Normal T Cells Express Two T Cell Antigen Receptor Populations, One of Which Is Linked to the Cytoskeleton via \( \zeta \) Chain and Displays a Unique Activation-dependent Phosphorylation Pattern*

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The TCR couples antigen recognition and the transmission of activation signals. We report the expression of two TCR populations on the surface of T lymphocytes, one of which is linked to the cytoskeleton via the \( \zeta \) chain. We also demonstrate that assembly of the CD3 subunits with cytoskeleton-associated \( \zeta \) is necessary for their maximal localization to the cytoskeleton. The potential significance of these two receptor forms is underscored by differences observed in non-activated T cells; while detergent-soluble phosphorylated \( \zeta \) appears as a 21-kDa protein, phosphorylated cytoskeleton-associated \( \zeta \) appears as a 16-kDa form. This dichotomous phosphorylation pattern is rigidly maintained following activation, although each of the receptor populations undergoes different activation-dependent modifications: 1) levels of soluble phosphorylated 21-kDa \( \zeta \) are enhanced, while phosphorylated 16-kDa cytoskeleton-associated \( \zeta \) exhibits little change; 2) soluble non-phosphorylated 16-kDa \( \zeta \) translocates to the cytoskeleton; 3) activation-dependent ubiquitinated \( \zeta \) forms localize to both fractions, albeit with different kinetics. We also show that the protein tyrosine kinase Lck undergoes activation-dependent modifications and translocates to the cytoskeleton. The phosphorylation profiles of the dichotomous TCR populations in both non-activated and activated lymphocytes suggest that each population could regulate distinct cellular functions, possibly by select intermolecular associations.

The TCR1 is a multisubunit complex composed of the dimeric \( \alpha/\beta \) disulfide-linked heterodimer and the invariant disulfide-linked \( \zeta \) and/or \( \gamma/\delta \) dimers (1). While the \( \alpha/\beta \) heterodimers are involved in antigen-major histocompatibility complex recognition and binding, the invariant chains couple antigen recognition to various intracellular signal transduction pathways (1, 2). Cumulative evidence from various studies indicates that of the invariant chains, \( \zeta \) plays a key role in the transmission of activation signals in the process of T cell stimulation (3–6).

None of the TCR subunits possess intrinsic kinase activity, therefore association of the invariant subunits with various intracellular molecules appears to be crucial for mediating the signaling process. Little is known about such associations in non-activated T cells. Thus far, only the protein tyrosine kinase Fyn has been found to maintain a constitutive association with the non-phosphorylated \( \zeta \) chain in non-activated T cells (7, 8). However, recent reports (9, 10) have shown that freshly isolated, non-activated thymocytes and lymph node T cells express a basal level of tyrosine-phosphorylated \( \zeta \) chain, to which the ZAP-70 protein tyrosine kinase is constitutively associated in a phosphorylation dependent manner. Following TCR cross-linkage, Src family tyrosine kinases Fyn and Lck are activated, promoting augmented tyrosine phosphorylation of the \( \zeta \) and \( \epsilon \) subunits and enhanced recruitment of ZAP-70. The latter is then phosphorylated by the Src family tyrosine kinases, culminating in its activation. In vitro studies have shown that the phosphorylated \( \zeta \) chain can also interact with adapter proteins such as Shc (11) and/or Grb2 (12), resulting in coupling of the TCR to the Ras signaling pathway. It remains to be determined whether additional intracellular molecules interact with the TCR invariant subunits and what role(s) such interactions play, particularly in non-activated T cells.

We have previously shown that 10–40% of the TCR \( \zeta \) chains are linked to the cytoskeleton in non-activated T cells, and that this linkage is dependent upon the integrity of the actin microfilament system (13). A recent study by Rozdzial et al. (14) supports our findings and shows that \( \zeta \) chain can be co-immunoprecipitated with actin. The potential significance of the association of various cell surface receptors with the cytoskeleton is reflected by a number of recent studies (reviewed in Ref. 15). Such associations have been observed for the following cell surface-expressed molecules: epidermal growth factor receptors (16, 17), integrin receptors (18), CD2 (19), the tyrosine phosphatase CD45 (20), the B cell antigen receptor (21–24), and the high affinity receptor for immunoglobulin E (FcR1) (25–27). However, the physiologic relevance of most of these interactions is at present unclear.

In the current study we extend our understanding of the interactions between \( \zeta \) chain, the CD3 \( \gamma, \delta \), and \( \epsilon \) subunits and the cytoskeletal matrix in non-activated T cells. We demonstrate that cytoskeleton-associated \( \zeta \) (cska-\( \zeta \)) chain is assembled within a complex containing the TCR subunits in normal mouse lymphocytes. Furthermore, we have shown in this study that the localization of CD3 to the Triton-insoluble fraction is largely dependent on the presence of the cska-\( \zeta \) chain. Our results suggest that there are two cell surface-expressed TCR populations: Triton-soluble receptors and Triton-insoluble receptors, of which only the latter are linked to the cytoskeleton via the TCR \( \zeta \) chain. We demonstrate that in non-activated mouse lymphocytes, the Triton-insoluble cska-\( \zeta \) chain differs from its Triton-soluble counterpart in its phosphorylation state.
and possibly also in its conformation. While these differentially phosphorylated \( \xi \) forms are maintained in their respective fractions subsequent to TCR-mediated triggering, we have established that various ubiquitinated phosphorylated forms are common to both soluble and cytoskeletal fractions, albeit with different kinetics. Finally, we provide data showing that Lck, a key protein tyrosine kinase involved in TCR-mediated signaling, displays a cytoskeletal localization which is dependent on the state of cellular activation. These results suggest that the cska-\( \xi \) form could play a unique role in mediating signal transduction events initiated by receptor cross-ligation and transphosphorylation, and possibly also in its conformation. While these differentially phosphorylated \( \xi \) forms are maintained in their respective fractions subsequent to TCR-mediated triggering, we have established that various ubiquitinated phosphorylated forms are common to both soluble and cytoskeletal fractions, albeit with different kinetics. Finally, we provide data showing that Lck, a key protein tyrosine kinase involved in TCR-mediated signaling, displays a cytoskeletal localization which is dependent on the state of cellular activation. These results suggest that the cska-\( \xi \) form could play a unique role in mediating signal transduction events initiated by receptor cross-ligation and transphosphorylation.

**EXPERIMENTAL PROCEDURES**

Animals—BALB/c female mice were bred in our SPF facility. Cells and Antibodies—Thymocytes were isolated from mice aged 4–8 weeks and splenocytes were derived from mice aged 12–16 weeks. The antigen-specific T-cell hybridoma B24 and \( \xi \)-deficient T-cell hybridomas 5.8 were grown as described (28). The 145C211 (231) hamster monoclonal antibody is directed against the murine CD3 \( \varepsilon \) chain (29). Anti-CD3-\( \xi \) and anti-\( \xi \)-polyclonal antibodies were generated in rabbits as described (30, 31). Anti-CD3\( \gamma \) and anti-CD3\( \varepsilon \) polyclonal antibodies were generated in rabbits immunized with denatured protein eluted from SDS-PAGE. The monoclonal anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). Immunoprecipitations were done using antibodies bound to protein A-Sepharose beads (Pharmacia).

**RESULTS**

Cska-\( \xi \) Chain Forms a Complex with the CD3 \( \gamma \), \( \delta \), and \( \varepsilon \) Subunits Which Is Expressed on the Cell Surface of Non-activated Normal Lymphocytes—An important issue concerning the putative function of cska-\( \xi \) chain in non-activated lymphocytes is whether it assembles with the TCR complex or is expressed on the cell surface independently of the remainder of the TCR subunits. Thus, we first determined whether any of the CD3 subunits could be detected in the detergent-insoluble cytoskeletal fraction, together with the cska-\( \xi \) chain. Accordingly, we utilized a detergent-based lysis solution containing the cytoskeleton-preserving buffer MES to minimize dissociation of loosely bound cytoskeleton-associated molecules (Refs. 35 and 36; see also “Experimental Procedures”). Upon lysis of freshly isolated thymocytes, the TCR \( \xi \) chain was detected in both the Triton-soluble and Triton-insoluble fractions (Fig. 1A).

Increasing the lysis time or temperature from 4 to 22°C (data not shown) had no effect on the level of \( \xi \) chain in each fraction; 30–40% of the \( \xi \) chain was Triton-insoluble and 60–70% was Triton-soluble. Analysis of CD3 subunit localization under similar conditions revealed that there is a hierarchy for the association of the TCR subunits with the cytoskeleton (Fig. 1A). While the highest Triton-insoluble to Triton-soluble protein ratio (I/S ratio) is maintained by the \( \xi \) chain, the CD3 \( \gamma \) and \( \delta \) chains can also be detected in the Triton-insoluble cytoskeletal fraction. The CD3 \( \varepsilon \) chain also appears to be weakly associated with the cytoskeleton and has the lowest I/S ratio. Similar results were also obtained following the analysis of mouse splenocytes (data not shown).

We next analyzed whether cell surface-expressed cska-\( \xi \) chain is associated with the Triton-insoluble CD3 (\( \gamma \), \( \delta \), and \( \varepsilon \)) subunits and is part of the TCR complex. For this purpose, we performed immunoprecipitations of the Triton-insoluble and Triton-soluble fractions of freshly isolated thymocytes following cell-surface labeling with biotin-ester (Fig. 1B). To retain potential associations between non-covalently linked proteins in the Triton-insoluble cytoskeletal fraction, we extracted the proteins in the Triton-insoluble pellets with DNase I, and avoided the use of denaturing agents. This enzyme digests the DNA in the nuclei which are localized to the Triton-insoluble fraction and also induces in vitro depolymerization of the actin microfilaments (37). Following this procedure, anti-\( \xi \) antibodies clearly immunoprecipitated not only surface labeled \( \xi \) chain, but also the CD3 \( \gamma \), \( \delta \), and \( \varepsilon \) subunits (Fig. 1B) as well as the Ti...
Triton-soluble (lysedin the modified MES-lysis buffer (see “Experimental Procedures”); the detergent-insoluble cytoskeleton. Freshly isolated thymocytes were other is detergent-soluble and not associated with the activated T cells; one is linked to the cytoskeleton while the z chain resides on the cell surface, or whether both α and β chains (data not shown). Although the relative level of Triton-insoluble χ chain is greater than that of the CD3 subunits, when biotinylated, the latter appear to have a greater representation in the Triton-insoluble fraction. This discrepancy arises from the fact that the CD3 subunits possess large extracellular domains with 4-9 lysine residues (targets for biotinylation) as opposed to a single lysine residue in the short extracellular domain of χ chain. Although we cannot rule out the possibility that some of the cell surface-expressed cska-χ chain is physically associated with Triton-insoluble CD3 γ, δ, and ε subunits. These results reveal that there are two TCR populations expressed on the cell surface of non-activated T cells; one is linked to the cytoskeleton while the other is detergent-soluble and not associated with the cytoskeleton.

Localization of CD3 γ, δ, and ε Subunits to the Triton-insoluble Fraction Is Enhanced Upon Their Association with Cska-χ—To determine whether the localization of the CD3 subunits to the Triton-insoluble fraction depends upon the expression of cska-χ chain, we examined the levels of Triton-insoluble CD3 chains in the 5.8 χ-deficient T cell hybridomas. Although 5.8 cells do not express the χ chain, they synthesize the remaining TCR subunits, most of which are degraded in the lysosome with a small portion of χ-deficient TCR expressed on the cell surface (38). Scanning densitometric analysis consistently showed that upon lysis of 5.8 cells, relatively low levels of the CD3 subunits were retained in the Triton-insoluble fraction. The CD3 I/S ratio in 5.8 cells was 3–5-fold lower than that of the parental 2B4 hybridoma cells, which express the χ chain and both the detergent-soluble and cytoskeleton-linked TCR populations were retained in the Triton-insoluble fraction. The CD3 I/S ratio of the glycosylated forms.

### Table I

| Cell line | TCR subunit | γ | δ | ε |
|-----------|-------------|---|---|---|
| 2B4       | 0.670       | 0.380 | 0.310 | ND |
| 5.8       | 0.075       | 0.075 | ND | ND |
| 5.8/FL χ | 0.500       | 0.220 | 0.220 | ND |
| 5.8/Tac χ | 0.430–0.700 | 0.081 | 0.081 | ND |

*ND, not done.*

The Tac χ I/S ratio is presented as a range due to the wide variation of the glycosylated forms.

Western blot analysis was used to determine the I/S ratios of the TCR invariant chains. Quantitative densitometric analysis was performed on multiple film exposures using the Bio-rad Molecular Analyst System. The results presented as I/S ratios are representative of a series of five independent experiments.

The Tac χ is a chimeric molecule which is expressed on the cell surface independently of the rest of the TCR chains and does not “drag” them to the cell surface (34). We demonstrate here that while surface-expressed Tac χ was found both in the Triton-insoluble cytoskeletal fraction and the Triton-soluble fraction, it did not reconstitute the levels of Triton-insoluble CD3 (Table I) and was unable to invoke TCR cell surface expression (data not shown). This is in contrast to what was observed following transfections with full-length Tac χ cDNA (5.8/FL Tac χ) (Table I) and was unable to invoke TCR cell surface expression (data not shown). This is in contrast to what was observed following transfections with full-length Tac χ cDNA.

Consequently, the assembly of cska-χ chain with the CD3 subunits appears necessary for optimal Triton-insoluble CD3 localization and the CD3-cytoskeleton association appears to be mediated via the cska-χ chain.

The Two TCR Populations in Non-activated T cells Possess Distinct Forms of χ Chain—The results demonstrating that there are two TCR populations, one of which is linked to the cytoskeleton via χ chain, raised the issue of whether the χ chains associated with each population exhibit any differences. Our earlier studies (13) have demonstrated that following cell surface iodination using lactoperoxidase, cska-χ chain was preferentially labeled while only trace levels of iodinated soluble χ chain were detected. It was unclear at that time whether only the cska-χ chain resides on the cell surface, or whether both forms are cell surface-expressed but differentially iodinated due to specific structural differences. To resolve this issue, we compared the labeling of the two χ forms by using two different labeling methods, iodination and biotinylation. While the former targets tyrosine residues, the latter binds to the free ε amino groups, such as those on lysine residues. As shown in Fig. 2, A-D, cell surface iodination of freshly isolated thymo-

Fig. 1. A, differential localization of the TCR invariant subunits to the detergent-insoluble cytoskeleton. Freshly isolated thymocytes were lysed in the modified MES-lysis buffer (see “Experimental Procedures”); Triton-soluble (SOL) (2.5 x 10⁷ cells) and Triton-insoluble (INS) (5 x 10⁷ cells) fractions were denatured and separated by two-dimensional non-reducing/reducing SDS-PAGE. After transfer to nitrocellulose filters, the filters were incubated with polyclonal anti-α, anti-δ, and anti-γE antibodies and proteins were detected (see “Experimental Procedures”). To visualize the TCR subunits simultaneously, autoradiograms were superimposed. B, the detergent-insoluble CD3 subunits are associated with the cska-χ chain in normal lymphocytes. Freshly isolated mouse thymocytes were cell surface biotinylated (see “Experimental Procedures”). Labeled cells were lysed with MES-lysis buffer and Triton-soluble (SOL) and insoluble (INS) fractions were obtained. These samples were immunoprecipitated with anti-α antibodies, separated by two-dimensional non-reducing/reducing SDS-PAGE, and transferred to nitrocellulose filters. Streptavidin-horseradish peroxidase and enhanced chemiluminescence were used to detect biotinylated proteins on the filters.
mouse horseradish peroxidase (HRP) detected using either protein A-HRP (for anti-ζ-antibodies) or goat anti-phosphotyrosine antibodies (for anti-pTyr). Protein detection was performed as described. Open arrow indicates the position of the soluble 21-kDa phosphorylated ζ form, and filled arrow indicates the position of the 16-kDa ζ form in both fractions.

Distinct Activation-dependent Modifications of the Detergent-insoluble Fraction of Cytoskeletal-associated TCRs—To examine the putative function of the cytoskeleton-associated TCR population and its possible involvement in early receptor-mediated signaling events, we studied the kinetics of activation-dependent changes for each of the receptor populations. For this purpose, we activated normal mouse splenocytes for various time intervals with anti-CD3 antibodies cross-linked by antigen presenting cells also derived from the splenic population. As indicated in Fig. 3A, within 5 min of stimulation, there was a dramatic increase in the level of 21-kDa phosphorylated ζ form, and this increase was associated with an increase in the level of the 16-kDa ζ form.
changes, indicative of TCR-mediated activation. However, even after 30 min of stimulation, the 21-kDa phosphorylated ζ form was localized exclusively to the Triton-soluble fraction and was never observed in association with the cytoskeleton. In contrast, the 16-kDa phosphorylated form remained unique to the cytoskeletal fraction, even after 30 min of stimulation (Fig 3A). Moreover, the phosphorylated 16-kDa cskα-ζ form was not enhanced upon TCR-mediated activation, and in several experiments a slight activation-dependent decrease was observed after 15–30 min of activation. Despite little change in the level of 16-kDa phosphorylated cskα-ζ form, the total protein level of cskα-ζ (detected with anti-ζ antibodies) showed an activation-dependent increase which generally peaked between 15 and 30 min (Fig 3A). Under these conditions, a slight decrease in Triton-soluble non-phosphorylated 16-kDa ζ was observed. Differences were also observed in the CD3 ε chains of the two receptor populations. Despite an activation-induced increase in the level of tyrosine phosphorylation of soluble ε chains and changes in migration using two-dimensional non-reducing/reducing SDS-PAGE (Fig 3B), only trace amounts of ε could be detected in the detergent-insoluble cytoskeletal fraction both prior to and after activation (data not shown). Even after 30 min of stimulation, no phosphorylated or unphosphorylated ε could be detected in the cytoskeletal fraction.

In the process of studying early activation-dependent modifications of the two receptor populations, we analyzed the ubiquitination state of soluble and cskα-ζ. Cenciardi et al. (39) have previously reported that TCR-triggering induces ubiquitination of ζ chain, but in this study only the Triton-soluble fraction was analyzed. Therefore, we determined whether ubiquitinated ζ forms could also be detected in the Triton-insoluble cytoskeletal fraction. In these experiments splenocytes were stimulated as described above and the presence of ζ chain in the various fractions was analyzed. While the level of detergent-soluble ubiquitinated ζ forms reached a peak after about 30 min of stimulation, the level of ubiquitinated cskα-ζ forms showed a dramatic increase which peaked within 5 min of stimulation (Fig 4A). Moreover, both the 24-kDa ubiquitinated cskα-ζ and soluble ζ forms were phosphorylated (Fig 4B), with peak phosphorylation levels appearing within 5 min of activation for both forms. However, the level of phosphorylation of the 24-kDa cskα-ζ form was consistently higher than its soluble counterpart. Maintenance of the dichotomy of phosphorylated soluble and cskα-ζ forms even after activation, together with the differences in kinetics shown here, suggest that each of the two TCR populations may play select roles in receptor-mediated activation events.

TCR-mediated Activation of Normal Splenocytes Induces Changes in the Detergent-solubility of Lck—Theactivation-dependent kinetics and differences observed in the phosphorylation patterns of detergent soluble and cskα-ζ chains strongly suggest that the cytoskeleton-associated TCR population may be involved in early signaling events. Therefore, we determined whether additional molecules which are known to play a role in early TCR-mediated signaling events are also modified upon TCR triggering and localize to the detergent-insoluble cytoskeletal fraction. In these experiments, we performed a kinetic analysis to assess the detergent-insoluble cytoskeletal localization of Lck upon T cell activation. Our results show that in non-activated splenocytes, two forms of Lck could be detected; the 56-kDa form present primarily in the detergent-soluble fraction and a 60-kDa Lck form localized mainly to the detergent-insoluble fraction. The two Lck forms were detected by immunoblotting of total lysates (Fig. 5), or of immunoprecipitated samples (data not shown). Subsequent to 1 min of TCR triggering, the detergent-insoluble 60-kDa Lck form was no longer detected and after 5 min of activation this 60-kDa form was also absent in the detergent-soluble fraction (Fig. 5). However, after 30 min of activation we observed a reappearance of the 60-kDa Lck form, in both the detergent-soluble and detergent-insoluble cytoskeletal fractions. While the 60-kDa Lck form is localized to both soluble and insoluble fractions with specific activation-dependent kinetics, the 56-kDa Lck form localizes only to the detergent-soluble fraction regardless of the state of activation. These results again demonstrate that in our experimental system, there are molecules which are unique to either the detergent-soluble or the detergent-insoluble cytoskeletal fraction. Similar studies on Fyn and ZAP-70 protein tyrosine kinases did not disclose significant levels of detergent-insoluble bands, at least within the time frame utilized for Lck. Although the functional significance of the two Lck forms is not yet understood, the transient activation-dependent kinetics of Lck-cytoskeleton association strengthen the claim that each of

![Fig. 4. Distinct activation-dependent ubiquitination kinetics of the detergent-soluble and cskα-ζ forms.](image)

**FIG. 4.** Distinct activation-dependent ubiquitination kinetics of the detergent-soluble and cskα-ζ forms. Mouse splenocytes were treated and processed as described in the legend to Fig. 3. Soluble (SOL) and insoluble (INS) samples (5 × 10⁷ cells each) were subjected to two-dimensional non-reducing/reducing SDS-PAGE, transferred to nitrocellulose filters, and incubated with anti-ζ antibodies (A) or anti-phosphotyrosine antibodies (B). Proteins were detected as described. I, indicates the position of the 24-kDa mono-ubiquitinated ζ form. II, indicates the position of the 32-kDa ubiquitinated ζ form. III, indicates the position of the 40-kDa ubiquitinated ζ form.

![Fig. 5. Lck exhibits changes in detergent-insolubility and apparent molecular weight following TCR-mediated activation.](image)

**FIG. 5.** Lck exhibits changes in detergent-insolubility and apparent molecular weight following TCR-mediated activation. Mouse splenocytes were activated with 2C11 ascites (1:250) for the indicated times after rest. Samples (2.5 × 10⁷ cells) were separated to detergent-soluble (SOL) and insoluble (INS) fractions, reduced, and subjected to 8% SDS-PAGE. After transfer to nitrocellulose filters, detection of Lck was achieved by incubation with polyclonal anti-Lck antibodies, protein A-horseradish peroxidase, and enhanced chemiluminescence.
the two TCR populations may initiate distinct cellular signaling cascades, and suggest that additional signaling molecules may be involved in mediating these functions.

**DISCUSSION**

In the current study we demonstrate that non-activated T cells express two TCR forms on the cell surface. While one receptor form is localized to the Triton-insoluble fraction and is associated with the cytoskeleton via the ζ chain, the other is Triton-soluble with no anchorage to the cytoskeleton. Our study focuses on the differences between these two receptor forms and on the inter-relation between the CD3 subunits and the ζ chain within the cytoskeleton-associated receptors. In addition, we assess the potential physiologic significance of these interactions in normal mouse lymphocytes, by comparing the activation-dependent kinetics of phosphorylation and ubiquitination of the Triton-soluble and Triton-insoluble cska-ζ forms.

Although previous studies described the localization of the TCR to detergent-insoluble cytoskeletal fractions of cell lysates, little data was available pertaining to the nature of such associations. Cumulative evidence suggests that the association of the TCR ζ chain with the cytoskeleton is mediated via the actin microfilaments: 1) whereas interactions with microtubules are temperature sensitive, the ζ-cytoskeleton association is insensitive to a range of temperatures. 2) Our previous results (13) describe the loss of Triton-insoluble ζ chain following treatment of cells with the actin microfilament depolymerizing agent cytochalasin B; a recent study by Rozdzial et al. (14) confirmed our results using cytochalasin D, and also showed that ζ chain and actin can be co-immunoprecipitated under certain conditions. 3) As shown in the current study, DNase I, which is also an actin depolymerizing agent (37), dissociates the TCR ζ chain from the Triton-insoluble pellet in vitro.

A crucial issue concerning the physiologic importance of cska-ζ in non-activated T cells is whether it associates with the TCR complex on the cell surface or is independently expressed. Although the cytoskeletal localization of the CD3 subunits (Fig. 1A) hinted that cska-ζ chain likely interacts with the TCR complex, previous studies have shown that in T cells, the ζ chain is not necessarily limited to the TCR, but can exist as part of other receptor complexes with different functions. For example, it was recently shown that ζ chain physically associates with the transferrin receptor and undergoes phosphorylation upon activation via this receptor (42). Moreover, it has been shown that the cell surface expressed TCR ζ chain undergoes internalization and recycling independently of the other TCR subunits (43). By co-immunoprecipitation analysis of the ζ chain in the Triton-insoluble fraction, we provide strong evidence that the cska-ζ chain is associated with the rest of the TCR subunits (Fig. 1B). These results indicate that there are two cell-surface expressed TCR complexes: one is linked to the cytoskeleton while the other is devoid of such an association.

Our analysis of ζ-deficient 5.8 cells together with transfections which reconstitute ζ chain expression in these cells provide compelling evidence that maximal CD3 detergent insolvibility is likely due to the bridging of CD3 to the cytoskeleton via ζ chain. From this analysis (Table I), it appears that the assembly of cska-ζ with the CD3 subunits is crucial for their detergent insolvibility. Moreover, since cska-ζ chain links the CD3 subunits to the cytoskeleton and the CD3 subunits display a hierarchy of localization to the Triton-insoluble cytoskeletal fraction, it is possible that the degree of CD3 cytoskeletal localization reflects the degree of their interaction with the cska-ζ chain. Several models have been suggested depicting the interactions between the receptor subunits (44), but in each case the ζ chain was arbitrarily placed. Since the I/S ratio follows the pattern: δ > γ > ε, our data suggests that the association between cska-ζ and the CD3 subunits may be mediated primarily via the γ and δ chains, in the cytoskeleton-linked receptor population. However, a fuller understanding of the complex interactions between the various receptor subunits awaits a more detailed study. These results suggest that the cska-ζ chain is the main distinguishing feature between these two receptor populations.

It is not known whether there are differences between the two TCR ζ forms which might account for their differential interactions with the cytoskeleton or alternatively, result from such interactions. A difference in the amino acid sequence of the two ζ forms was excluded since ζ chain is localized to both Triton-soluble and Triton-insoluble fractions following transfection of wild type ζ cDNA to ζ-deficient cells. In addition, several monoclonal and polyclonal antibodies directed at different epitopes recognize both ζ forms. Another possibility is that cska-ζ chain appears in a different conformation than the soluble ζ chain. Indeed, our studies utilizing two different labeling techniques (Fig. 2, A-D) revealed that following biotinylation both cska-ζ and soluble ζ chains are expressed on the cell surface, but are differentially labeled by iodination. These results suggest that either a difference in ζ chain conformation or occlusion of the tyrosine residue due to steric interference by neighboring subunits could result in poor iodination of the detergent-soluble ζ chain. Alternatively, association with the cytoskeleton could lead to enhanced exposure of the only tyrosine residue localized to the interface between the transmembrane and extracellular domains. An example of such changes in receptor conformation induced by association with the cytoskeleton comes from a recent report by Gronowski and Berths (17). Their study shows that a cytoskeleton-associated epidermal growth factor receptor population binds its ligand with a greater affinity than the soluble receptor counterparts, suggesting that this enhanced affinity could result from changes in receptor conformation.

A clue to the putative differential function of the two ζ forms in non-activated lymphocytes can be derived from our observations demonstrating their differential state of phosphorylation (Fig. 2, E and F). Despite being phosphorylated in non-activated T cells, the cska-ζ chain maintains an apparent molecular mass of 16 kDa, while its soluble 16-kDa counterpart is non-phosphorylated. Furthermore, the soluble fraction of freshly isolated thymocytes and splenocytes often contains a 21-kDa phosphorylated ζ form (typical of activated T cells and indicative of basal activation in vivo) which is not observed in the Triton-insoluble cytoskeletal fraction. The dissimilarity in the apparent molecular masses of the two phosphorylated ζ forms could result from variations in the number of phosphorylated tyrosine residues and/or from differences in the site of phosphorylation. The unique phosphorylated cska-ζ form could mediate signaling pathways different from those mediated by the 21-kDa soluble phosphorylated ζ form.

Much effort has been made to elucidate the function of receptor-cytoskeleton associations (reviewed in Ref. 15). In this study, we provide evidence that the two receptor populations may well mediate different intracellular signaling cascades leading to distinct cellular effects. We have shown that the activation-dependent ubiquitination of ζ chain, which was previously reported by Cenciarelli et al. (39), is common to both TCR populations. However, there are definite differences in the kinetics of this modification (Fig. 4A). The functional significance of these differential kinetics may be difficult to assess; a
recent study by Hou et al. (45) did not observe any changes in the half-life of \( \zeta \) chain in cells stably transfected with a mutated \( \zeta \) chain which is unable to undergo ubiquitination. However, the role of the differential kinetics of ubiquitination in the soluble and cytoskeletal fractions awaits a systematic study in normal lymphocytes.

One of our more intriguing observations is that the two major phosphorylated \( \zeta \) forms, the 21-kDa soluble form and 16-kDa cska-\( \zeta \) form, each remain in their respective fractions even following 30 min (Fig. 3) and 50 min (data not shown) of stimulation. This dichotomy is maintained despite various activation-dependent modifications, including enhancement of the soluble 21-kDa phosphorylated form. The mode of phosphorylation of each of these \( \zeta \) forms could be of major importance in determining their function. For example, while Zap-70 has been shown to utilize its tandem SH2 domains in a cooperative interaction to bind to paired phosphorylated tyrosine residues in the intracellular region of \( \zeta \) chain (46), Fyn has been shown to bind to the first \( \zeta \)-immune receptor tyrosine-based activation motif when it contains a single phosphorylated tyrosine residue (47). Since the Triton-soluble phosphorylated 21-kDa \( \zeta \) chain apparently differs in its phosphorylation pattern from that of the cska-\( \zeta \) chain, there could be important differences between the two \( \zeta \) forms in the binding to Zap-70, Fyn, and other intracellular signaling molecules.

A recent report by Rozdzial et al. (14) is in agreement with our earlier findings showing that resting lymphocytes contain levels of cska-\( \zeta \). Moreover, both their study and our current work show that there is an activation-dependent translocation of \( \zeta \) chain to the cytoskeleton. However, our study clearly shows that while the level of phosphorylated soluble 21-kDa \( \zeta \) chain increases after 5–30 min of stimulation, no parallel increase is observed in the level of 16-kDa phosphorylated cska-\( \zeta \) form. This contrasts with the above-mentioned study (14) which claims that the level of phosphorylation of cska-\( \zeta \) is greatly enhanced upon activation. How can these results be reconciled? One possibility is that the stimulation of T cell hybridomas transfected with \( \zeta \)-chimeric molecules induces a different phosphorylation pattern from the one we observed using normal mouse splenocytes. Another possibility relates to the type of analysis utilized: by non-reducing/reducing two-dimensional SDS-PAGE, we were able to differentiate between the various \( \zeta \) forms, including the soluble 21-kDa phosphorylated form, the 16-kDa phosphorylated cska-\( \zeta \) form, and the phosphorylated ubiquitinated forms. Since analysis in non-reducing one-dimensional SDS-PAGE is less informative, the authors (14) may have observed the sum total of all the insoluble phosphorylated forms: the 16-kDa form which does not increase, and the 24-kDa phosphorylated ubiquitinated form which we find is enhanced upon TCR-stimulation (Fig. 4B), particularly in the cytoskeletal fraction (Fig. 4B).

Our results, which provide information regarding the kinetics and mode of phosphorylation of cska-\( \zeta \), suggest a unique function for this form in TCR-mediated activation, possibly by differential interactions with various intracellular signaling molecules. It is possible that the cytoskeleton serves as a matrix for the recruitment and concentration of signaling molecules, which facilitates molecular communications. Indeed, recent studies on platelets (reviewed in Ref. 48) show that various kinases, including those of the src family, are translocated to the cytoskeleton after cell activation. Moreover, a recent study in T cells depicts the translocation of Zap-70 as well as the Grb2 and PLC\( \gamma \)1 molecules to a detergent-insoluble spectrin-enriched fraction subsequent to TCR-mediated activation (49). In our study, we demonstrate that Lck, a key src family kinase involved in TCR-mediated signaling, also undergoes activation-dependent translocation to the cytoskeleton and a shift in its apparent molecular weight. Ascertaining whether detergent-insoluble Lck binds to cska-\( \zeta \) remains a priority for future studies, but may necessitate the use of sophisticated cross-linking analysis. Although the role of kinase-cytoskeletal localization is not yet clear, evidence points to significant differences in kinase activity for enzymes linked to the cytoskeleton. For example, it was recently demonstrated that upon receptor ligation, the kinase activity of the cytoskeleton-associated epidermal growth factor receptors is greater than that of the soluble receptors (50). Although no evidence is yet available concerning the putative function of the 60-kDa detergent-insoluble Lck form (present in non-activated and activated T cells), there is a possibility that this form may also possess enhanced kinase activity and play a role in the phosphorylation of cska-\( \zeta \).

What is the role of cska-\( \zeta \) and the cytoskeleton-linked TCR? From our results, it is tempting to speculate that the cytoskeleton-linked receptor population could mediate distinct intracellular signaling cascades. If the actin-based microfilament system is required for TCR-mediated activation as suggested (51, 52), then it is possible that the 16-kDa phosphorylated cska-\( \zeta \) form could perhaps maintain the cells in a state of partial activation where they can respond swiftly to stimulation, either by their ability to sequester phosphorylation-dependent associated kinases, or by undergoing additional phosphorylation and translocation to the soluble fraction as a 21-kDa phosphorylated \( \zeta \) form. However, a study by DeBell et al. (53) suggests that the cytoskeleton could play a role in curbing TCR-mediated signaling events. The 16-kDa phosphorylated cska-\( \zeta \) form and its associated TCR subunits might negatively regulate TCR-mediated signaling as observed in anergy. Recent studies (54, 55) discuss the potential significance of the 18- and 21-kDa phosphorylated \( \zeta \) forms in the detergent-soluble fraction, and the possibility that the ratio of these forms plays a role in binding different kinases and determining whether receptor ligation ultimately culminates in activation or anergy. Although the role of cska-\( \zeta \) and its putative relation to anergy was beyond the scope of this work, such studies may eventually shed light on the function of the phosphorylated 16-kDa cska-\( \zeta \) form. Additional studies will be required to discern the roles of the various phosphorylated \( \zeta \) forms associated with the two TCR populations in initiating or braking the molecular machinery involved in TCR-mediated signal transduction.

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