Differential Src Family Kinase Activity Requirements for CD3 ζ Phosphorylation/ZAP70 Recruitment and CD3ε Phosphorylation

Tara L. Lysechko and Hanne L. Ostergaard


http://www.jimmunol.org/content/174/12/7807

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**  This article cites 38 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/174/12/7807.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852 Copyright © 2005 by The American Association of Immunologists All rights reserved. Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Differential Src Family Kinase Activity Requirements for CD3ζ Phosphorylation/ZAP70 Recruitment and CD3ε Phosphorylation

Tara L. Lysechko and Hanne L. Ostergaard

The current model of T cell activation is that TCR engagement stimulates Src family tyrosine kinases (SFK) to phosphorylate CD3ζ. CD3ζ phosphorylation allows for the recruitment of the tyrosine kinase ZAP70, which is phosphorylated and activated by SFK, leading to the phosphorylation of downstream targets. We stimulated mouse CTLs with plate-bound anti-CD3 and, after cell lysis, recovered proteins that associated with the CD3 complex. The protein complexes were not preformed, and a number of tyrosine-phosphorylated proteins were inducibly and specifically associated with the TCR/CD3 complex. These results suggest that complex formation only occurs at the site of TCR engagement. The recruitment and tyrosine phosphorylation of most proteins were abolished when T cells were stimulated in the presence of the SFK inhibitor PP2. Surprisingly, CD3ζ, but not CD3ε, was inducibly tyrosine phosphorylated in the presence of PP2. Furthermore, ZAP70 was recruited, but not phosphorylated, after TCR stimulation in the presence of PP2, thus confirming the phosphorylation status of CD3ζ. These data suggest that there is a differential requirement for SFK activity in phosphorylation of CD3ζ vs CD3ε. Consistent with this possibility, ZAP70 recruitment was also detected with anti-CD3-stimulated, Lck-deficient human Jurkat T cells. We conclude that TCR/CD3-induced CD3ζ phosphorylation and ZAP70 recruitment do not absolutely require Lck or other PP2-inhibitable SFK activity, but that SFK activity is absolutely required for CD3ε and ZAP70 phosphorylation. These data reveal the potential for regulation of signaling through the TCR complex by the differential recruitment or activation of SFK.


Phosphorylation of CD3ζ is one of the earliest detectable events that occurs after TCR stimulation of T cells (1). Ample evidence suggests that the Src family kinases (SFK), Lck and, to a lesser extent, Fyn, phosphorylate the ITAMs of CD3ζ (2–4). ZAP70 is recruited to CD3ζ, binding to dually phosphorylated ITAMs, via its tandem Src homology 2 (SH2) domains (2, 5–7). Once recruited, ZAP70 is phosphorylated by Lck or Fyn, which renders it catalytically active (2, 3, 5, 8, 9), leading to phosphorylation of downstream substrates, including the adapter protein, linker for activation of T cells (10). Tyrosine phosphorylation of linker for activation of T cells, in turn, allows for the recruitment of a number of proteins to the signaling complex (10).

We have developed a biochemical method to capture and analyze proteins found complexed with the TCR after TCR stimulation. In this approach, an immobilized stimulatory Ab is used to both stimulate T cells and, after cell lysis, capture protein complexes regardless of how they are associated with the complex. This immobilized Ab capture approach is similar to the solid phase immunoisolation assay described by Tamura et al. (11), in which Abs were immobilized to plastic to capture labeled proteins from cell lysates. This solid phase immunoisolation was shown to be highly specific, with low background binding (11). We have adapted this technique to use anti-TCR/CD3 stimulatory Ab to capture protein complexes formed in live T cells to study TCR-proximal signaling events. This approach can also be used as a tool to identify novel proteins that are associated with such protein complexes.

Using solid phase immunoisolation, we have found that TCR-associated CD3ζ can be phosphorylated and recruits ZAP70 in the presence of the SFK inhibitor PP2, a potent inhibitor of Lck and Fyn activity in T cells (12). By contrast, PP2 did block both CD3ε and ZAP70 phosphorylation and all additional downstream tyrosine phosphorylation events. These data suggest that there is a differential SFK requirement for CD3ζ and CD3ε/ZAP70 phosphorylation. Furthermore, we observed CD3ζ phosphorylation and ZAP70 recruitment in Lck-deficient Jurkat cells. Our results indicate that during TCR stimulation, the PP2-inhibitable SFK, Lck, and Fyn, are not uniquely capable of mediating CD3ζ phosphorylation, resulting in ZAP70 recruitment. However, Lck and/or another SFK are absolutely required for CD3ε and ZAP70 phosphorylation and recruitment of additional proteins to the CD3 complex, suggesting that the levels of active SFK needed for phosphorylation of the various CD3 subunits differ substantially.

Materials and Methods

Cells

The murine CD8⁺ CTL clone AB.1 has been described previously (13). The clones were stimulated weekly with irradiated allogeneic C57BL/6 splenocytes and IL-2 and were used for experiments 5–6 days after stimulation. The degranulation assay has been described in detail previously (14). Con A blasts were generated by culturing C57BL/6 splenocytes with...
2 μg/ml Con A for 48 h. Human Jurkat T cells and the Lck-deficient variant JCaM1.6 were obtained from American Type Culture Collection.

Abs and reagents
The source and purification of Abs from hybridomas producing 145-2C11 (anti-CD3ε), H57–597 (anti-TCRβ), I3/2.3 (anti-CD45, pan-specific), MB23G2 (anti-CD45RB), MB4B4 (anti-CD45RB), M1/42.3.9.8 (anti-MHC class I), OKT3 (anti-CD3ε), and P72.10.5 (anti-phosphotyrosine) have been described previously (15). The hybridoma producing H146-968A was obtained from Dr. M. C. Miceli (University of California, Los Angeles, CA) (16). Polyclonal anti-Lck (C437) used for Lck immunoprecipitations was generated in our laboratory using a BSA-coupled peptide based on aa 476–509 of Lck. Anti-ERK2, anti-Lck, anti-Nck, and anti-ZAP70 were purchased from BD Biosciences. Anti-CD3ε (M-20), anti-CD3γ (6B10.2), ant-Nck1/2 (C-19), anti-α p21-activated kinase (PAK) (N-20), and anti-goat IgG-HRP were purchased from Santa Cruz Biotechnology. Anti-mouse IgG-HRP were purchased from Jackson ImmunoResearch Laboratories. Protein A-HPB was purchased from Pierce. The Src family inhibitor, PP2, and the inactive analog, PP3, were purchased from Calbiochem. Myelin basic protein (MBP) was purchased from Sigma-Aldrich.

Cell stimulation with immobilized Abs and recovery of associated complexes
For immobilizing Abs on plastic, 60-mm, non-tissue culture-treated plates were incubated with 10 μg/ml 145-2C11 or other mAb overnight at 4°C. The plates were washed twice with PBS, blocked with 2% BSA in PBS for 45 min at 37°C, and washed three times with PBS, then 1 × 106 cells were added to the plates. Where indicated, the cells were pretreated with PP2 or PP3 for 15 min on ice before addition to the plates. The cells were stimulated at 37°C for the indicated time, after which the cells were lysed in the plates for 20 min with lysis buffer (1% Nonidet P-40, 10 mM Tris (pH 7.5), 150 mM NaCl, and 1 mM sodium vanadate). Lysates were recovered from the plates, and a sample containing 5 × 106 cell equivalents was saved for further analysis. The plates were vigorously washed three times with lysis buffer to ensure that all cellular debris was removed, then the immobilized Abs and associated proteins were eluted from the plate by adding reducing sample buffer and heating the plates to 55°C. The entire sample of eluted protein was loaded into a single lane of an SDS-PAGE gel. When used, PP2 was not added to the lysis buffer used to wash plates, because we have found that its inclusion in the washes does not impact the quality or quantity of phosphoproteins recovered to the TCR/CD3 complex.

Western blotting
Cell lysates and proteins eluted from the mAb-coated plates were run on 12 or 15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using the appropriate HRP-coupled Ab and was visualized by ECL (NEN).

Lck in vitro kinase assays
AB.1 clones were washed three times with PBS, and 1 × 106 cells were resuspended in RPMI 1640. Cells were pretreated with DMSO, PP2, or PP3 for 15 min on ice. Cells were lysed with 1% Nonidet P-40 lysis buffer with or without PP2/PP3. Lck or normal rabbit serum (NRS) immunoprecipitations were performed on postnuclear lysates. Immunoprecipitates were washed twice with radioimmunoprecipitation assay (RIPA) buffer (32) with or without PP2/PP3 and once with HEPES kinase buffer (10 mM HEPES (pH 7.1) and 10 mM MnCl2) with or without PP2/PP3. Kinase reactions contained Lck or NRS immunoprecipitates, 5 μg of MBP substrate, 1 mM ATP, and either PP2 or PP3 in 50 μl. Kinase reactions proceeded for 30 min at 32°C, at which time the beads containing immunoprecipitates were subjected to centrifugation, and 40 μl of the supernatant containing the substrate was transferred to a new tube. Substrate-containing supernatant was loaded onto a 15% SDS-PAGE gel. Proteins were transferred, and the membrane was probed with anti-phosphotyrosine. A portion of the sample was stained with Coomassie Blue to detect MBP protein loading. Lck immunoprecipitates were run on an 8.5% gel and transferred, and the membrane was blotted for Lck as a loading control.

Results
Stimulation of CTL clones with immobilized anti-CD3 results in specific recruitment of a number of tyrosine-phosphorylated proteins to TCR/CD3 complex
We have established a biochemical approach to evaluate proteins recruited to the TCR complex by capturing the TCR complex and associated proteins after cell stimulation with immobilized mAb. Proteins that remain bound to the plate are recovered after cell lysis and removal of cellular debris. We demonstrate that numerous tyrosine-phosphorylated proteins are enriched after stimulation of the CTL clone AB.1 and recovery of the proteins bound to the plate (Fig. 1A). A number of tyrosine-phosphorylated proteins that cannot be detected in the cell lysate prepared from stimulated cells are enriched after capturing the complexes on the plate. As expected, the SFK Lck and Syk family kinase ZAP70 was found to be associated with CD3ε (Fig. 1A). We were unable to detect Fyn in the CD3ε complexes; however, these CTL clones express very low levels of Fyn (data not shown). The SH2/SH3 adapter Nck was also found in the CD3ε complexes (Fig. 1A).

We have performed a number of experiments to validate this approach. To determine whether this method results in the recovery of preformed complexes or whether we are indeed detecting inducible recruitment, we prepared lysates of unstimulated CTL clones, added the lysates to the plate, recovered CD3ε, and analyzed associated proteins. There is no specific recruitment of tyrosine-phosphorylated proteins or of Lck, Nck, or ZAP70 from lysates of unstimulated cells (Fig. 1B), confirming that we are observing TCR-induced complex formation. Also of note, the recovery of associated proteins is essentially identical after stimulation with either anti-CD3ε or anti-TCRβ (Fig. 1B).

Because cells spread on the anti-CD3 and become tightly adhered to the mAb, it could be argued that many of these proteins are nonspecifically recovered, perhaps as a result of trapping large membrane fragments or cellular debris. To control for this possibility, we plated cells on immobilized anti-CD45, which results in dramatic cell spreading (17), and analyzed captured protein for the presence of CD45 or CD3ε. CD3ε is only captured when cells are plated on anti-CD3ε, and CD45 is only captured when cells are plated on anti-CD45 (Fig. 2A). These results clearly demonstrate that there is specificity to the protein capture, and proteins do not nonspecifically adhere to, or become trapped on, the plates. As an additional control, we plated cells on anti-class I MHC to compare class I MHC-captured proteins with those captured with anti-CD3ε and anti-TCRβ. Very few tyrosine-phosphorylated proteins were recovered with immobilized anti-class I MHC (Fig. 2B) compared with anti-CD3ε and TCRβ. As well, no ZAP70 (Fig. 2B), Lck, or Nck (data not shown) complexed with class I MHC in these cells.

Because we wanted to use this procedure for the isolation of intracellular proteins that associate with the TCR complex, we needed to demonstrate that intracellular proteins do not nonspecifically associate with the complex. We examined two kinases that become activated upon TCR stimulation, but have not been shown to associate with the TCR complex: ERK2 and p38. Immunoblots with anti-ERK2 and anti-p38 demonstrate that these proteins are not recovered with anti-CD3ε and TCRβ complexes after stimulation, even though both proteins are clearly present in the lysate (Fig. 2B). We also examined a number of additional intracellular proteins and did not observe recruitment of these proteins (data not shown). These results demonstrate that there is specificity to the recruitment of proteins.

CD3ε, but not CD3α, is tyrosine phosphorylated in the presence of the SFK inhibitor PP2
The phosphorylation cascade induced by T cell activation is generally accepted to be initiated by SFK (18). To confirm in our system that the bulk of the tyrosine-phosphorylated proteins that are recruited to the TCR/CD3 complex during immobilized mAb stimulation are dependent on the activation of SFK, we examined
the effect of inhibiting SFK activity with PP2, a potent and selective inhibitor of SFK, particularly Lck and Fyn (12). We first assessed the effect of PP2 on a CTL functional response, specifically degranulation. At concentrations of 5–20 μM, PP2 completely inhibited TCR-triggered degranulation, whereas the inactive analog, PP3, had no impact on degranulation (Fig. 3A). To ensure that these concentrations of PP2 effectively inhibited SFK activity, we examined the ability of Lck to phosphorylate a protein substrate (MBP) on tyrosine in the presence of PP2 or PP3. We were unable to

FIGURE 1. Several tyrosine-phosphorylated proteins are recruited to the TCR/CD3 complex upon stimulation of CTL clones with immobilized anti-CD3. A, AB.1 CTL clones were stimulated with plate-bound anti-CD3 (145-2C11) for the indicated time or were left unstimulated by plating on BSA as a control. Cells were then lysed in the plates, the lysate was removed, and a sample was saved for analysis (indicated as lysate). The proteins associated with the immobilized mAb were recovered as described in Materials and Methods. Samples were run on SDS-PAGE gels, transferred to membranes, and probed with the indicated mAb. The dot designates the Ig H chain. B, AB.1 clones were exposed to BSA or immobilized anti-CD3ε or anti-TCRβ (indicated as cells) as in A, or lysates from unstimulated AB.1 clones were added to plates precoated with anti-CD3ε or anti-TCRβ, and complexes from the lysate that bound to the plate were recovered and analyzed (indicated as lysate).

FIGURE 2. Specific complexes form in association with CD3 after stimulation of CTL clones with plate-bound anti-CD3. A, Cells were plated on immobilized anti-CD3 or anti-CD45 for the indicated time, and complexes were recovered as described in Fig. 1A. Samples were probed with anti-CD3ε or anti-CD45RB. B, CTL clones were stimulated on immobilized anti-class I MHC, anti-CD3ε, or anti-TCRβ for 20 min, and complexes were recovered as described in Fig. 1A. A sample of the lysate containing the unbound proteins, which was removed from the plate, is also shown. Immunoblots for phosphotyrosine, ERK2, αPAK, and ZAP70 are shown.
FIGURE 3. Effect of the SFK inhibitor, PP2, on CTL functional responses, Lck kinase activity, and tyrosine phosphorylation after TCR stimulation. A, Serine esterase release (degranulation) was measured from AB.1 in response to 10 μg/ml immobilized anti-CD3 in the presence of different concentrations of PP2 and its inert analog, PP3. B, Kinase activity of Lck immunoprecipitated from control and PP2-treated cells was assessed by an in vitro kinase assay using MBP as a substrate. AB.1 clones were pretreated with PP2 or PP3 and lysed. Lck immunoprecipitations were performed, mixed with the MBP substrate and reaction mix, and incubated for 30 min. The Lck-bound beads were subjected to centrifugation, and a sample of the MBP-containing supernatant was removed. A phosphotyrosine blot (top panel) shows the degree of MBP phosphorylation, and a Coomassie stain (middle panel) is an indication of the amount of MBP in the assay. An Lck blot of the immunoprecipitate is shown in the bottom panel. An NRS immunoprecipitate was included as a control for background phosphorylation of MBP. C, Anti-phosphotyrosine blot of postnuclear cell lysates from AB.1 stimulated with immobilized anti-CD3ε in the absence or the presence 10 μM PP2 or PP3.

to examine Fyn activity because its expression was barely detectable in these cells (data not shown). Lck was immunoprecipitated from unstimulated AB.1 clones in the presence of PP2 or PP3 with an anti-C-terminal Ab, which results in maximal Lck activity (data not shown), probably by binding in such a way that the Lck is in an open and active conformation. The Lck immunoprecipitates were then used in an in vitro kinase reactions with MBP as a substrate in the presence of PP2 or PP3. After the reaction, Lck immunoprecipitates were recovered by centrifugation, and the supernatant containing MBP was used to analyze the phosphorylation status of MBP. In the presence of PP3, Lck is active and phosphorylates MBP on tyrosine residues (Fig. 3B). However, with concentrations of PP2 ranging from 10 to 20 μM, MBP was not phosphorylated, indicating that Lck kinase activity was effectively inhibited in this assay system. An NRS control immunoprecipitate was used in an identical kinase reaction to detect any kinase activity that may be nonspecifically associated with protein A-coated beads. A Coomassie stain of a gel containing a fraction of the substrate supernatants showed that equal amounts of MBP were present in each sample. The Lck proteins bound to the protein A-coated beads were also loaded onto gels and probed with anti-Lck to demonstrate that similar amounts of Lck were present in the kinase assays. We also examined the effect of SFK inhibition on general tyrosine phosphorylation and recruitment of proteins to the TCR/CD3 complex after stimulation with immobilized anti-CD3. It is clear that 10 μM PP2 is sufficient to abolish nearly all detectable anti-CD3-induced tyrosine phosphorylation in the lysate (Fig. 3C).

The impact of SFK inhibition on the recruitment of tyrosine-phosphorylated proteins after CD3 stimulation was next assessed. As shown in Fig. 1, a number of tyrosine-phosphorylated proteins were captured with the plate-bound Ab in the absence of any inhibitor (Fig. 4). With the inactive analog PP3, a similar profile of tyrosine-phosphorylated proteins was recruited to the TCR/CD3 complex (Fig. 4). In the presence of PP2, this recruitment was almost totally abolished, except, surprisingly, for CD3ζ, which remained phosphorylated (Fig. 4). After stimulation, with or without the inert analog PP3, a substantial fraction of phosphorylated CD3ζ appeared as a 19-kDa form, which was previously described (19), in addition to the 21- and 23-kDa forms of CD3ζ (Fig. 4). In the presence of PP2 we detected primarily the 19-kDa and low levels of the 21-kDa form, but never the 23-kDa form, of CD3ζ (Fig. 4 and data not shown). These results suggest that CD3ζ is subject to tyrosine phosphorylation in the absence of detectable Lck activity.

The detection of CD3ζ phosphorylation in the presence of the SFK inhibitor PP2 was surprising and was investigated further. As an additional indirect confirmation of CD3ζ phosphorylation, we determined whether ZAP70 was recruited to the complex in the presence of PP2. Interestingly, ZAP70 recruitment could easily be detected in the captured complexes isolated from cells treated with PP2 (Fig. 4). The reduced recruitment in the presence of PP2 could stem from the inability of cells to strongly adhere to the immobilized mAb in the presence of PP2, thereby not allowing for optimal association of the cells with the immobilized mAb. Although ZAP70 was clearly recruited to the complex, it was not phosphorylated in the presence of PP2 (Fig. 4), thus confirming that SFK activity was indeed effectively inhibited. Furthermore, unlike CD3ζ, CD3ε was not phosphorylated in the presence of PP2 (Fig. 4). Similar results were seen in Con A blasts prepared from C57BL/6 spleen cells (Fig. 5). We clearly detected CD3ζ phosphorylation in the captured CD3ε complexes in the presence of PP2. ZAP70 recruitment was reduced in these cells, but was still detectable (Fig. 5). Thus, there is a differential requirement for
either SFK family members or levels of active SFK in the phosphorylation of CD3 components, because CD3ζ can be phosphorylated in the presence of PP2, whereas CD3ε phosphorylation is completely abolished by this SFK inhibitor.

CD3ζ is phosphorylated after TCR/CD3 stimulation in Lck-deficient Jurkat T cells

To further explore the apparent Lck-independent phosphorylation of CD3ζ, we examined whether CD3ζ is phosphorylated in JCaM1.6, Jurkat cells deficient in Lck expression (20). We used different anti-CD3ε concentrations to stimulate Jurkat and JCaM1.6 cells and found, as previously documented (20), that induction of tyrosine phosphorylation in the lysates of JCaM1.6 cells stimulated with anti-CD3 is severely compromised (Fig. 6 A). We then examined recruitment of tyrosine-phosphorylated proteins to the TCR/CD3 complex by capturing complexes with the stimulatory anti-CD3 on plastic. Surprisingly, we found that there are a number of tyrosine-phosphorylated proteins that are recruited to the complex in JCaM1.6 cells, although the degree of protein recruitment was significantly lower than that in wild-type Jurkat cells (Fig. 6B). Similar to what was found for the CTL clones treated with PP2, phosphorylated CD3ζ (Fig. 6B) and ZAP70 recruitment (Fig. 6C) could be detected in CD3ε complexes obtained from JCaM1.6 cells, confirming that CD3ζ is indeed phosphorylated in the absence of Lck (Fig. 6C). There are lower levels of ZAP70 recruited in these cells; however, we found that there were also substantially lower levels of expression (data not shown) and recovery of CD3ζ (Fig. 6C) in these cells compared with Jurkat cells. These results indicate that CD3ζ is phosphorylated in the absence of Lck in JCaM1.6 cells. Combined with the results obtained using the SFK inhibitor with the CTL clones, CD3ζ appeared to be phosphorylated in an Lck-independent, and possibly a Fyn-independent, manner.

Discussion

In this study we document a novel approach to analyze proteins associated with the TCR complex after T cell activation. This method differs from that described by Harder and Kuhn (21), in which TCR-associated membrane fragments are isolated from homogenates. We use detergent to disrupt the cells and to remove proteins that are nonspecifically bound to the complexes captured on the plates. This approach will probably preserve interactions that would presumably be disrupted during standard immunoprecipitation procedures.

We found that the process of stimulating CTL on immobilized Ab and then capturing complexes significantly enriches for engaged TCR signaling complexes as the cells spread on the immobilized Ab. Stimulation with immobilized Ab also more closely mimics stimulation by APC or target cells than does stimulation with soluble cross-linked Ab. For example, immobilized Ab stimulation generates more sustained signaling, provides a polarized stimulus, and leads to degranulation of CTL (22), as is observed with target cells. Unlike stimulation with APC, in which engaged molecules can move freely within membranes, immobilized Ab
probed with Abs to CD3 and anti-phosphotyrosine. Described in Fig. 1B, run on a gel, and probed with anti-phosphotyrosine. B, Complexes remaining on the mAb-coated plates after removal of lysate from A were recovered as described in Fig. 1A. C, Membrane from B was probed with Abs to CD3ε, CD3ζ, ZAP70, and Lck. The arrowhead indicates the position of CD3ζ.

We have used a number of controls to demonstrate the specificity of the proteins recruited to the TCR/CD3 complex with our novel assay. We show that virtually no proteins bind to BSA-coated plates and that very few proteins (other than the protein to which the Ab is specific) within an unstimulated lysate will bind to immobilized anti-CD3ε or anti-TCRβ (Fig. 1B). We have also performed experiments in which stimulated lysates were plated onto immobilized anti-CD3ε and found, as with an unstimulated lysate, that there are very few proteins that can bind the immobilized Ab (data not shown). These results clearly demonstrate that intact cells must be stimulated to induce complex formation around the TCR/CD3 complex. As well, we have shown that proteins do not nonspecifically adhere to or become trapped on the plates as the cells spread on the immobilized stimulatory Ab (Fig. 2A). Control experiments performed with immobilized anti-class I MHC demonstrate that a different and less complex set of proteins is recruited to the immobilized Ab (Fig. 2B and data not shown). In addition to detecting proteins known to associate with the TCR/CD3 complex, we were unable to detect proteins that would not be expected to associate with the TCR/CD3 complex (Fig. 2B). We therefore conclude that our method of immunosolation is highly specific and physiologically relevant.

Our most surprising finding is that CD3ζ can become phosphorylated in the absence of detectable Lck activity. We found that both CD3ζ phosphorylation and ZAP70 recruitment can occur in normal T cells after treatment with the SFK inhibitor, PP2, even though phosphorylation of CD3ε and ZAP70 is completely inhibited under these conditions (Figs. 4 and 5). We could also detect CD3ζ phosphorylation and ZAP70 recruitment in JCaM1.6, a Lck-deficient variant of Jurkat cells (Fig. 6). JCaM1.6 cells still express Fyn, albeit at low levels (27); therefore, we cannot rule out a role for Fyn in the phosphorylation of CD3ζ in these cells. However, the results from the CTL clones and Con A blasts treated with PP2 suggest that neither SFK is responsible for this phosphorylation. Therefore, our results suggest that another kinase(s), in addition to Lck or Fyn, is capable of phosphorylating CD3ζ. It is possible that the kinase responsible for CD3ζ phosphorylation is a SFK that is not inhibited by PP2. This is nonetheless interesting, because this would imply that this other SFK, or perhaps reduced levels of SFK activity, could function to phosphorylate CD3ζ, but is not able to compensate for the lack of Lck activity with respect to the phosphorylation of CD3ε or ZAP70. This study seems to contradict other studies that concluded that Lck is required for CD3ζ phosphorylation; however, this approach has not been used previously, and we may be significantly enriching for TCR signaling complexes, thus improving the ability to recover and detect phosphorylated CD3ζ.

What is the nature of the CD3ζ phosphorylation that is detected using this approach? It has been shown previously that in 30–40% of the cell surface-expressed TCR/CD3 complexes CD3ζ can associate with the cytoskeleton through the interaction of CD3ζ with monomeric actin (28–30). This pool of CD3ζ that can interact with the cytoskeleton is detergent insoluble and has been designated cytoskeleton-associated ζ (cska-ζ). Cska-ζ differs from detergent-soluble CD3ζ in that it is phosphorylated in resting and activated T cells, and phosphorylation does not change the mobility of cska-ζ during electrophoresis (30). With the method used in this study to recover the TCR-associated complexes, all proteins bound to the immobilized Ab are collected; therefore, it is conceivable that we may be capturing some insoluble cytoskeletal components. The predominant species of phosphorylated CD3ζ that we recover

**FIGURE 6.** CD3ζ phosphorylation and ZAP70 recruitment occur after CD3 stimulation of JCaM1.6 cells. A, Jurkat and JCaM1.6 cells were stimulated with the indicated concentration of plate-bound anti-CD3 (OKT3) for 10 min, after which a sample of the cell lysate was removed from the plate, subjected to SDS-PAGE, and probed with anti-phosphotyrosine. B, Complexes remaining on the mAb-coated plates after removal of lysate from A were recovered as described in Fig. 1A, run on a gel, and probed with anti-phosphotyrosine. C, Membrane from B was probed with Abs to CD3ε, CD3ζ, ZAP70, and Lck. The arrowhead indicates the position of CD3ζ.
from cells stimulated in the presence of PP2 migrates at 19 kDa. This 19-kDa phosphorylated species has also been observed in a study examining the cytoskeletal recruitment of CD3ζ (19). We can detect a minor, less phosphorylated form of CD3ζ that seems to migrate just below the p19 species and appears to have a low level of constitutive phosphorylation that may correspond to cska-ζ. We cannot exclude the possibility that p19 is, in fact, cska-ζ; nevertheless, two pieces of evidence argue against the likelihood of this species being cska-ζ. First, the amount of cska-ζ is increased after T cell activation (29, 30), and we do not detect an increase in the amount of complex-associated, 19-kDa ζ protein after activation. Second, as mentioned previously, cska-ζ is basally phosphorylated (30), whereas the 19-kDa CD3ζ phosphorylation that we are detecting is clearly inducible, with little or no pre-existing phosphorylation in unstimulated cells. Furthermore, we can detect ZAP70 with captured protein complexes, which is presumably associated with phosphorylated CD3ζ, and it has not yet been determined whether ZAP70 is capable of interacting with phosphorylated cska-ζ. Interestingly, Caplan et al. (31) found that cska-ζ can be phosphorylated by a kinase other than Lck in vivo, because treatment of cells with PP1, an SFK inhibitor, had no effect on cska-ζ phosphorylation. However, the authors were unable to identify which kinase(s) was responsible.

There are two major phosphorylated intermediates of soluble CD3ζ that form upon T cell activation, which migrate at 21 and 23 kDa. It has been shown that the 21-kDa (p21) intermediate is constitutively phosphorylated in thymocytes and peripheral T cells, and it associates with nonphosphorylated ZAP70 as a result of in situ TCR interactions with peptide/MHC complexes (32, 33). van Oers et al. (34) have elegantly demonstrated that p21 is tyrosine phosphorylated on the two membrane-distal ITAMs. ZAP70 can associate with p21; however, this associated ZAP70 does not become phosphorylated (34, 35). After activation through the TCR, p21 becomes phosphorylated at the most membrane-proximal ITAM, giving rise to p23, and the associated ZAP70 becomes phosphorylated (34). We did not detect basally phosphorylated CD3ζ or ZAP70 association with the recovered complexes before TCR stimulation. The major phosphorylated form of CD3ζ that we detected after TCR stimulation in both the presence and the absence of PP2 inhibition was ~19 kDa (Fig. 4). We hypothesize that we may be capturing and detecting an earlier tyrosine-phosphorylated intermediate than p21. Indeed, it has been shown that the sites of CD3ζ phosphorylation affect the migration of CD3ζ during electrophoresis (34). In the absence of PP2, we can detect both the p21 and p23 species, and in the presence of PP2, upon longer exposures, a band migrating at ~21 kDa can clearly be detected; however, no p23 seems to be present in any of our experiments (data not shown), suggesting that we are not fully phosphorylating CD3ζ in the presence of PP2.

We do not yet know which protein tyrosine kinase (PTK) is phosphorylating the CD3ζ in the presence of the SFK inhibitor; however, this approach lends itself to proteomic analysis to identify potential kinases that are recruited to the complex. We have found Itk and Pyk2 in the TCR complexes in the presence of PP2 (data not shown). Whether these PTK or yet to be identified PTKs are responsible for mediating the early CD3ζ phosphorylation remains to be determined.

There are a number of possible reasons why non-SFK might phosphorylate CD3ζ. It is not clear how Lck is recruited to the TCR complex, particularly under conditions where coreceptor engagement does not occur. Another kinase(s) might phosphorylate CD3ζ to prime the complex to allow for rapid initiation of the response once enough Lck accumulates at the complex after TCR stimulation, perhaps via binding of its SH2 domain to the phosphorylated CD3ζ. It has been shown previously that the SH2 domain of Lck can interact with phosphorylated CD3ζ (36, 37) and that a functional SH2 domain of Lck is required for the earliest signaling responses (37).

Another explanation for why CD3ζ might undergo Lck-independent phosphorylation is based on the differential signaling model proposed by Pitcher and van Oers (38). In this model, the γ-, δ-, and ε-chains of the TCR/CD3 complex perform distinct functions from the CD3ζ chain. CD3ζ-γ, -δ, and -ε are responsible for mediating activation events (signaling, proliferation, and effector functions), whereas CD3ζ may mediate distinct signaling pathways and TCR expression and recycling. This model is based on observations that CD3ζ can interact with distinct proteins that do not bind to the other invariant chains through ITAM and non-ITAM sequences (38). That we found that CD3ζ can be phosphorylated by another PTK(s) supports the hypothesis that TCRζ could be involved in a distinct signaling pathway.

In this study we provide evidence that CD3ζ can be phosphorylated and recruit ZAP70 in the presence of an SFK inhibitor and in the absence of detectable Lck activity. In contrast, the SFK inhibitor completely blocked CD3ε and ZAP70 recruitment, implying that there is a differential SFK requirement for CD3ε and CD3ζ phosphorylation. Our results suggest that Lck and possibly Fyn, because it is also effectively inhibited by PP2, may not be essential for CD3ζ phosphorylation and that perhaps another SFK or another PTK may be able to phosphorylate CD3ζ, but not CD3ε. Alternatively, if SFK are still required for CD3ζ phosphorylation, the levels and/or types of active SFK differ substantially from those required for CD3ε phosphorylation. Taken together, our data suggest that current models of membrane-proximal, TCR-mediated signaling events may be incomplete, and that additional studies are required to understand the apparent differential phosphorylation requirements for CD3ε and CD3ζ.

Acknowledgments
We thank Dr. Kevin Kane for critical review of the manuscript and helpful discussions.

Disclosures
The authors have no financial conflict of interest.

References


