lacking GFP (labeled 0 on the x-axis of each graph) exhibited a basal level of adhesion to fibronectin. Stimulation of the CD3/TCR complex via cross-linking with the anti-CD3 mAb OKT3 failed to enhance the adhesion of GFP-negative cells above the level seen with unstimulated cells. In contrast, stimulation for 10 min at 37°C with the phorbol ester PMA, which bypasses defects in proximal TCR signaling in P116 T cells, resulted in enhanced adhesion of P116 T cells to fibronectin. Similar enhancing effects were observed with the βi integrin-specific activating mAb TS2/16 (data not shown). These results indicate that βi integrins expressed on P116 T cells can respond to appropriate integrin-activating signals. In P116 T cells expressing wild-type ZAP-70, CD3/TCR stimulation resulted in increased adhesion to fibronectin when compared with unstimulated cells expressing comparable levels of ZAP-70 (Fig. 1). Furthermore, the magnitude of the increase in adhesion following CD3 stimulation was dependent on the amount

Each tyrosine to phenylalanine mutation was made in a HA-tagged form of wild-type ZAP-70 expressed in a GFP bicistronic plasmid expression vector (41). ZAP-70-deficient P116 Jurkat T cells were transiently transfected with these ZAP-70 expression vectors, and transfection efficiency, as assessed by GFP expression, varied between 20% and 40% (data not shown). GFP expression correlates with expression of HA-tagged ZAP-70 (Ref. 41 and data not shown). Immunoprecipitation and Western blotting were performed as previously described. Lysates were preincubated in the indicated amount of inhibitor (U73122 or U73343) for 3 min, and lysed by adding an equivalent volume of lysis buffer (1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.002 M EDTA, 0.01 M sodium phosphate, pH 7.2, 2 mM sodium vanadate, 20 mM µg/ml leupeptin, 20 µg/ml aprotinin, and 2 mM PMSF). Lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4°C. Immunoprecipitations were performed as previously described using anti-HA (3F10) or anti-LAT-coated protein G beads (Zymed Laboratories, San Francisco, CA) for 2 h at 4°C as previously described. After washing with lysis buffer, proteins were removed from beads by boiling. Samples were separated on 7.5% or 10% SDS polyacrylamide gels, and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) and immunoblotted with anti-LAT, anti-HA (16B12), or anti-pTyr (4G10) Abs (1/1000 dilution in PBS/5% milk), followed by appropriate dilutions of HRP-conjugated secondary Abs (1/50,000 for donkey anti-rabbit IgG and 1/50,000 for goat anti-mouse IgG). Membranes were developed by ECL (Pierce, Rockford, IL) according to the manufacturer’s instructions. Stripping and reprobing of membranes was performed as previously described.

To assess LAT expression and phosphorylation in J.Cam2 T cells expressing wild-type and mutant forms of LAT, T cells were stimulated for 0 or 5 min at 37°C using anti-CD3 beads. The cells were lysed using an equivalent volume of 2× lysis buffer (as previously described). Immunoprecipitation and Western blotting were performed as previously described.

**PI3-K assays**

Cells were stimulated with the anti-CD3 mAb OKT3 as previously described for 10 min at 37°C. Unstimulated and CD3-stimulated cells were lysed, and lysates were immunoprecipitated with the anti-phosphotyrosine mAb 4G10 as previously described. PI3-K activity was measured by in vitro phosphorylation of the PI3-K substrate phosphatidylinositol 4,5-bisphosphate and detection by TLC as previously described (50, 51).

**Results**

**Effects of ZAP-70 mutations on CD3-induced adhesion of Jurkat T cells to fibronectin.**

We have previously demonstrated that increases in βi integrin-mediated adhesion to the ECM protein fibronectin following CD3/TCR stimulation are impaired in the ZAP-70-deficient Jurkat T cell line P116 (41). However, CD3/TCR signaling to βi integrins can be restored in this cell line upon expression of wild-type, but not kinase-inactive, ZAP-70 (41). To further define the mechanism by which ZAP-70 regulates CD3/TCR-mediated increases in βi integrin function, we mutated three key tyrosine residues in ZAP-70 to phenylalanine and determined the effect of these mutations on CD3/TCR signaling to βi integrins. Two of these tyrosine residues, at positions 292 and 315, lie in the interdomain B region of ZAP-70 (21, 24). The third tyrosine, at position 493 in the kinase domain, is phosphorylated by lck and is critical to the kinase activity of ZAP-70 (19).
of ZAP-70 expressed in the cell. ZAP-70 expression did not alter either the basal or PMA-induced adhesion of P116 T cells to fibronectin. In contrast to the results obtained with wild-type ZAP-70, expression of a mutant form of ZAP-70, in which tyrosine 493 has been mutated to phenylalanine (Y493F), failed to restore CD3/TCR signaling to β1 integrins, even when Y493F ZAP-70 was expressed at high levels (Fig. 1). This result indicates a critical function for tyrosine 493 in ZAP-70-dependent regulation of β1 integrin function by the CD3/TCR.

When compared with wild-type ZAP-70, CD3/TCR signaling to β1 integrins was also impaired following expression of ZAP-70 in which tyrosine 315 in the interdomain B region of ZAP-70 has been mutated to phenylalanine (Y315F) (Fig. 1). However, unlike Y493F ZAP-70, expression of Y315F ZAP-70 was able to partially restore CD3/TCR-mediated increases in β1 integrin function. In contrast to the results obtained with both Y493F ZAP-70 and Y315F ZAP-70, mutation of tyrosine 292 in the interdomain B region of ZAP-70 to phenylalanine (Y292F) resulted in an enhanced ability to restore CD3/TCR signaling to β1 integrins when compared with wild-type ZAP-70 (Fig. 1). We also assessed adhesion induced by CD3 stimulation induced by the OKT3 mAb in the absence of secondary cross-linking. Similar results were observed in that OKT3 in the absence of additional cross-linking induced higher levels of adhesion of P116 T cells expressing Y292F ZAP-70 compared with P116 T cells expressing wild-type ZAP-70. However, the overall levels of adhesion were lower compared with OKT3 with cross-linking, and thus expression of the Y315F ZAP-70 construct failed to restore adhesion above that observed with the Y493F kinase-inactive ZAP-70 mutant (data not shown). Similar results were observed when we used a different anti-CD3 mAb, 38.1. Thus, these studies have identified tyrosine residues that have both positive (Y493 and Y315) and negative (Y292) regulatory effects on ZAP-70-dependent coupling of the CD3/TCR to β1 integrin functional activity.

Effects of ZAP-70 mutations on CD3-mediated activation of PI3-K

Activation of the lipid kinase PI3-K has been implicated in the regulation of βι integrin-dependent adhesion by the CD3/TCR complex (39). Therefore, we investigated the role of ZAP-70 in coupling CD3/TCR to PI3-K. PI3-K activity in anti-phosphotyrosine immunoprecipitates, as assessed by in vitro kinase assays using phosphatidylinositol 4,5-bisphosphate as a PI3-K substrate, was determined in unstimulated and CD3-stimulated ZAP-70-deficient P116 T cells. CD3 stimulation of P116 T cells failed to enhance PI3-K activity above that observed with unstimulated P116 T cells (data not shown). However, CD3 stimulation of P116 T cells transfectants expressing wild-type ZAP-70 resulted in an ∼4-fold increase in PI3-K activity (Fig. 2). The magnitude of the increase in PI3-K activity observed upon re-expression of wild-type ZAP-70 was observed in several independent subclones of P116 T cell transfectants and was similar to the increase seen following CD3 stimulation of wild-type Jurkat T cells (Fig. 2 and data not shown). In contrast to the results obtained with wild-type ZAP-70, CD3 stimulation of P116 transfectants expressing the kinase-dead Y493F mutant ZAP-70 construct did not result in enhanced PI3-K activity. Expression of Y315F ZAP-70, which partially restores CD3/TCR signaling to βι integrins, also partially restored CD3/TCR-mediated activation of PI3-K (Fig. 2). Similar to the results obtained in adhesion assays, expression of Y292F ZAP-70 resulted in CD3/TCR-mediated activation of PI3-K that was greater than that observed upon expression of similar levels of wild-type ZAP-70 (Fig. 2).

Effects of ZAP-70 mutations on CD3-induced tyrosine phosphorylation of LAT

Because ZAP-70-dependent tyrosine phosphorylation of the adapter protein LAT plays a major role in TCR signaling (31), we next explored the role of LAT in regulating CD3/TCR signaling to βι integrins. CD3-induced tyrosine phosphorylation of LAT was assessed in P116 T cells expressing wild-type and mutant forms of ZAP-70. In the absence of ZAP-70, CD3 stimulation failed to induce tyrosine phosphorylation of LAT, as detected with the anti-phosphotyrosine mAb 4G10 (Fig. 3A). However, tyrosine phosphorylation of LAT was detected following CD3 stimulation of P116 T cells expressing wild-type ZAP-70. Expression of kinase-dead Y493F ZAP-70 did not restore CD3-mediated tyrosine phosphorylation of LAT, consistent with the requirement for ZAP-70 kinase activity in mediating TCR-induced phosphorylation of this adapter protein. Although the Y315F mutation impairs CD3-induced activation of βι integrin function and activation of PI3-K, overall levels of tyrosine phosphorylation of LAT following CD3 stimulation were comparable in P116 T cells expressing wild-type ZAP-70 and Y315F ZAP-70. In contrast, expression of Y292F ZAP-70 resulted in enhanced CD3-mediated tyrosine phosphorylation of LAT when compared with P116 T cells expressing wild-type ZAP-70 (Fig. 3A).

Because the anti-phosphotyrosine mAb 4G10 assesses total tyrosine phosphorylation of LAT, we used phosphorylation-specific anti-LAT Abs to determine whether mutations in ZAP-70 that altered TCR-dependent βι integrin activation specifically altered tyrosine phosphorylation of key tyrosine residues in the LAT cytoplasmic domain. We used two Abs that recognize tyrosine phosphorylated residues 171 and 191 in the LAT cytoplasmic domain. Tyrosines 171 and 191 are part of key SH2 domain binding sites in LAT that have been implicated in interactions of tyrosine phosphorylated LAT with Gb2, Gads, PLC-γ1, SLP-76, and the regulatory p85 subunit of PI3-K (31, 34, 52). CD3 stimulation of P116 T cells expressing control vector or the Y493F ZAP-70 resulted in minimal phosphorylation of tyrosines 171 and 191 in...
in intracellular calcium and expression of IL-2 (36). CD3 stimulation of the J.CaM2 T cell line also results in dramatically impaired adhesion to fibronectin (Fig. 4). The adhesion response of J.CaM2 cells to CD3 stimulation was only slightly higher than the adhesion of unstimulated J.CaM2 cells to fibronectin. However, β1 integrin expression and function is normal in J.CaM2 cells, as J.CaM2 cells express αβ1 and αδβ1 integrins at levels comparable to wild-type Jurkat T cells (data not shown), and stimuli that increase adhesion independently of CD3/TCR stimulation, such as PMA and the activating β1 integrin-specific mAb TS2/16, enhance adhesion of J.CaM2 cells to fibronectin to levels comparable to wild-type Jurkat T cells treated with the same activation stimuli (Fig. 4).

To verify that the loss of LAT is responsible for reduced adhesion, an HA-tagged form of wild-type LAT was re-expressed in J.CaM2 cells. CD3 stimulation of LAT+ J.CaM2 transfectants resulted in increases in adhesion to fibronectin that were comparable to CD3 stimulation of wild-type Jurkat T cells (Fig. 4). Flow cytometric analysis revealed no changes in expression of CD3, αβ1 integrin and αδβ1 integrin on the LAT+ J.CaM2 transfectants compared with J.CaM2 or wild-type Jurkat T cells (data not shown). These results suggest a critical role for LAT in coupling the CD3/TCR to β1 integrin function.

**Mutation of tyrosines 171 and 191 in the LAT cytoplasmic domain impairs CD3/TCR signaling to β1 integrins**

Because mutant ZAP-70 constructs that failed to restore CD3/TCR signaling to β1 integrins in P116 T cells also did not restore CD3-mediated tyrosine phosphorylation of residues 171 and 191 in the LAT cytoplasmic domain, we determined whether mutation of these tyrosine residues to phenylalanine altered the ability of LAT to restore CD3/TCR-mediated activation of β1 integrins. We analyzed transfectants of J.CaM2 T cells expressing three different mutant forms of LAT: Y171F LAT, in which tyrosine 171 was mutated to phenylalanine; Y191F LAT, in which tyrosine 191 was mutated to phenylalanine; and Y171/191F LAT, in which both tyrosines were mutated to phenylalanine. Although CD3 stimulation of J.CaM2 T cells expressing wild-type LAT enhanced T cell adhesion to fibronectin to levels comparable to that observed with PMA stimulation (Fig. 4 and Fig. 5A) or β1 integrin Ab stimulation (Fig. 4 and data not shown), CD3 stimulation of J.CaM2 T cells expressing any of the three LAT mutants (Y171F LAT, Y191F LAT, or Y171/191F LAT) failed to enhance adhesion above what

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**FIGURE 3.** Effects of ZAP-70 mutations on CD3-mediated tyrosine phosphorylation of LAT. P116 cells were transfected with the indicated ZAP-70 constructs and either left unstimulated or stimulated for 3 min at 37°C with the anti-CD3 mAb OKT3 and goat anti-mouse IgA. A, LAT was immunoprecipitated from 1 × 10^6 GFP-positive cells, and immunoprecipitates were analyzed by Western blotting with the phosphotyrosine mAb 4G10 (top). Membranes were subsequently stripped and reprobed with an anti-LAT Ab (middle). Western blotting analysis of anti-HA immunoprecipitates with an anti-HA mAb also was performed with each set of transfected cells to verify expression of HA-tagged ZAP-70 (bottom). B, Whole cell lysates (1 × 10^6 GFP-positive cell equivalents) were analyzed by Western blotting with the anti-phospho LAT Y171 Ab (top) or the anti-phospho LAT Y191 Ab (middle). Membranes were subsequently stripped and reprobed with an anti-LAT Ab (bottom). Western blotting analysis of anti-HA immunoprecipitates with an anti-HA mAb was also performed with each set of transfected cells to verify expression of HA-tagged ZAP-70.

LAT (Fig. 3B). In contrast, expression of wild-type ZAP-70 in P116 T cells resulted in clearly detectable tyrosine phosphorylation of both tyrosine 171 and tyrosine 191 following CD3 stimulation. Similar to the results observed with the anti-phosphotyrosine mAb 4G10, CD3 stimulation of P116 T cells expressing the Y292F ZAP-70 mutant resulted in decreased phosphorylation of tyrosines 171 and 191 when compared with CD3-stimulated P116 T cells expressing wild-type ZAP-70. In contrast, a specific effect of the Y315F ZAP-70 mutation on CD3-mediated tyrosine phosphorylation of LAT tyrosines 171 and 191 was observed. Although overall tyrosine phosphorylation of LAT in CD3-stimulated P116 T cells expressing Y315F ZAP-70 was comparable to CD3-stimulated P116 T cells expressing wild-type ZAP-70, expression of Y315F ZAP-70 did not restore CD3-stimulated tyrosine phosphorylation of LAT residues 171 and 191 (Fig. 3B). These results suggest that Y292 and Y315 in ZAP-70 are critical for regulating TCR-mediated tyrosine phosphorylation of amino acids 171 and 191 in the LAT cytoplasmic domain.

**CD3/TCR signaling to β1 integrins requires LAT**

We next used the LAT-deficient Jurkat T cell variant J.CaM2 to investigate the role of LAT in signaling from the CD3/TCR to β1 integrins (31, 41, 53). J.CaM2 T cells exhibit impaired TCR signaling, as measured by responses such as Ras activation, increases.

**FIGURE 4.** CD3/TCR signaling to β1 integrins is dependent on LAT. Adhesion of Jurkat T cells, the LAT-deficient Jurkat T cell line J.CaM2 and a stable transfectant of J.CaM2 expressing a HA-tagged form of wild-type LAT (J.CaM2/LAT+) to fibronectin was assessed following no stimulation (UNSTIM.), or following stimulation for 10 min at 37°C with 10 ng/ml PMA (PMA STIM.), the anti-CD3 mAb OKT3 (CD3 STIM.), or the activating β1 integrin-specific mAb TS2/16 (β1 STIM.). The data are represented as the mean percentage of adhesion of triplicate wells ± SD. Results shown are from one of at least three representative experiments.
The LAT cytoplasmic domain are critical for TCR-mediated in-
B 5). Together, these results suggest that tyrosines 171 and 191 in
slightly above the levels observed with unstimulated cells (Fig.
eels of tyrosine phosphorylation of this LAT mutant that were only
mutated to phenylalanine, CD3 stimulation induced very low lev-
LAT mutants. CD3-induced tyrosine phosphorylation of the
in reduced tyrosine phosphorylation of both the Y171F and Y191F
with the results obtained in P116 T cells, CD3 stimulation resulted
LAT constructs following CD3 stimulation (Fig. 5
also analyzed tyrosine phosphorylation of wild-type and mutant
LAT mutants, CD3/TCR signaling to integrins involves PLC-
was observed following CD3 stimulation of J.CaM2 T cells ex-
pressing the control vector (Fig. 5A). All transfectants tested ex-
pressed similar levels of CD3, αβ, integrins, and αγβ, integrins,
and responded comparably to stimulation by the β1 integrin activ-
ating Ab or PMA stimulation (Fig. 5A and data not shown). We
also analyzed tyrosine phosphorylation of wild-type and mutant
LAT constructs following CD3 stimulation (Fig. 5B). Consistent
with the results obtained in P116 T cells, CD3 stimulation resulted
in reduced tyrosine phosphorylation of both the Y171F and Y191F
LAT mutants. CD3-induced tyrosine phosphorylation of the
Y191F LAT mutant was consistently lower than phosphorylation of
the Y171F LAT mutant. When both Y171 and Y191F were
mutated to phenylalanine, CD3 stimulation induced very low lev-
els of tyrosine phosphorylation of this LAT mutant that were only
slightly above the levels observed with unstimulated cells (Fig.
5B). Together, these results suggest that tyrosines 171 and 191 in
the LAT cytoplasmic domain are critical for TCR-mediated in-
creases in β1 integrin function.

**CD3/TCR signaling to β1 integrins involves PLC-γ1**

Because TCR-mediated tyrosine phosphorylation of LAT ty-
rosines 171 and 191 is critical to TCR-mediated activation of
PLC-γ1 (31, 36), we also explored the role of PLC-γ1 in regulat-
ing CD3-mediated increases in β1 integrin function. To address
this issue, we analyzed the effect of the PLC-γ1 inhibitor U73122
on the adhesion of purified mouse lymph node T cells to VCAM-1.
The results in Fig. 6 show that U73122, but not the inactive ana-
logue U73343, inhibited CD3-induced increases in adhesion of
primary mouse T cells to purified VCAM-1. Similar results were
also obtained when T cell adhesion to ICAM-1 was analyzed (data
not shown). The same concentration of U73122 that completely
inhibited CD3-mediated increases in T cell adhesion to VCAM-1
had only a partial effect on PMA-induced adhesion (Fig. 6), con-
sistent with other studies (43, 54). These studies suggest that CD3/
TCR signaling to integrins involves PLC-γ1.

**Discussion**

In this report, we provide several new insights into the intracellular
signaling pathways used by the CD3/TCR complex to regulate β1
integrin functional activity in Jurkat T cells. First, in addition to
a requirement for the kinase activity of ZAP-70 to couple CD3/TCR
to β1 integrins, we have identified tyrosine residues in the inter-
domain B region that positively (tyrosine 315) and negatively (ty-
rosine 292) regulate CD3/TCR signaling to β1 integrins and CD3/
TCR-mediated activation of PI3-K. Second, these ZAP-70 tyrosine
residues specifically regulate the efficiency with which ZAP-70
mediates phosphorylation of tyrosine residues 171 and 191 in the
LAT cytoplasmic domain. Third, we observed defective β1 inte-
grin activation by the CD3/TCR in LAT-deficient Jurkat T cells.

**FIGURE 5.** CD3/TCR signaling to β1 integrins is dependent on tyrosines 171 and 191 in LAT. A. Adhesion of J.CaM2 T cells expressing vector, wild-type LAT, the Y171F LAT mutant, the Y191F-LAT mutant, or the Y171/191F LAT mutant to various concentrations of immobilized fibronectin was assessed following no stimulation ( ), or following stimulation for 10 min at 37°C with the anti-CD3 mAb OKT3 ( ) or PMA ( ). The data are represented as the mean percentage of adhesion of triplicate wells ± SD. Results shown are from one of at least three representative experiments. B. LAT was immunoprecipitated from unstimulated and CD3-stimulated wild-type Jurkat T cells, and J.CaM2 T cells expressing vector, wild-type LAT, Y 171F LAT, Y 191F LAT, or Y171/191F LAT with an anti-LAT Ab. Western blots were probed with either the anti-phosphotyrosine mAb 4G10 (top) or with an anti-LAT Ab (bottom).

**FIGURE 6.** CD3/TCR signaling to β1 integrins in primary mouse T cells involves PLC-γ1. Lymph node T cells were purified from B6 mice and assessed for adhesion to VCAM-1 as described in Materials and Methods following no stimulation ( ), or following stimulation for 10 min at 37°C with the anti-CD3 mAb 2C11 ( ), or PMA ( ) in the presence of no inhibitor, 2 μM U73122, or 2 μM U73343. The data are represented as the mean percentage of adhesion of triplicate wells ± SD. Results shown are from one of at least three representative experiments.
Re-expression of wild-type LAT, but not mutant LAT constructs in which tyrosines 171 and/or 191 have been mutated to phenylalanine, restores CD3/TCR signaling to β1 integrins.

Previous studies of TCR-mediated signal transduction have demonstrated negative regulatory properties for tyrosine 292 in the interdomain B region of ZAP-70 (24). Our results analyzing CD3/TCR-mediated activation of β1 integrins are consistent with this previous work. When compared with wild-type ZAP-70, expression of Y292F ZAP-70 in ZAP-70-deficient P116 T cells resulted in enhanced CD3/TCR-mediated increases in β1 integrin-dependent adhesion to fibronectin, as well as enhanced CD3/TCR-induced tyrosine phosphorylation of LAT and activation of PI3-K. Studies in mice expressing Y292F ZAP-70 also indicate that tyrosine 292 dampens proximal TCR signaling events and cytokine production induced by CD3/TCR stimulation (28). These negative regulatory effects of tyrosine 292 are likely due to the binding of the Cbl adapter protein to ZAP-70 following phosphorylation of this residue (55, 56) because TCR-inducible binding of Cbl to ZAP-70 may be involved in Cbl-mediated ubiquitination of the TCRζ subunit (26).

In contrast to the negative regulatory effects of tyrosine 292, tyrosine 315 in the interdomain B region of ZAP-70 appears to positively regulate CD3/TCR signaling to β1 integrins. When compared with P116 T cells expressing wild-type ZAP-70, P116 T cells expressing the Y315F ZAP-70 exhibited reduced CD3/TCR-mediated increases in β1 integrin function and PI3-K activity. Previous studies of the effects of the Y315F mutation also indicated that it impaired Ag receptor signaling (21), although these studies were performed in DT40 B cells lacking the Syk tyrosine kinase. Binding of the Vav guanine nucleotide exchange factor to tyrosine 315 has been proposed to be a mechanism by which this tyrosine residue might regulate ZAP-70-dependent TCR signaling responses (21). This is particularly intriguing because CD3/TCR signaling to integrins is impaired in Vav-deficient mouse T cells (16, 57). However, studies in mice clearly show that the Y315F mutation in ZAP-70 does not have the same impact on T cell activation and development as the loss of Vav expression (28). For example, thymocytes expressing Y315F ZAP-70 exhibit relatively normal Ca2+ responses following TCR stimulation (28), whereas Vav-deficient T cells exhibit dramatically impaired TCR-induced Ca2+ signaling (58). In our studies using P116 T cells, the Y315F mutation clearly did not completely block CD3/TCR signaling to integrins, as some response above the basal level of adhesion with unstimulated T cells was observed. This was in contrast to the results obtained with the kinase-inactive Y493F ZAP-70 mutant, which did not restore CD3/TCR signaling to β1 integrins when expressed in P116 T cells. TCR stimulation of T cells from Vav-deficient mice also resulted in no increase in adhesion to fibronectin when compared with wild-type T cells (16), again suggesting a less severe effect of the Y315F mutation on TCR-mediated activation of β1 integrins when compared with eliminating Vav expression.

Although expression of the Y315F ZAP-70 mutant restored CD3-induced tyrosine phosphorylation of LAT when assessed with an anti-phosphotyrosine mAb, we observed that this mutant had a selective effect on tyrosine phosphorylation of two key residues in the LAT cytoplasmic domain, tyrosines 171 and 191. Coupled with our finding that CD3/TCR signaling to β1 integrins is LAT-dependent and requires tyrosines 171 and 191 in the LAT cytoplasmic domain, our findings suggest that ZAP-70-dependent tyrosine phosphorylation of these residues is critical to TCR-mediated activation of integrin function. Thus, although the Y315F mutation in ZAP-70 does not impair overall ZAP-70 kinase activity (21), it selectively alters the efficiency with which ZAP-70 phosphorylates two key tyrosine residues in LAT. Other studies in mice suggest that the Y315F mutation may attenuate TCR signaling via structural changes that alter the ability of ZAP-70 to bind tyrosine phosphorylated ITAMs (28, 59). However, it should be noted that such effects of the Y315F mutation on the binding of ZAP-70 to ITAMs were not noted when Y315F ZAP-70 was expressed in DT40 cells (21).

Although TCR stimulation leads to increases in PI3-K activity (60), the mechanism by which the TCR is coupled to PI3-K activity has remained unclear. Our studies suggest that ZAP-70 is a critical intermediate between the TCR and PI3-K because CD3/TCR-mediated activation of PI3-K requires ZAP-70 kinase activity. In addition, the Y315 and Y292 residues in ZAP-70 mediate positive and negative regulatory effects on CD3/TCR-mediated activation of PI3-K. LAT appears to play a central role in ZAP-70-dependent regulation of PI3-K by the TCR because the Y315 and Y292 residues regulate ZAP-70-mediated phosphorylation of LAT tyrosines 171 and 191, which are known to mediate binding of LAT to the p85 regulatory subunit of PI3-K upon TCR stimulation (31). These results are consistent with previous studies that have implicated PI3-K in the regulation of integrin function by the CD3/TCR (16, 61).

Based on the new findings in this study, we propose that CD3/TCR signaling to β1 integrins involves ZAP-70-dependent phosphorylation of tyrosine residues 171 and 191 in the LAT cytoplasmic domain. Although several functions of ZAP-70 and Syk overlap, we believe that CD3/TCR signaling to β1 integrins predominantly involves ZAP-70 because CD3 stimulation fails to enhance adhesion of ZAP-70-deficient T cells above that observed in the absence of stimulation. Tyrosines 292 and 315 in the ZAP-70 interdomain B region regulate the efficiency with which ZAP-70 phosphorylates LAT tyrosines 171 and 191 and thereby regulate the strength of integrin activation induced by CD3/TCR stimulation. As with other TCR signaling responses, activation of integrins is dependent on the formation of LAT-nucleated signaling complexes mediated to a large extent by tyrosine residues 171 and 191, which are part of key SH2 domain binding sites in the LAT cytoplasmic domain. We propose that these residues are critical in promoting PI3-K-dependent pathways that regulate integrin function. PI3-K recruitment to LAT signaling complexes may be critical to the regulation of other tyrosine kinases such as Itk because Itk activity is regulated by PI3-K (39, 62–64) and Itk inducibly associates with LAT following TCR stimulation (65). Because Itk also regulates CD3/TCR signaling to β1 integrins (39) and Itk-deficient T cells exhibit defective Ag-dependent interactions with APCs (66), LAT may play a central role in promoting integrin activation via Itk. Our finding that a PLC-γ1 inhibitor can block TCR-mediated increases in mouse T cell adhesion to VCAM-1 and ICAM-1 is consistent with this model, as LAT tyrosines 171 and 191 are involved in coupling the TCR to PLC-γ1 (34, 52) and Itk is a critical regulator of PLC-γ1 activity (67–69). Our studies are also consistent with recent reports that U73122 blocks mouse T cell adhesion to fibronectin (43). Other tyrosine residues in LAT besides 171 and 191 that have been implicated in regulation of PLC-γ1 activity (34, 52), such as LAT tyrosine 132, are also likely to be involved in CD3/TCR signaling to integrins. Finally, it is important to note that other signaling intermediates that associate with LAT via tyrosines 171 and 191, such as Grb2 and Gads/SLP-76, may also participate in LAT-dependent signaling to integrins. The role of these other LAT-associated proteins in regulating integrin function is currently under investigation.
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