Architectural Changes in the TCR:CD3 Complex Induced by MHC:Peptide Ligation

Nicole L. La Gruta, Haiyan Liu, Smaroula Dilioglou, Michele Rhodes, David L. Wiest and Dario A. A. Vignali

_J Immunol_ 2004; 172:3662-3669;
doi: 10.4049/jimmunol.172.6.3662
http://www.jimmunol.org/content/172/6/3662

**References**

This article cites 54 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/172/6/3662.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
Architectural Changes in the TCR:CD3 Complex Induced by MHC:Peptide Ligation

Nicole L. La Gruta, Haiyan Liu, Smaroula Dilioglou, Michele Rhodes, David L. Wiest, and Dario A. A. Vignali

A hallmark of T cell activation is the ligation-induced down-modulation of the TCR:CD3 complex. However, little is known about the molecular events that drive this process. The CD3ζ-chain has been shown to play a unique role in regulating the assembly, transport, and cell surface expression of the TCR:CD3 complex. In this study we have investigated the relationship between CD3ζ and the TCRαβCD3εγ complex after ligation by MHC:peptide complexes. Our results show that there is a significant increase in free surface CD3ζ, which is not associated with the TCR:CD3 complex, after T cell stimulation. This may reflect dissociation of CD3ζ from the TCRαβCD3εγ complex or transport of intracellular CD3ζ directly to the cell surface. We also show that MHC:peptide ligation also results in exposure of the TCR-associated CD3ζ NH2 terminus, which is ordinarily buried in the complex. These observations appear to be dependent on Src family protein tyrosine kinases, which are known to be critical for efficient T cell activation. These data suggest a mechanism by which ligated TCR may be differentially down-modulated and selectively down-modulated. The Journal of Immunology, 2004, 172: 3662-3669.

Received for publication May 2, 2003. Accepted for publication January 5, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Department of Immunology, St. Jude Children’s Research Hospital, Memphis, TN 38105; 2 Graduate Program in Pathology and 1 Department of Pathology, University of Tennessee Medical Center, Memphis, TN 38163; and 5 Fox Chase Cancer Center, Philadelphia, PA 19111

Copyright © 2004 by The American Association of Immunologists, Inc.
Materials and Methods
Generation of CD3ζ mutant constructs
All CD3ζ mutants were made by recombinant PCR, using murine CD3ζ cDNA as a template (gift from L. Samelson, National Institutes of Health, Bethesda, MD) and cloned into pCMneo (Promega, Madison, WI) (27), pCMV-internal ribosome entry sequence (IRES)-green fluorescent protein (GFP), or murine stem cell virus (MSCV)-IRES-GFP, which is an MSCV-based retroviral vector. The latter two of these vectors contain an encephalomyocarditis virus IRES and GFP. IRES allows for translation of the test protein and GFP from the same mRNA, thus facilitating verification of expression levels while avoiding attachment at the protein N terminus, CD3ζ-FLAG was produced by attaching the FLAG peptide to the CD3ζ N terminus. The first two amino acids were duplicated to ensure correct signal sequence cleavage. Thus, the sequence of the extracellular domain was SQDYKDDDDKQSPOLLDPK (duplicated residues in italics, FLAG peptide in bold, native CD3ζ extracellular sequence underlined). CD3ζ-K9R and K9S mutants were produced using degenerate primers that contained an AAA to AGA mutation (K9R) or an AAA to AGC mutation (K9S). Details of the construction strategy and oligos used will be provided on request (dario.vignali@stjude.org).

Transfection of T cell hybridomas
After sequence verification, CD3ζ-wild type (CD3ζ-WT) and CD3ζ-FLAG were transfected into CD3ζ loss variants of 3A9 (3A9.K9-7 and 3A9.K9-4, hen egg lysozyme (HEL) K9-specific), H-2A k restricted) (21) and 2B4 (MA5.8, pigeon cytochrome c (PCC)-specific, H-2E b restricted) (15) by electroporation (27, 28). Transfectants were selected with G418 and in some cases cloned or bulk sorted by FACS.

Retroviral transduction of T cell hybridomas
CD3ζ WT, K9R and K9S DNA constructs were cloned into MSCV.IRES.GFP. Ecotropic retrovirus was produced in 293T cells by FuGENE-mediated transfection. Briefly, 293T cells (provided by D. Baltimore) were transfected into CD3ζ wild type (CD3ζ-WT) and CD3ζ-FLAG were transfected into CD3ζ loss variants of 3A9 (3A9.K9-7 and 3A9.K9-4, hen egg lysozyme (HEL) K9-specific), H-2A k restricted) (21) and 2B4 (MA5.8, pigeon cytochrome c (PCC)-specific, H-2E b restricted) (15) by electroporation (27, 28). Transfectants were selected with G418 and in some cases cloned or bulk sorted by FACS.

Surface biotinylation and immunoprecipitation (IP)
Assays were performed as previously described with some modifications (5). Briefly, T cells (5 × 10^6/96-well) were cultured at 37°C with APC (L3K5.2 ≥ 10 μM HEL; 5 × 10^5/96-well) for 5 or 24 h. In some experiments LK35.2 cells expressing GFP were used to facilitate enumeration and equalization of T cells (GFP) between samples. In Src family protein tyrosine kinase (PTK) inhibition studies, T cells and APCs were incubated separately for 2 h at 37°C in the presence or the absence of 10 μM 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) (Calbiochem, San Diego, CA) before being cultured together for 24 h at 37°C. Cells were surface-biotinylated with 1 mg/ml sulfo-N-hydroxysuccinimide (NHS)-biotin (Pierce, Rockford, IL) in HBSS for 30 min on ice. Excess biotin was quenched with 20 mM β-lysine/HBSS, and the cells were washed three times. Dead cells were removed by density gradient centrifugation using Lymphocyte Separation Medium (Cappell/ICN Pharmaceuticals, Costa Mesa, CA). Cells were lysed in 1% digitonin lysis buffer (1% digitonin (Wako Biochemicals, Osaka, Japan; Calbiochem, San Diego, CA), 0.05 M Tris (pH 7.4), 0.15 M NaCl, 2 mM Pefabloc (Roche Applied Science, Indianapolis, IN), 40 μg/ml aprotinin, and 20 μg/ml leupeptin) at 4°C for 30 min, and the lysate was precleared with heat-killed formalin fixed Staphylococcus aureus (Pansorbin) cells (Calbiochem) for 1 h at 4°C. The lysate was then immunoprecipitated with protein A-Sepharose (Amersham Pharmaucia Biotech, Piscataway, NJ) precoated with anti-TCR-β (H57-597), twice sequentially (1 h each) at 4°C, followed by protein A-Sepharose precoated with anti-CD3ζ (H146-968) for 1 h at 4°C. Sepharose beads were washed with 0.2% digitonin buffer (three times) and immunoprecipitated proteins eluted in an equal volume of 2× SDS sample buffer at 75°C. Purification of biotinylated proteins was performed by guest on April 14, 2017 http://www.jimmunol.org/ Downloaded from

Purification of biotinylated proteins
Biotinylated proteins were purified from cell lysates using SoftLink Soft Release Avidin resin (Promega). Briefly, resin was washed with 0.1 M NaCl (10 min) and preadsorbed in 5 mM biotin/0.1 M NaPO₄ (pH 7.0). For the final wash, the beads were incubated for 30 min at 4°C with rocking to allow avidin to refold. Lysate containing biotinylated proteins was then added to the resin and incubated at 4°C for 30 min with rocking. The resin was washed with three times with 1 mL of 0.2% digitonin wash buffer, and biotinylated proteins were eluted with 10 mM biotin in 1% digitonin lysis buffer (three times, 500 μl).
Results

CD3ζ extracellular domain becomes exposed upon TCR ligation

CD3ζ loss variants of the 3A9 and 2B4 T cell hybridomas (3A9,ζ− and 2B4,ζ−) were transfected with either CD3ζ-WT or an N-terminal FLAG-tagged CD3ζ (CD3ζ-FLAG) to follow the fate of CD3ζ after TCR ligation with MHC:peptide complexes. Both CD3ζ constructs restored surface expression of the TCR:CD3 complex as well as function, as determined by IL-2 production, to levels comparable to those in the parental 3A9 and 2B4 T cell hybridomas (Fig. 1, A and B, and data not shown). Mock transfections with empty vector failed to restore TCR expression or function (data not shown). As TCR is not stably expressed on the cell surface in the absence of CD3ζ (13, 15, 16, 21), it is reasonable to assume that all surface TCR-CD3 complexes on the CD3ζ-FLAG transfectants contain FLAG-tagged CD3ζ. Indeed, both CD3ζ-WT and CD3ζ-FLAG restored TCR expression comparably (Fig. 1A). However, minimal FLAG staining was observed, suggesting that the epitope is concealed within the complex (Fig. 1A). As expected, both the 3A9 and 2B4 transfectants down-modulated TCR following stimulation with Ag-pulsed B cells (Fig. 1C). Surprisingly, there was a substantial increase in FLAG staining, which rose ~25-fold in the 2B4 transfectants and ~400-fold in the 3A9 transfectants 10–12 h poststimulation. These data imply that after TCR ligation, the TCR-CD3 complex undergoes structural alterations, such that the extracellular portion of CD3ζ-FLAG becomes exposed for Ab binding. The high level of CD3ζ-FLAG staining compared with TCR staining at 12 h poststimulation suggests that although TCR ligation induces down-modulation of the TCR-CD3 complex, CD3ζ may remain on the cell surface. Interestingly, the increase in FLAG staining was delayed relative to maximal TCR down-modulation. Although 3A9 TCR down-modulation was maximal after 3 h, CD3ζ-FLAG increase was not evident until 5 h. The reason for this delay is unclear, but it is possible that some time is required before a sufficient quantity of free CD3ζ has accumulated to a detectable level by flow cytometry. Alternatively, CD3ζ may be down-modulated with the ligated TCR complex, separated in an intracellular compartment, and thus not be seen as free CD3ζ until it was recycled back to the cell surface. This would be synonymous with our previous suggestions regarding the transport of nonligated TCR (5). Of course, such a lag may also be a reflection of the appearance of newly synthesized CD3ζ after TCR ligation.

FIGURE 1. Increased accessibility to the CD3ζ NH2 terminus after TCR ligation. CD3ζ loss mutants of 2B4 and 3A9 (2B4,ζ− and 3A9,ζ−) were transfected with CD3ζ-WT and CD3ζ-FLAG. A, Unstimulated cells were analyzed by flow cytometry with anti-CD3ε and anti-FLAG (cation-independent M2 mAb). B, The functional capacity of the CD3ζ-FLAG transfectants was evaluated by IL-2 production after stimulation with LK35-2 B cells plus the peptides indicated. C, Modulation of TCR and CD3ζ-FLAG after stimulation with Ag-pulsed B cells was determined by flow cytometry. In all cases, homogeneous down-modulation was observed. Data are either representative of two experiments (A) or are the mean of three experiments (B and C).

TCR ligation induces an increase in CD3ζ NH2-terminal biotinylation

The relationship between CD3ζ and the TCRαβCD3εζγ8 complex was studied further using surface biotinylation and sequential IP. The CD3ζ-WT and CD3ζ-FLAG transfectants of the 3A9ζ- hybridoma were surface-biotinylated 5 and 24 h after incubation with B cells precultured with or without Ag. Dead cells were removed to ensure that only surface-biotinylated proteins from viable cells were included. Cell lysates were immunoprecipitated sequentially with anti-TCR-ζβ three times to remove TCR complex-associated CD3ζ, followed by anti-CD3ζ to determine the amount of free surface-biotinylated CD3ζ.

In unstimulated CD3ζ-WT transfectants, there was some labeling of TCR-associated CD3ζ; however, very little free CD3ζ was observed. In the CD3ζ-FLAG transfectants, some biotinylated free CD3ζ was seen (Fig. 2). This increased biotin label on free CD3ζ may be due to increased biotinylation efficiency, as the FLAG tag contains two additional lysine residues. The relatively small amount of free CD3ζ-FLAG is consistent with the minimal surfaceFLAG staining observed on resting T cells (Fig. 1A). Interestingly, no TCR-associated CD3ζ-FLAG biotinylation was detected in unstimulated cells, suggesting that the tag is buried in the complex. Efficient biotinylation may also have been precluded if the extracellular lysine residues had formed stable interactions with adjacent residues in the complex. It is important to note that the lack of CD3ζ-FLAG biotinylation on resting cells is not due to an absence of associated protein, as the presence of CD3ζ-FLAG was confirmed by subsequent immunoblot analysis using an anti-CD3ζ Ab (data not shown and Figs. 3 and 4). After antigenic stimulation, two changes in the pattern of TCR-CD3 biotinylation were observed. First, there was an increase in the TCR-associated CD3ζ-FLAG staining observed on resting T cells (Fig. 1A).
biotin signal (Fig. 2). Second, the free CD3ζ biotin signal increased significantly, which was particularly evident after 24 h. In contrast, there was little change in the level of CD3γδζζ biotinylation.

Based on these observations, three key issues arose. Firstly, it was possible that the increased signal intensity at ~16 kDa was due to recruitment of another protein(s) to the TCR:CD3 complex after TCR ligation. To assess this possibility, CD3ζ-FLAG transfectants of the 3A9 CD3ζ loss mutant were incubated with LK35.2 B cells or LK35.2 prepulsed with 10 μM HEL. After 5 and 24 h, cells were surface-biotinylated with NHS-biotin and lysed in 1% digitonin. Cell lysates were immunoprecipitated three times sequentially with anti-TCR-Cζ (only the first and third are shown), followed by anti-CD3ζ. Non-specific binding of streptavidin-HRP to the anti-CD3ζ L chain is shown (IgL) and was also seen in lanes containing the IP Ab alone (data not shown). The results of two representative experiments with CD3ζ-FLAG transfectants and one experiment with CD3ζ-WT are shown. Comparable results were obtained using a fluorescence-based detection system (ECF, Amersham Pharmacia Biotech) and direct quantitation on a phosphorimager (N. La Gruta and D. A. A. Vignali, unpublished observations).

Secondly, as visualization of proteins in this system is dependent on their ability to be biotinylated, it was unclear whether the increased CD3ζ signal intensity represented an increase in the amount of CD3ζ protein or an increase in the biotinylated status of CD3ζ. Differentiation between these possibilities required selective quantitation of cell surface CD3ζ protein. Cells were surface-biotinylated and lysed as described; however, before IP with anti-TCR-Cζ, biotinylated proteins were purified from the lysate using a SoftLink monomeric avidin system. This allowed for the selective IP of cell surface proteins. The eluted biotinylated proteins were then sequentially immunoprecipitated with anti-TCR-Cβ and anti-CD3ζ as described above. Upon probing with streptavidin-AP, the characteristic pattern of increased TCR-associated and free CD3ζ signal intensity was observed after Ag stimulation (Fig. 4A, upper panel; compare lanes 1 and 4, and lanes 3 and 6), with densitometric analyses revealing 2.4- and 8.5-fold increases in TCR-associated and free CD3ζ, respectively (Fig. 4B, upper panels). The blots were then stripped and reprobed with a mixture of anti-CD3ζ and anti-CD3ζζζ Abs (Fig. 4A, lower panel). The anti-CD3ζζζ Ab was included to enable normalization of the CD3ζζζ signal intensity to the rest of the TCR:CD3 complex. Densitometric analyses of both TCR-associated and free CD3ζζζ protein revealed no increase in TCR-associated CD3ζζζ protein. However, a 3-fold increase in free CD3ζζζ protein was detected after Ag stimulation. The reduced CD3γδζζζ-biotin and CD3ζζζ protein signal after...
FIGURE 4. The amount of CD3ζ protein associated with the TCR remains unchanged despite the increased biotin signal after Ag stimulation. CD3ζ-FLAG transfectants of a 3A9 CD3ζ loss mutant were incubated with LK35.2 B cells pulsed, or not, with 10 μM HEL. After 24 h, cells were surface-biotinylated and lysed in 1% digitonin buffer. Biotinylated proteins were purified from the cell lysates using SoftLink soft release avidin resin (Promega). Purified biotinylated proteins were immunoprecipitated twice sequentially with anti-TCR-Cβ, followed by anti-CD3ζ. Biotinylated proteins were analyzed by probing the Western blot with streptavidin-AP (A, upper panel). Membranes were stripped and reprobed with anti-CD3ζ and anti-CD3ε Abs, washed, and probed with protein A/G-AP (Amersham; A, lower panel). A fluorescence-based detection system was used (ECF, Amersham Pharmacia Biotech), and bands were visualized on a phosphorimager for direct quantitation of biotin and Ab signals. Graphs are expressed as the ratio of CD3ε and CD3ζ arbitrary densitometric units (B: upper graph, biotin signal; lower graph, Ab signal).

FIGURE 5. Biotinylation of the CD3ζ extracellular domain does not involve the K9 residue. 3A9 CD3ζ hybrids were retrovirally transduced with virus encoding WT CD3ζ, CD3ζ.K9R, or CD3ζ.K9S. A. The functional capacity of the CD3ζ transductants was evaluated by IL-2 production after stimulation with either plate-bound anti-TCRβ (H57; left graph) or HEL plus LK35.2 B cells (right graph). The IL-2 concentration was determined using a particle-based flow cytometric assay (30). B–D. The 3A9 cells were incubated for 24 h with LK cells precultured with or without Ag. Cells were then surface-biotinylated, lysed, and immunoprecipitated with anti-TCR-Cβ. Bound proteins were separated by SDS-PAGE, immunoblotted, and probed with streptavidin-AP (A). Membranes were stripped and reprobed with anti-CD3ζ and anti-CD3ε Abs, followed by protein A/G-AP (B). Quantitative analysis of the CD3ζ band intensity was performed as described in Fig. 4 (C).

ligation is due to TCR down-modulation. Thus, the increased CD3ζ biotin signal observed after Ag ligation corresponds to an increased biotinylation state of TCR-associated CD3ζ rather than a change in the amount of CD3ζ protein associated with the TCR complex. For free CD3ζ, the increased biotin signal appears to be the consequence of an increased amount of protein.

Thirdly, what residue(s) in CD3ζ is biotinylated? The CD3ζ-chain has an extremely short, nine-amino acid extracellular domain (NH2-QSFGLLDPK). The most likely candidate for biotinylation is the lysine residue at position 9 (K9). To determine whether enhanced biotinylation at K9 is responsible for the increased CD3ζ biotin signal after TCR ligation, CD3ζ constructs were generated in which the lysine residue was mutated to either arginine (K9R) or serine (K9S). 3A9 CD3ζ-hybridomas were transduced with retrovirus encoding the mutant CD3 ζ-chains. TCR expression and T cell function were comparable to those in T cell systems used. Transductants were then stimulated, surface-biotinylated, and immunoprecipitated with anti-TCR-Cβ as described. The data clearly show that mutation of K9 had no effect on CD3ζ biotinylation (Fig. 5B). After detection of biotinylated proteins, the blot was stripped and probed with a mixture of anti-CD3ζ and anti-CD3ε Abs (Fig. 5C). This blot clearly demonstrates that despite a weak biotin signal, CD3ζ is associated with TCR complexes in unstimulated cells. It also supports our previous finding that the increased biotin signal of TCR-associated CD3ζ is not due to an increased amount of protein. The relative increase in the CD3ζ-CD3ε biotin ratio between unstimulated and stimulated cells...
was essentially identical (densitometric analysis: CD3ζ-WT, 2.09-fold; CD3ζ-K9R, 2.2-fold; CD3ζ-K9S, 1.8-fold; Fig. 5D). Thus, it appears that the increase in CD3ζ biotinylation is not mediated via the K9 residue.

Taken together, our data strongly suggest that TCR ligation by MHC:peptide causes alterations in the TCR:CD3 complex, such that the short extracellular domain of CD3ζ becomes more accessible for biotinylation. Furthermore, ligation appears to cause an increase in the amount of free CD3ζ on the cell surface. There are two possible explanations for the latter. First, de novo synthesis of CD3ζ induced by TCR ligation may escape intracellular retention and be expressed on the cell surface. Second, free CD3ζ may result from dissociation between CD3ζ and the rest of the TCR:CD3 complex.

**TCR ligation-induced architectural changes in the TCR:CD3 complex are Src family PTK-dependent**

Two members of the Src family of PTKs, p56


complex.

kinase activation.

family PTKs and thus presumably occur after or concurrently with TCR:CD3 complex after TCR ligation are highly dependent on Src together, these data suggest that the structural changes induced in the almost completely abrogated these changes (Fig. 6). Taken to-

described previously (Fig. 6). Pretreatment of the cells with PP2, a selective inhibitor of Src family PTKs (34), resulted in exposure of the CD3ζ NH2 terminus, which is ordinarily buried in the complex. For discussion purposes, we have generally interpreted this event as reflecting architectural changes in the TCR:CD3 complex. We would also propose, based on increased free CD3ζ levels following stimulation, that such architectural changes may ultimately lead to dissociation of the CD3ζ dimer from the TCR:CD3 complex. However, the origin of the free cell surface CD3ζ has not yet been formally demonstrated, and thus we cannot rule out the possibility that this free CD3ζ derives from de novo synthesis induced by TCR ligation. This would be surprising, because it is generally thought that the free CD3ζ seen after stimulation are products of de novo synthesis and cannot escape intracellular retention (13, 15, 16, 21). Further studies are required to resolve this issue. Regardless, we propose that the architectural changes occurring within the TCR:CD3 complex after TCR ligat-

tion. How does the T cell recognize ligated TCR and subsequently direct these molecules to lysosomes for degradation? Our results suggest that MHC:peptide ligation of the TCR:CD3 complex results in exposure of the CD3ζ NH2 terminus, which is ordinarily buried in the complex. For discussion purposes, we have generally interpreted this event as reflecting architectural changes in the TCR:CD3 complex. We would also propose, based on increased free CD3ζ levels following stimulation, that such architectural changes may ultimately lead to dissociation of the CD3ζ dimer from the TCR:CD3 complex. However, the origin of the free cell surface CD3ζ has not yet been formally demonstrated, and thus we cannot rule out the possibility that this free CD3ζ derives from de novo synthesis induced by TCR ligation. This would be surprising, because it is generally thought that the free CD3ζ seen after stimulation are products of de novo synthesis and cannot escape intracellular retention (13, 15, 16, 21). Further studies are required to resolve this issue. Regardless, we propose that the architectural changes occurring within the TCR:CD3 complex after TCR ligat-

**Discussion**

The expression of TCR on the surface of T cells is a dynamic process. In resting T cells, for example, a cycling reservoir of TCR is constitutively internalized and recycled back to the cell surface (5, 13, 35). T cell stimulation also results in internalization of the TCR:CD3 complex. However, this is dependent on ligation of the TCR by Ab or MHC:peptide complexes and, unlike internalization in resting cells, results in a rapid and net reduction of TCR on the cell surface (36, 37). TCR ligation-mediated down-modulation is a consequence of TCR degradation within lysosomal compartments (5, 38, 39). Some studies have proposed that TCR ligation induces the down-modulation of nonengaged receptors (40), although others argue that only specific, ligated receptors are down-modulated after ligation (41). Support for the latter theory comes from a recent study involving mathematical modeling of MHC:peptide-induced TCR down-modulation (41). In it, the researchers propose that an activated TCR remains marked for internalization after dissociating from the MHC:peptide complex. This raises an important ques-

![FIGURE 6. Architectural changes within the TCR:CD3 complex after Ag stimulation are Src family PTK-dependent. 3A9 hybridomas (A) or the CD3ζ-FLAG transfectants (B) were incubated with KL35.2 B cells pulsed, or not, with 10 μM HEL in the presence or the absence of the Src family PTK inhibitor PP2 (10 μM). After 24 h, cells were surface-biotinylated and lysed in 1% digitonin buffer. Cell lysates were immunoprecipitated twice sequentially with anti-TCR-Cβ, followed by anti-CD3ζ. Biotinylated proteins were analyzed by probing the Western blot with streptavidin-AP.](http://www.jimmunol.org/Downloadedfrom)
motifs are involved in this process, as T cells lacking the cytoplasmic tail of CD3γ and CD3δ still down-modulate TCR after Ag stimulation (43). Indeed, an endocytosis signal in CD3ζ has recently been described (44). Thus, TCR down-modulation may be determined by the exposure of intracellular retention motifs that are normally concealed by CD3ζ.

Until recently, the idea that the TCR:CD3 complex undergoes conformational change after TCR ligation was speculative. However, a study by Gil and colleagues (45) demonstrated that ligation of TCR resulted in the exposure of a proline-rich sequence in CD3ζ, which, in turn, leads to recruitment of the adaptor protein, Nck. Interestingly, these events occurred before and independently of tyrosine kinase activation and appeared to be involved in early signaling processes. In our study the observed architectural changes initiated by TCR ligation were heavily dependent on Src family PTK activity, suggesting that the exposure of CD3ζ and its subsequent dissociation from the remainder of the TCR:CD3 complex are relatively late events and may occur downstream of CD3ζ exposure and Nck recruitment. It is therefore possible that the architectural changes observed in this study may not be directly involved in the signaling process, but, rather, are required for the downstream effects of TCR ligation, such as TCR down-modulation. Importantly, it has been shown that the expression of constitutively active p56(lck) induces TCR down-modulation (39).

An interesting question remains of which residue in the short extracellular domain of CD3ζ is biotinylated upon TCR ligation? Previous studies have suggested an important role for the K9 residue in the extracellular domain of CD3ζ in signal transduction as well as in stabilizing the association of CD3ζ with the TCR:CD3 complex (31). The NHS-ester used to label proteins with biotin preferentially labels lysine residues, but can also label the protein NH2 terminus. Although other residues, such as arginine, histidine, and cysteine, can be labeled, the frequency and efficiency of this reaction at physiological pH are very low. Thus, the K9 residue appeared to be the most likely candidate. We hypothesized that K9 may, in unligated TCR:CD3 complexes, be involved in interchain interactions with other amino acids in other TCR or CD3 chains, preventing its biotinylation. Upon TCR ligation, it is possible that this association is broken, exposing the K9 residue for biotinylation. However, our studies clearly showed that the K9R and K9S mutations had no effect on CD3ζ biotinylation. Although it remains to be determined which residue/moiety in the extracellular domain of CD3ζ is biotinylated, the most likely candidate is the NH2 terminus, although these are usually capped in eukaryotic cells.

An interesting question raised by our data is whether there are other multichain transmembrane receptors that undergo architectural changes and/or dissociation upon ligand binding. CD3ζ is part of a family of molecules that form an integral part of many different receptors (11). For instance, CD3ζ (a splice variant of CD3ζ) and the FcRy can substitute for CD3ζ in the TCR:CD3 complex (46–49). In addition, CD3ζ and FcRy form part of the FcyRIII (CD16) Ig FeR expressed on T and NK cells. FcRy is also incorporated into several other FeRs, such as FcγRI, FcγRII, and FcεRI, on macrophages, monocytes, neutrophils, mast cells, and basophils (49). Lastly, the CD3ζ-like membrane adapter proteins, DAP12 and DAP10 (KAP10), form NK cell-activating receptors with CD94/NKG2C and NKG2D, respectively (50–53). Thus, it will be interesting to determine whether the ligand-induced alteration and/or dissociation of such transmembrane adapter molecules from multichain receptor complexes are common events. Given the large number of receptors that incorporate CD3ζ-like transmembrane adapter molecules, which are represented in all leukocytes and parts of the CNS (49–54), our demonstration of ligand-induced architectural changes in the TCR may have broad implications for our understanding of the molecular events that initiate or perpetuate signal transduction via multichain receptor complexes.

Acknowledgments

We are very grateful to Ralph Kubo for the Abs, Mark Davis for the 3A9 TCR transgenic mice, Elio Vanin and David Baltimore for the 293T cells, Emil Unanue for M12.C3.F6, and Larry Samelson for the CD3ζ cDNA. We also thank Richard Cross and Mahnaz Pakstian for assistance with the flow cytometry, Janet Gatewood for performing the particle-based flow cytometric cytokine assay, Elio Vanin for assistance and reagents for establishing retroligand transduction in our laboratory, and members of the Vignali laboratory for constructive criticisms and comments.

References


48. Rodewald, H. R., A. R. Arulanandam, S. Koyasu, and E. L. Reinherz. 1991. The high affinity Fce receptor \( \gamma \) subunit (FceRI\( \gamma \)) facilitates T cell receptor expression and antigen/major histocompatibility complex-driven signaling in the absence of CD3\( \zeta \) and CD3\( \eta \). J. Biol. Chem. 266:15974.


