

# T Cell Receptor Engagement Leads to Phosphorylation of Clathrin Heavy Chain during Receptor Internalization

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## Abstract

T cell receptor (TCR) internalization by clathrin-coated vesicles after encounter with antigen has been implicated in the regulation of T cell responses. We demonstrate that TCR internalization after receptor engagement and TCR signaling involves inducible phosphorylation of clathrin heavy chain (CHC) in both CD4<sup>+</sup> and CD8<sup>+</sup> human T cells. Studies with mutant Jurkat T cells implicate the Src family kinase Lck as the responsible enzyme and its activity in this process is influenced by the functional integrity of the downstream signaling molecule ZAP-70. CHC phosphorylation positively correlates with ligand-induced TCR internalization in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and CHC phosphorylation as a result of basal Lck activity is also implicated in constitutive TCR endocytosis by CD4<sup>+</sup> T cells. Remarkably, irreversible CHC phosphorylation in the presence of pervanadate reduced both constitutive and ligand-induced TCR internalization in CD4<sup>+</sup> T cells, and immunofluorescence studies revealed that this inhibition affected the early stages of TCR endocytosis from the plasma membrane. Thus, we propose that CHC phosphorylation and dephosphorylation are involved in TCR internalization and that this is a regulatory mechanism linking TCR signaling to endocytosis.

Key words: T lymphocytes • endocytosis • signal transduction • Src family kinases • lymphocyte activation

## Introduction

The interaction of the TCR with peptide–MHC complexes on APCs plays a critical role in the development of an immune response to a foreign antigen. The TCR is an oligomeric complex composed of three functional units, the antigen-binding  $\alpha$  and  $\beta$  chains and the signal transducing CD3  $\delta$ ,  $\epsilon$ , and  $\gamma$  chains as well as the distinct TCR  $\zeta$  chain (1). Before encounter with antigen, TCRs are continuously internalized and recycled back to the cell surface (2–4). Once the TCR engages a peptide–MHC complex on the surface of an APC, the TCR undergoes down-modulation. This process reflects a combination of receptor internalization, recycling, and degradation and is regulated by signaling through the TCR (5). Thus, TCR endocytosis influences at least two functions during T cell activation (6). First, internalization of the TCR potentiates the activation signal by translocating the receptor from the plasma membrane to early endosomes enriched for additional signaling molecules

(7). Second, TCR down-modulation from the endosome attenuates signaling through this receptor by targeting the TCR to a lysosomal compartment for degradation (8–10). Clathrin has been implicated in TCR internalization at the plasma membrane (11, 12) and could also play a role in TCR down-modulation from the endosome. Here we investigate the relationship between TCR signaling and the clathrin-mediated pathway of TCR internalization.

The major structural components of the clathrin-coated vesicle (CCV) are two oligomeric molecules, clathrin and adaptor proteins (13). Clathrin is composed of three  $\sim$ 192-kD heavy chains bound to three  $\sim$ 25-kD light chains. Adaptor proteins are heterotetramers of  $\sim$ 270 kD that recognize signals in the cytoplasmic domain of receptors and direct their sequestration into a CCV at the plasma membrane. The CD3  $\delta$ ,  $\epsilon$ , and  $\gamma$  chains and the TCR  $\zeta$  chain each

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Abbreviations used in this paper: BCR, B cell receptor; CCV, clathrin-coated vesicle; CHC, clathrin heavy chain; EGFR, epidermal growth factor receptor; hTfR, human transferrin receptor; ITAM, immunoreceptor tyrosine-based activating motif; LAT, linker associated with T cell activation; PTP, protein tyrosine phosphatase.

contain one of three previously described adaptor-binding motifs (14), and TCR engagement with antigen likely exposes these motifs through phosphorylation allowing for CCV sequestration and TCR internalization at the plasma membrane (3). Clathrin has also recently been implicated in the trafficking of receptors to lysosomes through its association with the adaptor protein Hrs on endosomal membranes (15). Hrs recognizes ubiquitinated receptors and because the TCR becomes ubiquitinated upon antigen binding (16, 17), clathrin via its association with Hrs may regulate TCR down-modulation from endosomes to lysosomes. TCR internalization in CCVs is likely regulated first at the plasma membrane when signaling through the TCR exposes the adaptor binding motif in the receptor, as has been observed for epidermal growth factor receptor (EGFR; 18) and CTLA-4 (19). Recent studies of the EGFR and the B cell receptor (BCR) demonstrate that receptor signaling also modifies clathrin heavy chain (CHC) by phosphorylation, which plays a role in the regulation of clathrin-mediated endocytosis (20, 21). This signal-induced clathrin modification is a critical step for regulation of expression of these receptors and represents a second mechanism by which receptor signaling influences receptor down-modulation.

The TCR signaling cascade is well characterized, complex, and implicated in TCR down-modulation. The initiating event is the phosphorylation of the immunoreceptor tyrosine-based activating motifs (ITAMs) located in each of the CD3  $\delta$ ,  $\epsilon$ , and  $\gamma$  chains and in the TCR  $\zeta$  chain by the Src family protein tyrosine kinase Lck. Lck also phosphorylates the downstream Syk family protein tyrosine kinase ZAP-70 (22). Upon activation, ZAP-70 not only binds to the phosphorylated ITAMs in the CD3 and  $\zeta$  chains (23), but also phosphorylates the linker associated with T cell activation (LAT) transmembrane protein (24). LAT unites the proximal signaling events after TCR engagement with the activation of downstream molecules that ultimately leads to T cell proliferation and the development of effector functions (25). This entire pathway has been associated with TCR internalization by the observations that the inhibition or absence of Src family kinases prevents TCR endocytosis (26–29). Here we demonstrate that there is a link between the TCR signaling cascade and TCR internalization involving CHC phosphorylation. We further establish that Lck is the active kinase in clathrin modification by TCR signaling and we present evidence that the regulation of clathrin phosphorylation correlates with the pattern of TCR surface expression.

## Materials and Methods

**Cell Lines.** The acute human T cell leukemia line Jurkat and its derivatives, J.Cam1 (30), J.Cam1-Lck (31), J45.01 (32), P116 (33), P116.c39 (33), and J.Cam2 (34), were cultured in RPMI 1640 supplemented with 10% FBS and 10 mM Hepes. The Jurkat derivative DK33 (33) was maintained in the above culture medium supplemented with 500  $\mu$ g/ml G418. The P116, P116.c39, and DK33 cell lines were obtained from R. Abraham

(The Burnham Institute, La Jolla, CA). The J45.01 cell line was purchased from American Type Culture Collection.

**CTL.** The EBV-specific CTL clone LYcl48 was obtained from A. Rickinson (University of Birmingham, Birmingham, UK) and was derived and grown as previously described (35). In brief, PBMCs from an EBV<sup>+</sup> donor were cultured with  $\gamma$ -irradiated B95.8 EBV-transformed autologous lymphoblastoid cell lines at a responder to stimulator ratio of 40:1. Clone LYcl48 was derived from these activated populations by seeding in semisolid agarose or by limiting dilution cloning and was maintained by weekly stimulation with mitomycin C-treated (Sigma-Aldrich) autologous EBV-transformed lymphoblastoid cell lines in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 15 mM Hepes, 1% human serum, 25% supernatant of the IL-2-producing MLA-144 cell line, and 50 U/ml rIL-2 (Sigma-Aldrich).

**Antibodies and Inhibitors.** The mouse mAb specific for human CD3 was anti-Leu4 (BD Biosciences). The mouse mAb specific for the Jurkat TCR was C305 (36). The mouse mAbs specific for CHC were X22 (37) and TD.1 (38). The mouse mAb 4G10, specific for phosphotyrosines, was provided by A. DeFranco (University of California San Francisco, San Francisco, CA). The mouse mAb specific for human transferrin receptor (hTfR) was from Molecular Probes. The mouse mAb specific for the His<sub>6</sub> tag was from BD Biosciences. The rabbit polyclonal antiserum against the clathrin light chains was anti-consensus peptide (39). For immunoblotting, the polyclonal anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase were from Zymed Laboratories. For FACS<sup>®</sup>, the FITC-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse Ig was from DakoCytomation and the FITC-conjugated anti-CD3 mAb was from BD Biosciences. For immunofluorescence, rhodamine-conjugated goat anti-mouse IgM was from Jackson ImmunoResearch Laboratories. The Src kinase inhibitor PP1 was from BIOMOL Research Laboratories, Inc., and a 20-mM stock solution was prepared in DMSO. The serine/threonine kinase inhibitor H7 was also from BIOMOL Research Laboratories, Inc., and a 100-mM stock solution was prepared in DMSO.

**T Cell Activation and Receptor Internalization.** T cells were washed twice with cold PBS and then incubated with 10  $\mu$ g/ml anti-CD3 Ab or 4  $\mu$ g/ml anti-hTfR Ab at a concentration of 10<sup>7</sup> cells/ml for 30 min on ice. In some assays, Jurkat T cells were incubated with 6- $\mu$ m diameter polystyrene latex microspheres purchased from Polysciences Inc. and coated with anti-CD3 Ab. The Ab was adsorbed to the beads as previously described (40, 41) with slight modifications. In brief, 250  $\mu$ g purified anti-CD3 Ab was incubated with 10<sup>7</sup> polystyrene beads in a final volume of 500  $\mu$ l PBS for 2 h at room temperature and then overnight at 4°C with constant rotation. An aliquot of 10<sup>7</sup> beads was incubated in a final volume of 500  $\mu$ l PBS plus 1% BSA under the same conditions. The beads were then washed three times with cold PBS, resuspended at 10<sup>8</sup> beads/ml in cold PBS, and stored at 4°C. The microspheres were incubated with Jurkat T cells at a 2:1 ratio. After stimulation, the cells were washed twice with cold PBS, resuspended at 10<sup>7</sup> cells/ml in cold PBS, and incubated at 37°C for various periods of time. Internalization was stopped by incubating the cells on ice and adding cold PBS. The cells were then collected for either FACS<sup>®</sup> or immunoprecipitation. In some assays, Jurkat T cells were treated with a final concentration of 100  $\mu$ M pervanadate and then immediately incubated at 37°C for various periods of time. Internalization was stopped and the cells were collected for either FACS<sup>®</sup> or immunoprecipitation. The pervanadate stock solution was prepared by mixing 2.3  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich)

with 1 ml of 20 mM Na<sub>3</sub>VO<sub>4</sub> and allowing the mixture to react for 5 min at room temperature.

**Immunoprecipitation and Western Blotting.** Cells were lysed on ice for 30 min in buffer containing 0.5% Triton X-100, 500 mM Tris-HCl, pH 7.2, 20 mM EDTA, 30 mM NaF, 5 mM sodium orthovanadate, 30 mM sodium pyrophosphate decahydrate, and the following protease inhibitors: 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. For CHC immunoprecipitation, protein G-sepharose (Amersham Biosciences) precleared lysates were incubated with 5 μg/ml X22 mAb for 1 h at 4°C and then precipitated with protein G for 1 h at 4°C. Precipitated proteins were resolved on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane (Schleicher & Schuell), and immunoblotted using Abs specific for phosphotyrosine (4G10) or CHC (TD.1), followed by incubation with an anti-mouse secondary peroxidase-conjugated Ab. Blots were visualized with enhanced chemiluminescence (Amersham Biosciences). For quantification of band intensities, autoradiographs were scanned and analyzed using NIH Image software.

**In Vitro Phosphorylation of CHC.** Recombinant Lck, ZAP-70, and LAT were from Upstate Biotechnology. 10 or 25 ng Lck or ZAP-70 were incubated with 5 μg His<sub>6</sub>-tagged clathrin Hub fragment (residues 1074–1675), clathrin light chain b, or LAT in 10 mM MnCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 50 mM Tris, pH 8, 1 mM TCEP, and 10 mM ATP for 20 min at room temperature. Clathrin Hub and light chain were prepared as previously described (42). The phosphorylation reaction was stopped by the addition of SDS-PAGE sample buffer and boiled for 5 min. Proteins were resolved on a 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. The extent of tyrosine phosphorylation was analyzed by immunoblotting using mAb 4G10 as described above. Clathrin Hub was immunoblotted using the His<sub>6</sub> tag Ab followed by an anti-mouse secondary peroxidase-conjugated Ab. Clathrin light chains were immunoblotted using the anti-consensus peptide Ab followed by an anti-rabbit secondary peroxidase-conjugated Ab.

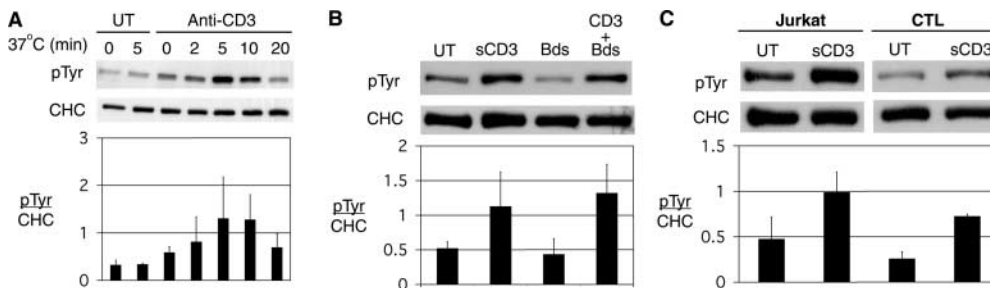
**Flow Cytometric Analysis.** After incubation with either anti-CD3 Ab or anti-hTfR Ab as described above, Jurkat T cells and derivatives, as well as the CTL clone LYcl48, were stained with FITC-conjugated anti-mouse Ig secondary Ab for 30 min on ice. T cells that were left untreated or incubated with pervanadate as described above were stained with FITC-conjugated anti-CD3 mAb for 30 min on ice. Cells were washed three times with PBS plus 1% BSA and then fixed in 1% paraformaldehyde. Flow cytometry was performed on a FACScan™ and the data were analyzed with CELLQuest™ software (BD Biosciences).

**Indirect Immunofluorescent Microscopy.** Jurkat T cells were washed twice with cold PBS and then incubated with either C305 supernatant or 50 μg/ml FITC-labeled transferrin (Molecular Probes) at a concentration of 10<sup>7</sup> cells/ml for 30 min on ice. After stimulation, the cells were washed twice with cold PBS, re-suspended at 10<sup>7</sup> cells/ml in cold PBS in the presence or absence of a final concentration of 100 μM pervanadate, and incubated at 37°C for various periods of time. Internalization was stopped by incubating the cells on ice and adding cold PBS. The cells were then fixed with 4% paraformaldehyde and plated on poly-L-lysine-treated (Sigma-Aldrich) coverslips. TCR was detected using a rhodamine-conjugated anti-mouse IgM secondary Ab. Cells were viewed using a DeltaVision Restoration Microscope, and a single section through the middle of the cell is shown (see Fig. 7). Images were processed using Adobe Photoshop software. The percent internalization was quantified from 200 images of cells for each treatment from three independent experiments.

## Results

### CHC Is Inducibly Phosphorylated in Activated T Cells.

The goal of this study was to investigate the molecular mechanisms linking TCR signaling with receptor internalization. Previous studies with the EGFR (20) and the BCR (21) had demonstrated that CHC becomes inducibly phosphorylated upon ligand binding and we sought to determine if CHC was also phosphorylated in T cells upon TCR engagement. The human CD4<sup>+</sup> T cell leukemia line Jurkat was treated with soluble anti-CD3 Ab and the cells were then incubated at 37°C to induce TCR activation and internalization. CHC was immunoprecipitated and levels of phosphorylation were measured by immunoblotting with a phosphotyrosine-specific Ab. In unstimulated Jurkat cells, no increase in the basal level of CHC phosphorylation was observed upon incubation at 37°C (Fig. 1 A). However, after 5 min of incubation, a noticeable increase (approximately fourfold) in CHC phosphorylation in activated Jurkat cells was detected above basal levels followed by a decrease in the signal at 20 min (Fig. 1 A). To more accurately mimic the activation of T cells in vivo, we also stimulated Jurkat cells with cell-sized beads that had been coated with anti-CD3 Ab. When Jurkat cells were in-

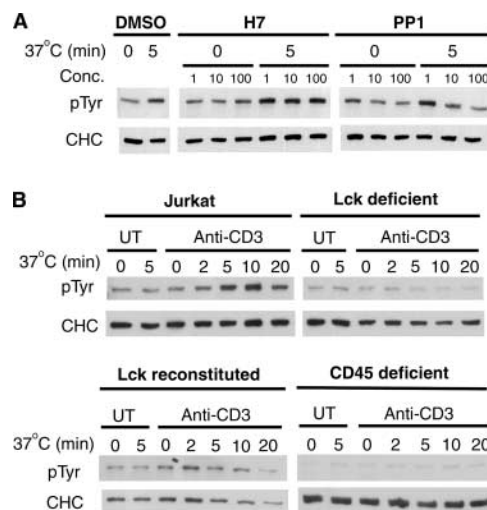


**Figure 1.** CHC phosphorylation in T cells after TCR engagement. (A) Jurkat cells were untreated (UT) or treated with anti-CD3 Ab at 4°C and incubated at 37°C for 0–20 min. CHC was immunoprecipitated from lysates, resolved by gel electrophoresis, and immunoblotted with an Ab to phosphotyrosine. Blots were stripped and reprobed with an Ab to CHC. To measure the extent of tyrosine phosphor-

ylation relative to the levels of CHC immunoprecipitated, immunoblot images were quantified using the NIH Image software and the ratio of phosphotyrosine to CHC signal was plotted. (B) Jurkat cells were untreated or treated with beads (Bds) alone and left at 4°C. Jurkat cells were also treated with anti-CD3 Ab or beads coated with anti-Q3 Ab (CD3 + Bds) at 4°C and incubated at 37°C for 10 min. CHC phosphorylation was assessed as described above. (C) Jurkat cells or CTL clone LYcl48 was untreated or treated with anti-CD3 Ab at 4°C and incubated at 37°C for 10 min. CHC phosphorylation was assessed as described above. Bars below each lane represent the average ratio obtained for each stimulation condition for three independent experiments.

cubated with uncoated beads, no increase in the basal level of CHC phosphorylation was observed upon incubation at 37°C (not depicted). However, after 10 min of incubation with coated beads at 37°C, a measurable increase in CHC phosphorylation was detected above basal levels (Fig. 1 B). These results demonstrate that CHC is phosphorylated in an inducible manner upon T cell activation by both soluble and bound ligands. Lastly, we examined CHC phosphorylation in nontransformed human CD8<sup>+</sup> peripheral T cells. The CTL clone LYcl48 was treated with soluble anti-CD3 Ab and the cells were then incubated for 10 min at 37°C to induce TCR activation and internalization. A measurable increase in CHC phosphorylation was detected above basal levels (Fig. 1 C). Thus, CHC phosphorylation occurs upon T cell activation in both the transformed CD4<sup>+</sup> Jurkat T cell line and normal human CD8<sup>+</sup> CTL.

**Clathrin Phosphorylation Requires the Activity of a Src Family Kinase.** The kinetics of CHC phosphorylation in activated T cells was slow compared with the total protein tyrosine phosphorylation in cell lysates (not depicted). This delay suggested that other signaling events are initiated before CHC phosphorylation. We had previously found that the activation of c-Src kinase or Lyn kinase, a Src kinase family member, was required for CHC phosphorylation after EGFR and BCR stimulation, respectively (20, 21). To address whether the activity of a Src family kinase was necessary for CHC phosphorylation in activated T cells, we treated Jurkat cells with various concentrations of the Src family kinase inhibitor PP1 before stimulation with soluble anti-CD3 Ab. At increasing concentrations of PP1, but not the serine/threonine kinase inhibitor H7, the level of inducible CHC phosphorylation was diminished (Fig. 2 A). Additionally, in the presence of PP1, the basal level of CHC phosphorylation in Jurkat cells before the induction of TCR internalization was also decreased, suggesting that basal Src family kinase activity was responsible for the basal level of CHC phosphorylation observed. To confirm these results, we examined CHC phosphorylation in J.Cam1 T cells, a mutant derived from Jurkat lacking the Src family kinase Lck (30, 31). In contrast to Jurkat cells, both basal and inducible CHC phosphorylation were significantly reduced in J.Cam1 cells (Fig. 2 B). This diminution in CHC phosphorylation was not the result of decreased TCR surface expression on the J.Cam1 cells (30 and confirmed by data not depicted). When J.Cam1 cells were reconstituted with Lck (J.Cam1-Lck; 31), basal and inducible CHC phosphorylation were restored after T cell activation (Fig. 2 B). Lck expression in the reconstituted J.Cam1 cells was ~20% of the expression in Jurkat cells, thus accounting for the lower levels of CHC phosphorylation in activated J.Cam1-Lck cells (unpublished data). Because the protein tyrosine phosphatase (PTP) CD45 induces Lck activity by dephosphorylating a tyrosine residue in the negative regulatory site of this kinase (43–45), we examined CHC phosphorylation in the CD45-deficient Jurkat T cell line, J45.01, in which Lck activity is attenuated (32). CHC phosphorylation was significantly reduced in J45.01 cells

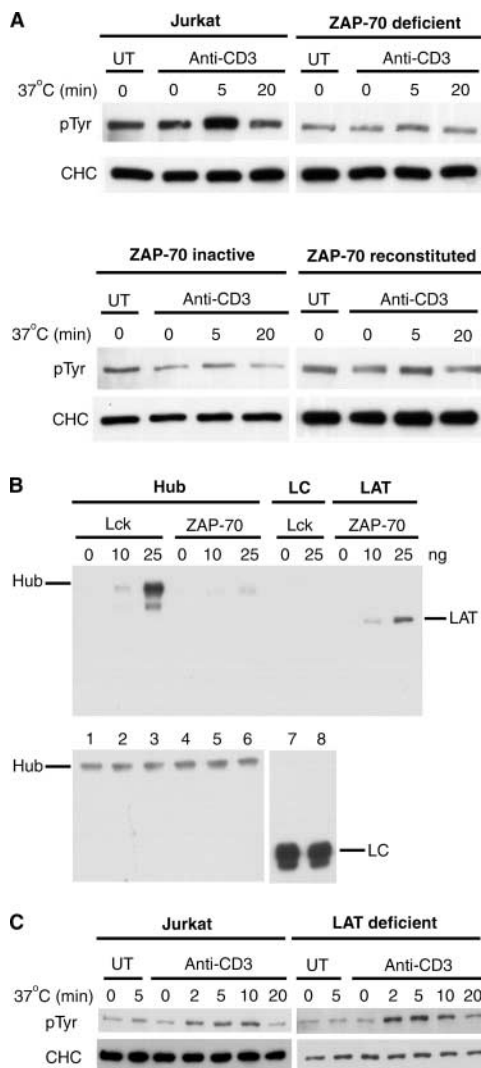


**Figure 2.** Lck activity is required for CHC phosphorylation. (A) Jurkat cells were pretreated with 1% DMSO or various micromolar concentrations of either the serine/threonine kinase inhibitor H7 or the Src kinase inhibitor PP1 in DMSO. Cells were then treated with anti-CD3 Ab in the continuous presence of DMSO ± inhibitors at 4°C and incubated at 37°C for 0 or 5 min. CHC phosphorylation was assessed as described in Fig. 1. The results are representative of two independent experiments. (B) Wild-type Jurkat cells and the indicated Jurkat mutants were untreated (UT) or treated with anti-CD3 Ab at 4°C and incubated at 37°C for 0–20 min. Cells tested were JCam.1 (Lck deficient), JCam.1-Lck (JCam.1 reconstituted with Lck), and J45.01 (CD45 deficient). CHC phosphorylation was assessed as described in Fig. 1. The results are representative of five independent experiments.

compared with Jurkat cells after activation (Fig. 2 B). These results indicate that the Src family kinase Lck plays a critical role in CHC phosphorylation in activated Jurkat cells.

**Proximal TCR Signaling Is Responsible for CHC Phosphorylation.** Because the induction of Lck activity after TCR stimulation initiates the signaling cascade in T cells (22), we next sought to determine the contribution of downstream TCR signaling events to CHC phosphorylation. Inducible CHC phosphorylation in response to anti-CD3 Ab treatment was reduced in P116 T cells lacking ZAP-70, a Syk family kinase activated by phosphorylation by Lck, and in DK33 T cells containing kinase-dead ZAP-70 (Fig. 3 A; reference 33). However, the reduction in basal and inducible CHC phosphorylation was not as extensive as that observed for the Lck-deficient J.Cam1 cells (Fig. 2 B). The diminution in CHC phosphorylation was not the result of decreased TCR surface expression on P116 or DK33 cells (not depicted). When P116 cells were reconstituted with ZAP-70 (P116.c39; reference 33), basal and inducible CHC phosphorylation were restored after T cell activation (Fig. 3 A). These results suggest that although ZAP-70 plays a role in CHC phosphorylation, its effects on these processes might be secondary to the activity of Lck.

To further define the roles of Lck and ZAP-70 in CHC phosphorylation, we incubated a recombinant fragment of the CHC (Hub residues 1074–1675) with either purified recombinant Lck or ZAP-70 in vitro and measured the extent of phosphorylation by immunoblotting with a phos-



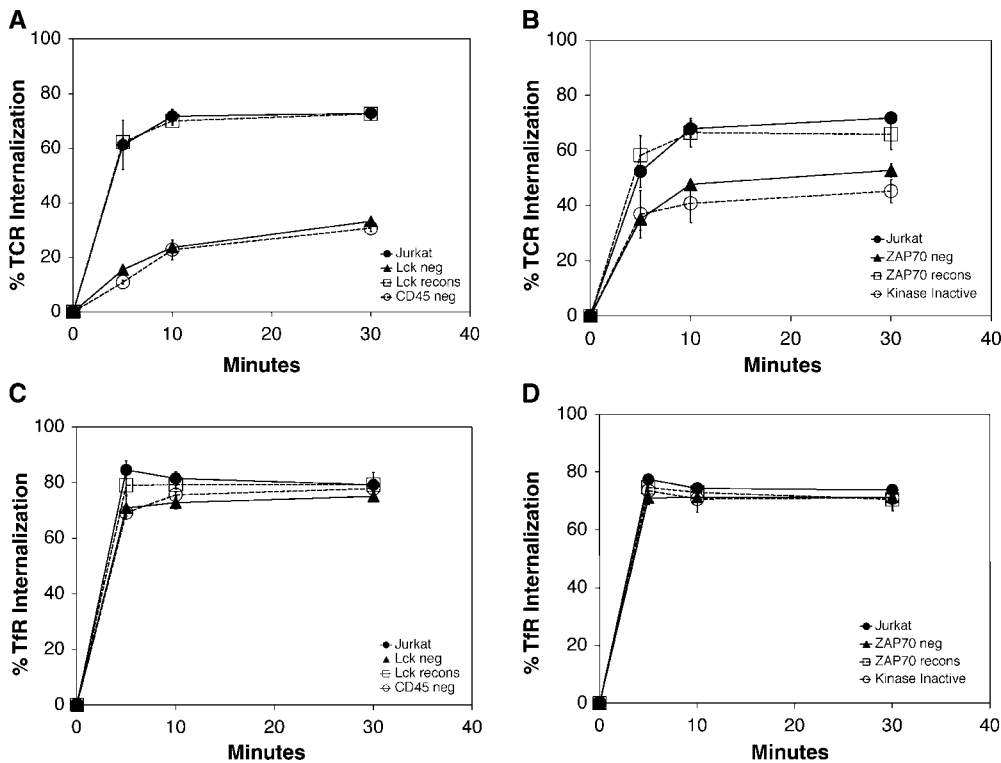
**Figure 3.** Proximal TCR signaling is responsible for CHC phosphorylation. (A) Wild-type Jurkat cells and the indicated Jurkat mutants were untreated (UT) or treated with anti-CD3 Ab at 4°C and incubated at 37°C for 0–20 min. Cells tested were P116 (ZAP-70 deficient), DK33 (ZAP-70 inactive), and P116.c39 (P116 reconstituted with ZAP-70). CHC phosphorylation was assessed as described in Fig. 1. (B) A bacterially expressed fragment of His<sub>6</sub>-tagged CHC Hub (residues 1074–1675) was incubated with either recombinant Lck or recombinant ZAP-70 in the presence of ATP for 20 min at room temperature. As controls, bacterially expressed clathrin light chain b (LC) was incubated with Lck and recombinant LAT was incubated with ZAP-70 in the presence of ATP for 20 min at room temperature. The proteins were resolved by gel electrophoresis and immunoblotted with an Ab to phosphotyrosine (top). The identical samples were resolved on a second gel and immunoblotted with an Ab to the His<sub>6</sub>-tag (lanes 1–6) or to LC (lanes 7 and 8; bottom). (C) Wild-type Jurkat and J.Cam2 (LAT deficient) cells were untreated (UT) or treated with anti-CD3 Ab at 4°C and incubated at 37°C for 0–20 min. CHC phosphorylation was assessed as described in Fig. 1. In A, B, and C, each set of results is representative of three independent experiments.

phosphotyrosine-specific Ab. We chose to test this segment of CHC because we had previously identified the site of CHC phosphorylation upon EGFR stimulation as tyrosine 1477 (20), which lies in a conserved region compatible with the Src family kinase consensus sequence (46). Lck

but not ZAP-70 phosphorylates Hub in vitro (Fig. 3 B). However, recombinant ZAP-70 was capable of phosphorylating its known in vivo substrate LAT. Furthermore, clathrin light chain b that lacks a Src consensus sequence (47) was not phosphorylated by Lck. These results demonstrated that Lck but not ZAP-70 directly phosphorylates CHC in vitro. Thus, the in vivo role of ZAP-70 in CHC phosphorylation after T cell activation must be due to its interaction with Lck, perhaps by a feedback mechanism. As a final confirmation of the importance of proximal TCR signaling in CHC phosphorylation, we examined J.Cam2 T cells deficient in LAT, a transmembrane protein linking proximal and distal TCR signaling events (34). In the absence of LAT, CHC was still inducibly phosphorylated in J.Cam2 cells after activation (Fig. 3 C). Taken together, the above data provide strong evidence that the phosphorylation of clathrin in activated Jurkat T cells is the result of proximal TCR signaling events that occur upstream of the molecule LAT and is most likely attributed to Lck.

*Clathrin Phosphorylation Links Proximal TCR Signaling Events to TCR Internalization.* The implication of Src family kinase activity, particularly that of Lck, in TCR internalization had previously been reported (26–29). By FACS<sup>®</sup> analysis, we also observed a significant impairment in the ability of the Lck-deficient J.Cam1 and the CD45-deficient J45.01 cell lines to internalize their TCR after Ab stimulation (Fig. 4 A). Reconstituting J.Cam1 cells with Lck restored TCR internalization to levels achieved in Jurkat cells after activation. TCR internalization was also slightly reduced in the ZAP-70-defective cell lines P116 and DK33 (Fig. 4 B), suggesting that ZAP-70 activity also influences Lck activity with respect to TCR internalization. These proximal TCR signaling events were influential for TCR internalization, but we detected no deficiency in the ability of the Lck signaling mutants J.Cam1 or J45.01 (Fig. 4 C), or the ZAP-70 signaling mutants P116 or DK33 (Fig. 4 D) to internalize the transferrin receptor. These results support a positive correlation between CHC phosphorylation and TCR internalization.

Having demonstrated that CHC was phosphorylated in the CD8<sup>+</sup> CTL clone LYcl48, we also investigated whether phosphorylation correlated with TCR internalization, as seen for CD4<sup>+</sup> Jurkat cells. By FACS<sup>®</sup> analysis, we observed ligand-induced TCR endocytosis in LYcl48 after stimulation with anti-CD3 Ab, although the internalization of this receptor was not as robust as seen with Jurkat cells (Fig. 5). Thus, stimulated Lck phosphorylation of CHC during TCR engagement correlates with receptor endocytosis in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Previous studies had demonstrated that before encounter with antigen, TCRs are constitutively internalized and recycled back to the cell surface (2–4). Next, we investigated whether basal levels of Lck activity might be involved in constitutive TCR internalization. Jurkat cells, the Lck-deficient J.Cam1 cells, and the LYcl48 CTL were incubated at 37°C and steady-state levels of surface TCR were measured by FACS<sup>®</sup> using FITC-conjugated anti-CD3 Ab. Reduction in these levels

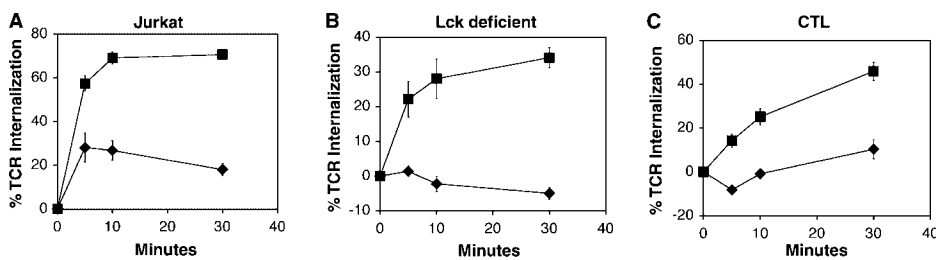


**Figure 4.** TCR but not transferrin receptor internalization is reduced in cells deficient in CHC phosphorylation. Wild-type Jurkat cells (●) and the indicated Jurkat mutants were treated at 4°C with anti-CD3 Ab (A and B) or an Ab against hTtR (anti-hTtR Ab; C and D), incubated at 37°C for 0–30 min, and then transferred to 4°C to stop receptor internalization. In A and C, cells tested were JCam.1 (Lck deficient; ▲), JCam.1-Lck (JCam.1 reconstituted with Lck; □), and J45.01 (CD45 deficient; ○). In B and D, cells tested were P116 (ZAP-70 deficient; ▲), P116.c39 (P116 reconstituted with ZAP-70; □), and DK33 (ZAP-70 inactive; ○). Cells were stained with a FITC-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse Ig to detect Ab remaining at the cell surface. The percent internalization represents the percentage of cells negative for surface Ab by FACS<sup>®</sup> analysis relative to staining at 4°C. The mean percent internalization from three independent experiments is shown. Note that the size of the symbols at some points masks the error bars.

relative to staining at 4°C was considered constitutive internalization. Interestingly, we only observed constitutive TCR internalization in Jurkat cells, and for these cells, it was completely abolished in the absence of Lck (Fig. 5 B). These results suggest that CHC phosphorylation is involved in both constitutive and ligand-induced TCR internalization and implicate Lck activity (basal or stimulated). However, they also demonstrate that the TCR does not always display constitutive endocytosis, perhaps reflecting differences between CD4<sup>+</sup> and CD8<sup>+</sup> T cells and/or levels of basal Lck activity.

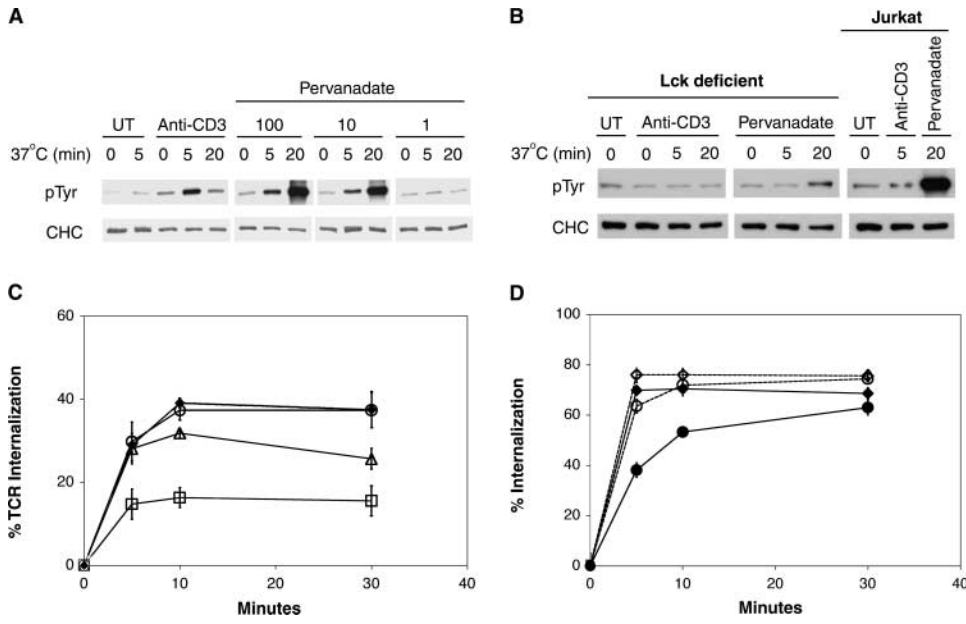
*Pervanadate Alters the Kinetics of CHC Phosphorylation and TCR Internalization.* Our results so far have suggested a positive correlation between CHC phosphorylation and TCR internalization in T cells after Ab stimulation and in

constitutive endocytosis. To investigate the mechanistic impact of CHC phosphorylation, we analyzed the effect of treating Jurkat cells with the PTP inhibitor pervanadate. Treatment of Jurkat cells with pervanadate has been documented to activate the signal transduction events usually observed upon ligand binding, despite its inhibition of the PTP CD45, which normally regulates Lck activity. This has been attributed to a net accumulation of phosphate on the tyrosine residue in the activation loop of Lck, bypassing the need for dephosphorylating the negative regulatory site of Lck (48). Even in the absence of TCR stimulation, CHC became substantially phosphorylated in Jurkat cells treated with concentrations of 10 μM and greater of pervanadate (Fig. 6 A). However, the kinetics of this phosphorylation was significantly different from that observed



**Figure 5.** Ligand-induced and constitutive TCR internalization in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Jurkat cells (A), J.Cam1 (Lck deficient) cells (B), or CTL clone LYcl48 (C) was untreated (◆) or treated (■) with anti-CD3 Ab at 4°C, incubated at 37°C for 0–30 min, and then transferred to 4°C to stop receptor internalization. Untreated cells were stained with a FITC-conjugated anti-CD3 Ab to detect reduction of TCR on the cell

surface relative to the level at 4°C, reflecting constitutive internalization. Stimulated cells were stained with a FITC-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse Ig Ab to detect remaining stimulatory Ab at the cell surface. The percent internalization represents the percentage of cells negative for surface Ab by FACS<sup>®</sup> analysis relative to staining at 4°C. The mean percent internalization from three independent experiments is shown. Note the different scales in A, B, and C.

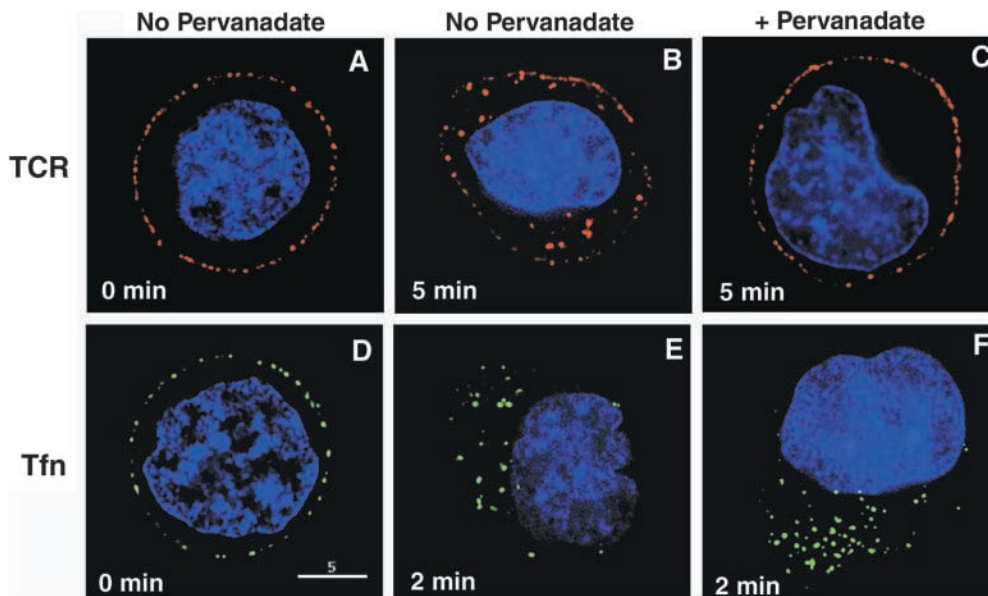


**Figure 6.** Pervanadate affects clathrin phosphorylation and TCR internalization but not TfR internalization. (A) Jurkat cells were untreated (UT) or treated with anti-CD3 Ab at 4°C or with various micromolar concentrations of pervanadate and incubated at 37°C for 0–20 min. (B) J.Cam1 (Lck deficient) or Jurkat cells were untreated (UT) or treated with anti-CD3 Ab at 4°C or with 100 μM pervanadate and incubated at 37°C for 0–20 min. CHC phosphorylation was assessed as described in Fig. 1. The results are representative of three independent experiments. (C) Jurkat cells were untreated (UT; ●) or treated with 100 (□), 10 (Δ), or 1 μM (○) pervanadate, incubated at 37°C for 0–30 min, and then transferred to 4°C to stop constitutive receptor internalization. Cells were stained with FITC-conjugated anti-CD3 Ab to detect reduction of TCR on the cell surface relative to the level at 4°C, reflecting constitutive

internalization. The percent internalization represents the percentage of cells negative for surface receptor by FACS® analysis relative to staining at 4°C. The mean percent internalization from three independent experiments is shown. (D) Jurkat cells were treated with anti-CD3 Ab at 4°C in the presence (●, solid lines) and absence (○, dashed lines) of 100 μM pervanadate or with anti-hTfR Ab in the presence (◆, solid lines) and absence (◇, dashed lines) of 100 μM pervanadate. Cells were incubated at 37°C for 0–30 min, transferred to 4°C to stop receptor internalization, and then stained with FITC-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse Ig to detect Ab remaining at the cell surface. The percent internalization represents the percentage of cells negative for surface Ab by FACS® analysis relative to staining at 4°C. The mean percent internalization from three independent experiments is shown. Note the different scales in C and D.

for Jurkat cells stimulated with Ab. Upon pervanadate treatment, CHC phosphorylation continued to accumulate through 20 min after incubation at 37°C, the latest time point we evaluated. To examine the kinase involved in this pervanadate-induced phosphorylation, we analyzed pervanadate effects on Lck-deficient J.Cam1 cells, in which CHC phosphorylation is negligible after TCR stimulation (Fig. 2). Although CHC was phosphorylated in J.Cam1

cells after 20 min of treatment with 100 μM pervanadate, the level of phosphorylation was reduced 15-fold compared with that observed in pervanadate-treated wild-type Jurkat cells (Fig. 6 B). These data suggest that pervanadate treatment and TCR engagement both activate the same signaling pathway leading to CHC phosphorylation and that CHC phosphorylation in both of these instances involves Lck activity.



**Figure 7.** Pervanadate affects TCR internalization at the plasma membrane. Jurkat cells were treated with anti-TCR Ab (A–C) or FITC-conjugated transferrin (D–F) at 4°C and incubated at 37°C for 0, 2, or 5 min in the presence or absence of 100 μM pervanadate. Anti-TCR Ab is detected using a rhodamine-conjugated anti-mouse IgM secondary Ab. The nucleus is stained with DAPI. The results are representative of at least three independent experiments and numbers are provided in Results. Bar, 5 μm for all panels.

Next, we investigated the effect of pervanadate on TCR internalization. Jurkat cells were treated with various concentrations of pervanadate, incubated at 37°C, and steady-state levels of surface TCR were measured by FACS® using FITC-conjugated anti-CD3 Ab. We observed a decrease in constitutive TCR internalization in Jurkat cells treated with 10 and 100 µM pervanadate (Fig. 6 C). We also examined the effect of pervanadate on ligand-induced TCR internalization. For Jurkat cells stimulated with anti-CD3 Ab in the presence of 100 µM pervanadate, we observed a reduction in TCR endocytosis (Fig. 6 D). The effect of pervanadate on inducible TCR internalization was equivalent to its effect on constitutive uptake because both processes were reduced to 50% of their level in the absence of the drug. The effect of pervanadate upon ligand-induced internalization was observed for the TCR, but we detected no deficiency in the ability of Jurkat cells treated with pervanadate to internalize the transferrin receptor (Fig. 6 D). We observed similar results when we examined the effect of pervanadate on ligand-induced TCR internalization by immunofluorescent microscopy (Fig. 7). Upon stimulation of Jurkat cells with anti-TCR Ab in the presence of 100 µM pervanadate, we observed a lack of TCR endocytosis in 41% of the cells ( $n = 200$  in each of three independent experiments; Fig. 7 C). In Jurkat cells stimulated with anti-TCR Ab in the absence of the drug, only 18% of the cells showed no TCR endocytosis with 82% of the cells internalizing this receptor ( $n = 200$  in each of three independent experiments; Fig. 7 B). Again, the effect of pervanadate upon ligand-induced internalization was observed for the TCR, but Jurkat cells treated with pervanadate were capable of internalizing FITC-conjugated transferrin to the same degree as untreated cells (Fig. 7, E and F). Taken together, the above results further implicate CHC phosphorylation in influencing the expression of TCR on the surface of Jurkat cells. They also suggest that although CHC phosphorylation is involved in TCR internalization, its subsequent dephosphorylation must be part of the mechanism for both constitutive and ligand-induced internalization.

## Discussion

In this study, we demonstrate that inducible CHC phosphorylation occurs as a result of TCR signaling after TCR engagement in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The Src family kinase Lck is apparently responsible for CHC phosphorylation and its activity is influenced by the integrity of the downstream signaling molecule ZAP-70. CHC phosphorylation positively correlates with ligand-induced TCR internalization in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and with constitutive TCR endocytosis in CD4<sup>+</sup> T cells. Pervanadate treatment causing irreversible CHC phosphorylation reduced both constitutive and ligand-induced TCR internalization in CD4<sup>+</sup> T cells, and this inhibition occurred at the early stages of ligand-induced TCR endocytosis from

the plasma membrane. By correlation, these studies implicate both CHC phosphorylation and dephosphorylation as influential in TCR internalization pathways.

Receptor-mediated endocytosis can be regulated both by modification of the cytoplasmic domains of internalized receptors and by receptor signaling effects on clathrin coat components. For a number of receptors including EGFR and CTLA-4, ligand binding causes tyrosine kinase activation, resulting in a structural change that exposes adaptor-binding motifs in the cytoplasmic domain of the receptors (18, 19). Furthermore, receptor signaling induces the modification of trafficking proteins, in particular the phosphorylation of the CHC (20, 21). It has been proposed that upon TCR engagement, the phosphorylation of the ITAMs within the cytoplasmic domains of the TCR-CD3 complex induces a conformational change exposing adaptor-binding motifs allowing for the assembly of the CCV and TCR internalization (10, 28). Here we demonstrate that TCR signaling also induces CHC phosphorylation in activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Similar to the EGFR and the BCR (20, 21), CHC phosphorylation upon TCR engagement is mediated by a Src family protein tyrosine kinase. Our results indicate that the Src family kinase Lck directly phosphorylates CHC in vitro, and in Jurkat cells deficient in Lck, inducible CHC phosphorylation is abolished. Although Lck plays a critical role in CHC phosphorylation, we demonstrate that its activity is influenced by the integrity of the proximal TCR signaling cascade. The reduction in CHC phosphorylation observed in Jurkat T cells defective in the downstream kinase ZAP-70 suggests that the activation of this kinase positively regulates Lck activity, thus explaining its influence on CHC phosphorylation and correlation of ZAP-70 activity with TCR internalization.

In this study, we demonstrate a correlation between CHC phosphorylation by Lck and TCR internalization after activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells by receptor engagement. Our data also suggest that basal Lck activity plays a role in constitutive TCR internalization. Interestingly, the CD8<sup>+</sup> CTL clone we examined here exhibited very minimal constitutive TCR internalization, and its ligand-induced TCR endocytosis was significantly less than the CD4<sup>+</sup> Jurkat cells despite having higher overall levels of the receptor on its surface. Recently, constitutive versus ligand-induced TCR down-modulation has been evaluated in murine CD4<sup>+</sup> T cells (10), but our study is the first to examine these processes in a human CD8<sup>+</sup> CTL clone. Although our findings may simply reflect intrinsic differences in normal versus transformed cells, we speculate that the discrepancies in constitutive versus ligand-induced TCR internalization in the CD4<sup>+</sup> and CD8<sup>+</sup> T cells might be attributed to the different specialized functions of these cells in vivo.

Previously, the site of clathrin phosphorylation upon EGFR signaling was mapped to a single tyrosine residue at amino acid position 1477 (20) in the region of the heavy chain that interacts with light chain and regulates assembly of the clathrin triskelion (38). Our preliminary studies sug-



gest that this tyrosine is also a target for Lck phosphorylation of CHC as well as adjacent tyrosine residues (unpublished data). It has been proposed that CHC phosphorylation could act either as a positive or a negative regulatory signal for clathrin function. For example, BCR signaling activates the Src family kinase Lyn necessary for CHC phosphorylation. This phosphorylation was shown to correlate with BCR internalization after signaling in rafts. The effect of CHC phosphorylation was compatible with positive stimulation of assembly of clathrin associated with rafts. It was, however, also compatible with a "negative" stabilization of preformed coated pits. In this latter scenario, phosphorylation might delay interactions with regulatory proteins required for the pit to vesicle transition to capture spent signaling receptors from rafts (21). In this study, we report the first data suggesting that CHC phosphorylation may indeed serve as a negative regulatory signal for CCV formation. Although our data show that CHC phosphorylation correlates with TCR internalization, we also demonstrate in experiments with pervanadate that if CHC dephosphorylation cannot occur, both constitutive and ligand-induced TCR internalization are reduced. This inhibition of TCR internalization occurs at the plasma membrane, reducing access of the TCR to early endosomes. Although pervanadate has pleiotropic effects on cells, we found that CHC phosphorylation in the Lck-deficient Jurkat cells treated with pervanadate was significantly reduced compared with wild-type Jurkat cells, suggesting that CHC phosphorylation after either pervanadate treatment or TCR engagement involves Lck activity. Furthermore, we demonstrated that the internalization of the transferrin receptor was not inhibited by pervanadate treatment, thus eliminating the possibility of pleiotropic effects of this drug on factors that affect endocytosis in general. Thus, we propose that CHC phosphorylation and dephosphorylation are relevant to signaling receptors whose internalization is induced by ligand but not those that are constitutively internalized.

Similar to its proposed role in BCR endocytosis, CHC phosphorylation could influence clathrin function during the initial TCR internalization from the plasma membrane once the receptors have completed their signaling in rafts. However, considering what is known about TCR pathways of down-modulation, it is also possible that CHC phosphorylation plays a role in the regulation of receptor levels at an additional intracellular site. Before encounter with antigen, TCRs are continuously internalized and recycled back to the cell surface (2–4). After TCR engagement with antigen, the TCR is internalized and trafficked to the lysosome for degradation (8–10). Clathrin is involved in targeting receptors destined for degradation from the endosome to lysosomes, and this pathway involves both ubiquitination of receptors and the adaptor molecule Hrs that interacts with ubiquitin and clathrin (15). CHC phosphorylation could be active during both internalization from the plasma membrane and in diversion from endosomes to lysosomes. Further analysis will be required to es-

tablish how this modification of CHC influences CHC-interacting proteins and affects either or both of these two clathrin-mediated pathways.

Recent studies have established the importance of TCR signaling (for review see reference 49) and TCR down-modulation (50) at the contact site between T cells and APCs, termed the immunological synapse. TCR down-modulation at this synapse serves two purposes. Internalization of the TCR from the plasma membrane to the endosome potentiates T cell activation by bringing the receptor in close proximity to critical molecules required for progression of the TCR signaling cascade (7). The ultimate result of these signaling events is the complete activation of the T cell and the development of an effective T cell response to pathogenic organisms, tumors, or self-ligands in cases of selection and autoimmunity. Second, TCR down-modulation attenuates signaling by targeting the TCR from the endosome to a lysosomal compartment for degradation (8–10). This process maintains a homeostatic balance between activation and down-modulation of the T cell response and may also function as a mechanism by which tumors induce lack of detection by the immune system (51). Clathrin has been implicated in both of these regulatory steps, and here we demonstrate that TCR signaling can influence clathrin modification after T cell activation through phosphorylation and dephosphorylation. We propose that this modification plays a role in both the development and the modulation of the T cell response.

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