Phosphorylation of the LFA-1 Integrin β2-Chain on Thr-758 Leads to Adhesion, Rac-1/Cdc42 Activation, and Stimulation of CD69 Expression in Human T Cells*

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Phosphorylation of the leukocyte function-associated antigen-1 (LFA-1) integrin β2-chain on Thr-758 occurs after T cell receptor stimulation and leads to 14-3-3 recruitment to the integrin, actin cytoskeleton reorganization, and increased adhesion. Here, we have investigated the signaling effects of β2 integrin Thr-758 phosphorylation. A penetratin-coupled phospho-Thr-758–β2 peptide (mimicking the part of the integrin β-chain surrounding Thr-758) stimulated adhesion of human T cells to the LFA-1 ligand intercellular adhesion molecule-1 (ICAM-1). Additionally, the peptide activated the small GTPases Rac-1 and Cdc42 in T cells. Constitutively active forms of Rac-1 and Cdc42, but not Rho, could compensate for the reduction of cell adhesion to ICAM-1 caused by the T758A mutation in the β2 integrin. Additionally, the active GTPases salvaged the cell-spreading defect of T758A integrin-transfected cells on coated ICAM-1. A dominant negative form of Cdc42, on the other hand, significantly reduced wild-type β2 integrin-mediated cell adhesion and spreading. In a T cell stimulation system, the pThr-758 penetratin peptide acted in a similar manner to coated ICAM-1 to increase T cell receptor-induced CD69 expression. These results show that Thr-758-phosphorylated LFA-1 is upstream of Rac-1/Cdc42, cell adhesion, and costimulatory activation of human T cells, thus identifying phosphorylation of Thr-758 in β2 as a proximal element in LFA-1 signaling.

T cells are circulating cells that need to interact with other cells to perform their immunological functions. T cell adhesion is required during contact with endothelium (when T cells exit the bloodstream during normal leukocyte recirculation and in inflammation) and in contacts between T cells and antigen-presenting cells during antigen recognition. Most of these cellular interactions of T cells are dependent on the integrin leukocyte function-associated antigen-1 (LFA-1, αLβ2, CD11a/CD18) binding to intercellular adhesion molecule-1 (ICAM-1) and other ICAM family members (1, 2).

The LFA-1 integrin is a heterodimeric transmembrane protein that mediates bidirectional signaling over the plasma membrane. In resting cells, the integrin is inactive and does not bind ligands. However, when T cells are stimulated, for example through the T cell receptor (TCR) or chemokine receptors, intracellular signaling pathways are activated, resulting in LFA-1 activation. This is termed inside-out signaling. Many different signaling pathways and proteins have been implicated in LFA-1 activation, including protein kinases (3–5), lipid kinases (6), calcium–binding proteins (7, 8), and small GTPases (3).

The activation mechanisms for integrins are under intense investigation. Both changes in integrin conformation/affinity and changes in clustering and cytoskeletal contacts are important for integrin activation (9–12). The actin cytoskeleton plays essential roles in LFA-1 regulation; both initial release from the cytoskeleton to allow ligand encounter and reattachment of ligand-bound integrins to the cytoskeleton are important in the regulation of integrin adhesiveness (10, 12). Binding of the cytoskeletal protein talin to the LFA-1 β-chain cytoplasmic domain leads to integrin conformational changes and activation (13, 14).

After ligand binding, integrins are able to transmit signals into the cell interior to change the cell behavior. This is termed outside-in signaling. LFA-1 has been intimately linked to T cell costimulation, whereby ligation of the integrin together with TCR recognition of cognate antigen leads to full T cell activation. Many signaling pathways are activated after LFA-1 ligation, including phospholipase Cγ (15), mitogen-activated protein kinase pathways (16, 17), phosphatidylinositol 3-kinase (17), and Rac-1 (18).

The integrin cytoplasmic domains are short and devoid of catalytic activity. However, they are crucial for the regulation of integrin inside-out and outside-in signaling, through the interaction with cytoplasmic factors. Phosphorylation of the integrin α and β cytoplasmic domains regulates the interactions between integrins and cytoplasmic molecules and, thus, integrin adhesiveness and signaling (11, 19). Constitutive phosphorylation of LFA-1 on the αL-chain Ser-1140 regulates integrin affinity (11). Phosphorylation of LFA-1 on the β-chain on several residues occurs after cell stimulation, and especially a triplet of threonines (Thr-758-Thr-760) has been shown to be essential for regulated cell adhesion through the integrin to the ligand ICAM-1 (reviewed in Ref. 19). Thr-758 becomes phosphorylated in T cells after phorbol ester treatment or T cell
receptor stimulation (20), and this leads to recruitment of 14-3-3 proteins to the integrin (5, 11). 14-3-3 proteins are adapter proteins that bind to phospho-serine- or phospho-threonine-containing sequences in proteins. The phospho-β2—14-3-3 association is required for actin cytoskeleton rearrangements, cell spreading, and adhesion to ICAM-1 (11). Additionally, Thr-phosphorylated LFA-1 is enriched in the cell cytoskeleton, implying that Thr phosphorylation may regulate LFA-1 cytoskeletal interactions (21). However, the downstream effectors of the β2 integrin-14-3-3 interaction have remained unknown.

Here, we have further investigated the role of Thr-758 phosphorylation of the integrin β2-chain in the regulation of adhesion and signaling through LFA-1. We show that treatment of T cells with Thr-758-phosphorylated cell-permeable integrin peptides leads to the activation of the small GTPases Rac-1 and Cdc42, well known reorganizers of the actin cytoskeleton (22). The phosphorylated integrin peptides also potently activated cell adhesion to ICAM-1. Active forms of Rac-1 and Cdc42 were able to compensate for the cell adhesion and actin rearrangement defects of the T758A-mutated β2-chain. Additionally, cell-permeable Thr-758-phosphorylated integrin peptides induced further signaling events, i.e. the surface expression of the T cell activation marker CD69, when used in conjunction with coated T cell receptor antibodies. This costimulation of T cells resembles the effect of coated ICAM-1. Thus, Thr-758 phosphorylation of the LFA-1 integrin β2-chain has a signaling function in T cells.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human ICAM-1 was obtained from R&D Systems, and the peptides (CLFKSATTVMN (T-β2), CLFKSAPTTTVMN (pT-β2), and CLKPLHEKDEPSGGKKD (pαL), where pS is phospho-serine and pT is phospho-threonine) were synthesized by Fmoc (N-(9-fluorenylethoxycarbonyl) chemistry (23). Activated penetratin peptide and biotin-penetratin peptide were purchased from Qbiogene and the Rac/Cdc42 assay reagent from Millipore. G418 was purchased from Calbiochem-Novabiochem and oka-diacid from Sigma. ICAM-1-Fc was produced by transient transfection of COS-1 cells. They were cotransfected with purified α2 subunit of leukocyte integrin have been described previously (25). The monoclonal antibodies against Rac-1 and Cdc42 were obtained from BD Biosciences, and the phospho-p44/42 MAPK antibody was purchased from Cell Signaling Technology. The filamin antibody was from Chemicon International. To produce antiserum to phospho-β2, the integrin β2 chain phosphopeptide (CLFKSAPTTTVMN) was conjugated to keyhole limpet hemocyanin (26). The complex was injected into rabbits. The antisera were purified by affinity chromatography using the phosphorylated peptide. EGFP-Rac-GTP, EGFP-Cdc42-GTP, EGFP-Rho-GTP, or EGFP-Cdc42-GDP constructs were kind gifts from P. Lappalainen (University of Helsinki).

Cell Lines, Transfections, and Penetratin Peptide Treatments—Buffy coats used for the isolation of T cells were obtained from the Finnish Red Cross Transfusion Service (23). The penetratin-coupled peptides were added in RPMI 1640 medium at 10-μM concentration or as indicated in Fig. legend 6 and incubated with the T cells for 1 h at 37 °C without serum.

COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics and used for transient expression of WT and mutant β2 integrins. They were cotransfected with purified α- and β-subunit cDNAs with or without activated Rho family GTPases (27) EGFP-Rac-GTP (V12), EGFP-Cdc42-GTP (V12), EGFP-Rho-GTP (V14), or an inactive (GDP) form of Cdc42, EGFP-Cdc42-GDP (27), using FuGENE 6 transfection reagent according to the manufacturer’s instructions (Roche Applied Science). G418 was added at 0.8 mg/ml 24 h after transfection. Flow cytometric analysis was used to quantify cell surface expression of integrins in the transfected COS-1 cells.

Cell Fractionation—T cell fractionation into soluble and detergent-insoluble fractions was done as described previously (8).

Rac/Cdc42 Pulldown Assay—Human T cells were treated with penetratin peptides or left untreated. Cells were lysed, and the Rac/Cdc42 assay reagent was used on the lysates according to the manufacturer’s instructions (Millipore). The bound proteins were analyzed by Western blotting with a Cdc42 antibody or Rac-1 antibody. MAPK activation in the cell lysates was investigated using a pMAP kinase antibody.

Cell Adhesion Assays—Recombinant soluble ICAM-1 (0.3 μg/well) was coated on flat bottom 96-well microtiter plates by overnight incubation at 4 °C. The wells were blocked with 1% dry milk (COS-1 cells) or 2% dry milk (T cells) for 1 h and 15 min at 37 °C. COS-1 cells were suspended in Dulbecco’s modified Eagle’s medium with 40 mM Hepes, 0.1% bovine serum albumin, and 2 mM MgCl2, binding medium and T cells in RPMI with 40 mM Hepes, 0.1% bovine serum albumin, and 2 mM MgCl2, binding medium. Cells were allowed to adhere for 25 min at 37 °C after which the unbound cells were removed by gentle washing. The binding was quantified by enzyme-linked immunosorbent assay.

Immunofluorescence Staining—In the transfected COS-1 spreading assays, 2 × 105 cells were seeded onto ICAM-1-coated coverslips and incubated for 65 min at 37 °C. Unbound cells were gently washed away, and adherent cells were fixed for 10 min with 1% formaldehyde/phosphate-buffered saline. Cells were labeled with TRITC-phalloidin in 0.1% saponin/1% fetal calf serum/phosphate-buffered saline for 30 min. For detection of integrin-penetratin peptides in T cells, biotin-penetratin peptide-treated T cells were seeded onto OKT3-coated coverslips and incubated for 30 min at 37 °C. After fixing as above, cells were incubated with streptavidin-fluorescein isothiocyanate (Immunotools) in saponin buffer for 30 min. After washing with phosphate-buffered saline, coverslips were mounted with Mowiol mounting medium, observed under a fluorescence microscope (model IX71; Olympus), and photographed with a camera (model DP70; Olympus). Images were analyzed and processed using the analysis program (Soft Imaging System GmbH) and Adobe Photoshop.
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T Cell Stimulation Assay—T cells were further purified with a MACS pan-T cell kit (Miltenyi Biotec) according to the manufacturer’s instructions. ICAM-1-Fc and hIgG was biotinylated according to standard protocols with biotinamidocaproate N-hydroxysuccinimide ester (Sigma), 100 μg/mg protein. The degree of biotinylation was examined by quantitation of binding to Streptavidin-Sepharose. For the solid phase stimulation system, avidin (Pierce Biotechnology Inc.) was coated onto 24-well plates at 50 μg/ml. After blocking with 1% bovine serum albumin/phosphate-buffered saline, 100 ng of biotin-UCHT1 (Ancell Corp., Bayport, MN) together with either 100 ng of biotin-ICAM-1-Fc or 100 ng of biotin-hIgG were added on the avidin-coated wells (the total amount of biotinylated protein in each well was 200 ng). Purified T cells were treated with penetratin peptides or left untreated, added onto the wells (0.5–1 million cells/well), and stimulated for 4–20 h. CD69 expression was examined by flow cytometry.

Flow Cytometry—Basic protocols were used for surface staining of purified T cells. The CD69-phycocerythrin conjugate was from ImmunoTools (Friesoythe, Germany). Flow cytometry analysis was performed on a FACScan (BD Biosciences). CD69 expression was reported as mean fluorescence intensity. Alternatively, the activated cell population (positive for CD69 expression) was gated and analyzed separately to determine the percentage of activated cells or mean fluorescence intensity of activated cells.

RESULTS

Thr-758 Is Phosphorylated Only in Stimulated T Cells—The phospho-Thr-758-β2–14-3-3 complex is essential for LFA-1-mediated adhesion, actin reorganization, and cell spreading (11). To be able to further study the Thr-758-phosphorylated integrin in cells, we generated a phospho-specific antibody capable of recognizing β2 only when it is phosphorylated on Thr-758. The antibody was purified using the phosphopeptide antigen. The specificity of the antibody was confirmed by Western blotting of lysates of COS-1 cells expressing WT β2 and Thr-758A-β2, where Thr-758 had been mutated into nonphosphorylatable alanine. The cells were either activated with okadaic acid and phorbol ester or left untreated. The antibody recognized only the WT β2 polypeptide after okadaic acid and phorbol ester treatment and did not bind to the mutated β2 polypeptide (Fig. 1A). Thus, the antibody is sequence- and phospho-specific.

This antibody was used on T cell lysates that were either treated with okadaic acid and OKT3 (monoclonal activating antibody against CD3) or left untreated. The phospho-specific antibody recognized the β2 polypeptide only from T cells that were stimulated with OKT3 (Fig. 1B), confirming specific phosphorylation of Thr-758 in T cells activated through the T cell receptor (20). Using the pThr-758-phospho-specific antibody, we investigated the distribution of Thr-758-phosphorylated integrins in detergent-soluble and -insoluble fractions from activated T cells. The Thr-758-phosphorylated integrins were found both in the soluble and insoluble (cytoskeletal) fractions of activated T cells (Fig. 1C), indicating that the phosphorylation does not directly regulate cytoskeletal attachment of activated integrins.

A Cell-permeable Phospho-Thr-758-β2 Peptide Induces T Cell Adhesion to ICAM-1—It has previously been shown that the β2 integrin phosphorylated on Thr-758 specifically binds to 14-3-3 proteins, resulting in reorganization of the actin cytoskeleton and increased cell adhesion to ICAM-1 (11). These functional studies were, however, conducted in COS-1 cells. Thus, we were interested to study the role of Thr-758-phosphorylated β2 integrins in T cells. To do this, we coupled integrin cytoplasmic peptides (pT-β2, T-β2, and pαL) to activated penetratin (PENA) peptides, which are able to carry coupled peptide sequences into cells. Intracellular localization of the peptides was confirmed by using biotin-penetratin-coupled peptides (Fig. 2A).

To examine the effect of the penetratin-coupled integrin peptides on adhesion, T cells that were treated with either penetratin peptides or phorbol ester or were left untreated were seeded on wells coated with the β2 integrin ligand ICAM-1. Phorbol ester–treated T cells adhered to ICAM-1 (Fig. 2B). The same effect could be seen with T cells treated with the phosphorylated Thr-758-β2 penetratin peptide (Fig. 2B). The pT-β2 penetratin peptide also increased adhesion of phorbol ester-stimulated cells (not shown). Specificity of pThr-758-β2 penetratin-induced adhesion to ICAM-1 was shown by treatment of cells with R7E4, a blocking LFA-1 antibody (Fig. 2B). A penetratin peptide coupled to an unphosphorylated β2 peptide (T-β2) did not induce adhesion of T cells to ICAM-1, and neither did a Ser-1140-phosphorylated αL peptide (pαL). These results confirm the importance of the Thr-758-phosphorylated β2–14-3-3 complex in the regulation of LFA-1-mediated T cell adhesion to the ligand ICAM-1.
FIGURE 2. Phosphorylated Thr-758 β2 peptide induces T cell adhesion to ICAM-1. A, intracellular localization of the integrin penetratin peptides in treated T cells was verified by immunofluorescence staining using the biotinylated penetratin (PENA) peptides and streptavidin-fluorescein isothiocyanate. Control, no peptide added; pT-β2-PENA, cells treated with 10 μM penetratin-biotin-coupled phosphorylated Thr-758-β2 peptide; T-β2-PENA, cells treated with 10 μM penetratin-biotin-coupled nonphosphorylated β2 peptide; pol-L-PENA, cells treated with 10 μM Ser-1140-phosphorylated L-β2 peptide. B, cell adhesion of T cells to coated ICAM-1 was examined as described under “Experimental Procedures.” T cells were pretreated with pT-β2 penetratin, T-β2 penetratin, or pol-L penetratin at a concentration of 10 μM in the presence or absence of LFA-1-blocking antibody 9E10 (20 μg/ml) or mixed with 200 nM PDBu before adding to the plate. The experiment was repeated three times in duplicate. Error bars represent S.D., and significant differences (p ≤ 0.001 for PDBu-treated samples and p ≤ 0.005 for pT-penetratin-treated samples) in bracketed comparisons are indicated by a single asterisk.

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Rac-1/Cdc42 Is Activated by the Thr-758-phosphorylated β2 Integrin—Next, we sought to determine the downstream effector of the phospho-Thr-758 β2 integrin in the regulation of cell adhesion. Because the large amounts of peptide introduced into cells induced, rather than inhibited, adhesion, the phosphorylation did not seem to play a role in linking the active integrin to the actin cytoskeleton through 14-3-3 binding, in which case the peptide would presumably have competed with endoge-
reorganization and cell spreading on ICAM-1 (11). Thus, we examined whether the constitutively active forms of Rac-1 and Cdc42 were able to compensate for the loss of the Thr-758 phosphorylation site in cell-spreading assays. Transfected COS cells were seeded onto coverslips coated with ICAM-1. The constitutively active Rac-1 and Cdc42 compensated for the T758A mutation of the β2 integrin, because the cells were able to attach and spread on ICAM-1 in a similar manner as WT β2 integrin-transfected cells (Fig. 4, B and C). In contrast, active Rho was not able to compensate for the loss of threonine 758 of the β2 integrin in the cell-spreading assay (Fig. 4, B and C).

The Adhesion and Spreading of WT β2 Integrin-transfected Cells to ICAM-1 Are Reduced by Dominant Negative Cdc42—To further investigate the involvement of small GTPases in LFA-1-mediated adhesion, we next went on to study the effect of dominant negative Cdc42 on the adhesion of WT β2 integrin to ICAM-1 (Fig. 5A). The dominant negative construct indeed caused a significant decrease in cell adhesion of WT β2-transfected COS cells to coated ICAM-1. Additionally, this construct reduced WT LFA-1-mediated cell spreading on coated ICAM-1 (Fig. 5B). We also examined the effect of dominant negative Cdc42 on pT-β2 penetratin-induced adhesion (Fig. 5C). Indeed, the dominant negative construct significantly reduced cell adhesion to ICAM-1 induced by the phosphorylated peptide. These results further confirm that this group of small GTPases is involved in regulating LFA-1 adhesion downstream of pThr-758. The level of GFP proteins and cell surface LFA-1 integrins in the EGFP-positive cells was equal in Cdc42-GDP cells, as examined by flow cytometry.

The pThr-758 Penetratin Peptide Can Induce Downstream Signaling in Conjunction with TCR Antibodies in a T Cell Stimulation System—Ligation of the T cell receptor alone is not enough to induce full T cell activation. Instead, a costimulatory signal is needed, to avoid T cell anergy (non-
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FIGURE 5. A dominant negative Cdc42 construct reduces both β2 integrin-dependent adhesion and spreading. A, transiently transfected COS-1 cells expressing WT β2 integrin with or without Cdc42-GDP, T758A-β2 integrin, or Cdc42-GDP alone were allowed to bind ICAM-1-coated wells as described under “Experimental Procedures.” The experiment was repeated three times in duplicate. Significant differences (p < 0.05) in bracketed comparisons are indicated by a single asterisk. B, spreading of the transfected COS-1 cells was examined by F-actin staining, and the spreading was quantified by counting the percentage of spread transfected cells. A total of 100 cells was counted/experiment, and the experiment was repeated three times. Significant differences (p < 0.005) in bracketed comparisons are indicated by a single asterisk. C, transiently transfected COS-1 cells expressing WT β2 integrin with or without Cdc42-GDP were incubated with the pT-β2 penetratin (PENA) peptide and thereafter allowed to bind ICAM-1-coated wells as described under “Experimental Procedures.” The experiment was repeated four times. Significant differences (p < 0.005) in bracketed comparisons are indicated by a single asterisk. Error bars represent S.D.

LFA-1 can act as a costimulatory receptor for T cell activation by activating downstream signaling events after binding to its ligands (16, 17, 30) (reviewed in Ref. 31). To study whether pThr-758 was involved in further downstream signaling events through the activation of Rac-1/Cdc42, we made use of a cell stimulation system, where T cell receptor antibodies (UCHT1) or control antibodies were coated onto wells and T cells treated with the different penetratin peptides were added to the wells. As a control of T cell costimulation, the LFA-1 ligand ICAM-1 was coated together with UCHT1 (Fig. 6A). After 4–20 h of incubation, the expression level of the CD69 receptor (an early marker of cell receptor antibodies already induced some increase in CD69 expression (Fig. 6D)). This may be the result of cell membrane changes that make T cells more unspecifically adhesive. However, compared with other integrin peptides coupled to penetratin, pThr-758-β2 showed a larger effect on CD69 levels in the activated T cell population (Fig. 6D), whereas the other peptides induced CD69 levels comparable with uncoupled penetratin.

DISCUSSION

In this study, we have investigated the signaling downstream of the Thr-758-phosphorylated LFA-1 in cells. We have shown that Thr-758 is indeed phosphorylated in T cells activated with OKT3 (a T cell receptor antibody), by using a novel phosphospecific antibody to this site. We have previously described that Thr-phosphorylated integrins are enriched in the cell cytoskeleton (21), arguing for a role of Thr-758 phosphorylation in LFA-1 cytoskeletal attachment and, thus, adhesion strengthening. Here we show that Thr-758-phosphorylated integrins are indeed enriched in, but not exclusively restricted to, the cell cytoskeleton. This result, as well as our failure to detect any significant differences between WT β2 and TTT/AAA-mutated β2 distribution in cytoskeletal and soluble fractions in transfected COS cells, seems to exclude a simple cytoskeletal linking function for the pThr-758-β2–14-3-3 complex in cell adhesion.

By using a penetratin-coupled Thr-758-phosphorylated β2-peptide, we have shown that this specific phosphorylation is important for regulating integrin-mediated T cell adhesion. The importance of the TTT region in adhesion has been previ-
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A.

B.

C.

D.

FIGURE 6. ICAM-1 and pT-β2 penetratin induce increased CD69 expression in T cells activated with UCHT1. A, the relative level of CD69 expression (mean fluorescence intensity of samples as compared with UCHT1-stimulated cells) in T cells stimulated with control antibody (hIgG), T cell receptor antibody (UCHT1) alone, or UCHT1 and ICAM-1 together was measured after 4 h of stimulation by flow cytometry with a CD69-phycocerythrin antibody. The experiment was repeated four times in duplicate. Significant differences (p ≤ 0.0001) in bracketed comparisons are indicated by a single asterisk. B, T cells were pretreated with penetratin (PENA) peptides at 5 μM concentration as described and added onto UCHT1-coated or hIgG-coated wells as described. After 21 h of stimulation, the relative levels of CD69 expression were measured as in panel A. Significant differences (p ≤ 0.01) in bracketed comparisons are indicated by a single asterisk. C, as in panel B, except that the relative amount of activated cells was measured (percentage of activated cells in penetratin-treated cell samples as compared with cell samples stimulated with UCHT1 alone). Significant differences (p ≤ 0.02) in bracketed comparisons are indicated by a single asterisk D, as in panel B, except the relative level of CD69 expression (mean fluorescence intensity of penetratin peptide-treated samples compared with mean fluorescence intensity of UCHT1 alone) only in the activated cell population was determined. Significant differences (p ≤ 0.01) in bracketed comparisons are indicated by a single asterisk. Experiments in panels B–D were repeated four to six times in duplicate. Error bars represent S.D.

Phosphorylation of Thr-758 in β2 integrins is crucial for the adhesion of T cells to ICAM-1. However, the role of this phosphorylation has not been widely studied. Here, we investigated the effect of constitutively active Rac-1 and Cdc42 constructs on integrin-mediated functions that are defective in T758A-β2-transfected cells. We found that the active forms of these GTPases could compensate for both the adhesion and cell-spreading defects of the mutated integrin in cells. Also, a dominant negative Cdc42 construct significantly reduced both constitutive and pThr-758-β2 penetratin-induced LFA-1-mediated adhesion to ICAM-1, as well as actin reorganization and cell spreading. Together, these results implicate Rac-1 and Cdc42 as downstream effectors of the Thr-758-phosphorylated integrin 14-3-3 complex in T cells. In light of the recently reported results on RhoA being downstream of the β2 threonine triplet in Mac-1(CD11b/CD18, αMβ2)-mediated monocyte phagocytosis (34), it is clear that small GTPases of the Rho family are essential downstream effectors of the leukocyte integrins in several β2 integrin-mediated functions and that the functionally important threonines in β2 mediate the activation of these GTPases.

To further study the effect of the phosphorylated integrin on T cell-mediated functions, we used a T cell stimulation system to study downstream signaling of the integrin in conjunction with T cell receptor antibodies. LFA-1 has been clearly implicated as a costimulatory receptor in T cells (16, 17) (reviewed in Ref. 31), and an additional phosphorylation site, Ser-745 (5), has been implicated in LFA-1 signaling (30). Here, we show that T cell receptor antibodies, together with the pThr-758-β2 penetratin peptide, can induce further downstream signaling (expression of the T cell activation marker CD69) in T cells in a similar manner to coated TCR antibody together with ICAM-1. The LFA-1-ICAM-1 interaction has previously been shown to induce Rac-1 activation in T cells (18), and Rac-1 has been implicated in the up-regulation of CD69 expression in T cells (35). Together, these data indicate that the pThr-758-β2 penetratin costimulatory effect may be due to Rac-1 activation. The large amount of phosphorylated integrin peptides introduced into cells by penetratin may mimic the clustering of LFA-1 integrins induced by ICAM-1 in other stimulatory systems leading to Rac-1 activation (18).

Thus, we have shown that 1) phosphorylation of Thr-758 in β2 leads to T cell adhesion to ICAM-1, 2) phosphorylation of
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Thr-758 leads to Rac-1/Cdc42 activation and further T cell signaling, and 3) active forms of Rac-1 and Cdc42 can compensate for the T758A mutation in cell adhesion and spreading assays, while a dominant negative construct of Cdc42 reduces WT LFA-1-mediated adhesion. These results clearly implicate Rac-1 and Cdc42 as downstream mediators of β2 Thr-758 phosphorylation in T cells (Fig. 7). The Thr-758-phosphorylated β2 peptide binds 14-3-3 in vitro and in cells (11). 14-3-3 proteins are dimers and are able to interact with two phosphorylated targets at the same time, thus functioning as adapter proteins (36, 37). Jin et al. (38) have shown that 14-3-3 proteins bind many proteins involved in small GTP-binding protein regulation in cells, for example, a Rac-1/Cdc42 guanine nucleotide exchange factor. Thus, it is conceivable that a phospho-β2–14-3-3 guanine nucleotide exchange factor complex works on the small GTPases in T cells, thereby mediating their activation. The activation of small GTPases downstream of the phosphorylated β2 integrins ultimately leads both to increased cell adhesion and further downstream signaling events through intermediate steps that remain to be established (Fig. 7).

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