Physical Dissociation of the TCR–CD3 Complex Accompanies Receptor Ligation

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Summary

Recent studies indicate that there may be functional uncoupling of the TCR–CD3 complex and suggest that the TCR–CD3 complex is composed of two parallel signal-transducing units, one made of γδε chains and the other of ζ chains. To elucidate the molecular mechanism that may explain the functional uncoupling of TCR and CD3, we have analyzed their expression by using flow cytometry as well as immunochemical means both before and after stimulation with anti–TCR-β, anti-CD3ε, anti-CD2, staphylococcal enterotoxin B, and ionomycin. We present evidence that TCR physically dissociates from CD3 after stimulation of the TCR–CD3 complex. Stimulation with anti-CD3 resulted in down-modulation of TCR within 45 min whereas CD3ε was still expressed on the cell surface as detected by flow cytometry. However, the cell surface expression of TCR and CD3 was not affected when cells were stimulated with anti–TCR-β under the same conditions. In the case of anti-CD3 treatment of T cells, the TCR down-modulation appeared to be due to the internalization of TCR, as determined by immunoelectron microscopy. Immunochemical analysis of cells after stimulation with either anti-TCR or anti-CD3 mAbs revealed that the overall protein levels of TCR and CD3 were similar. More interestingly, the dissociation of the TCR–CD3 complex was observed with both treatments and occurred in a manner that the TCR and the associated TCR-ζ chain dissociated as a unit from CD3. These results provide the first report of physical dissociation of TCR and CD3 after stimulation through the TCR–CD3 complex. The results also suggest that the signal transduction pathway triggered by TCR may differ from that induced by CD3.

T lymphocytes, which play an important role in immune responses, recognize antigens on the surface of APCs in the context of molecules encoded by the MHC by virtue of the heterodimeric TCR. For TCR-α/β T cells, the antigen recognition unit is composed of polymorphic α and β subunits and is expressed at the cell surface as a molecular complex with CD3 and members of the TCR-ζ chain family (1–4). The CD3 subunits γ, δ, and ε and the TCR-ζ chain are invariant and thought to be responsible for signal transduction after ligation of the TCR by antigen (5, 6). Recent studies have shown that the CD3 subunits in the antigen recognition ensemble are expressed as noncovalently associated γε and δε pairs (2–4). The TCR-ζ family includes the ζ and η chains and the γ chain of the high-affinity IgE receptor (FcεRI) and is expressed not only in T cells but also in other cell types (6–8). These ζ, η, and FcεRIγ subunits can exist as homodimers or can combine to form heterodimers (9). Thus, the minimal TCR complex is made up of at least eight polypeptide chains.

Biosynthetic labeling studies in mature T cells have demonstrated that TCR-α/β, CD3γ, CD3δ, and CD3ε chains are indispensable for expression of the antigen receptor complex on the cell surface (10, 11). Although TCR–CD3 complexes can be expressed on the cell surface without ζ-ζ or ζ-η subunits, surface expression is <10% of the full octameric TCR complex (α, β, δ, γ, ϵ_2, and ζ_2). Because TCR-ζ chain association with the TCR–CD3 complex is essential for signal transduction, the complete octameric TCR complex is thought to be required for optimal expression on the cell surface and receptor function.

Reports suggest the occurrence of functional uncoupling between TCR and CD3 (12–16), but no study has addressed the fate of the complex when ligation of the TCR takes place. During maturation of immature thymocytes, treatment with anti-CD3 mAb resulted in the elimination of all $CD4^+8^+$ thymocytes, whereas anti-TCR mAb treatment caused the deletion of only one-half of immature thymocytes. The cells that remained after anti-TCR mAb treatment failed to mobilize Ca^{2+} upon stimulation with anti-TCR but mobilized Ca^{2+} with anti-CD3, suggesting

that uncoupling occurred between TCR and CD3 in these cells (12, 13). In peripheral (mature) T cells, signal transduction through CD4 with $p56^{lck}$ inhibits the proliferation induced by the ligation of TCR-α/β but is unable to affect the induction of growth through the CD3 complex (15). These results led us to suspect that TCR-α/β could physiologically dissociate from CD3 upon ligation of the receptor complex. In this study, we demonstrate this uncoupling through analysis of the expression of TCR and CD3 before and after stimulation with anti–TCR-β, anti–CD3ε, and anti-CD2 mAbs, staphylococcal enterotoxin B (SEB)[1], and ionomycin. Using flow cytometric analysis, we found that stimulation with anti-CD3 mAb resulted in the down-modulation of the TCR, although CD3 surface expression was at normal levels, whereas the cell surface expression of TCR and CD3 was not affected when cells were stimulated with the anti–TCR-β mAb. The fate of the TCR complex after stimulation of T cells with either anti-TCR or -CD3 was also analyzed by immunoelectron microscopy and by immunoprecipitation and immunoblot analysis. We demonstrate here that dissociation of the TCR from CD3 can occur after stimulation via the TCR–CD3 complex and suggest that the signal transduction pathway triggered by TCR may differ from that induced by CD3 ligation.

Materials and Methods

Animals, Cells and Antibodies. C3H/HeN (C3H) and C57BL/6 (B6) mice were produced in our animal facility (Research Institute for Biological Sciences, Science University of Tokyo, Chiba, Japan). Anti–H-Y TCR Vβ8 transgenic mice were established by Drs. H. von Boehmer and M. Steinmetz (17) and were provided by Dr. Alfred Singer (National Cancer Institute, Bethesda, MD). LK35.2, a B cell hybridoma (FcR^+ and MHC class II^+), was used as a cross-linker of mAbs or the APC for superantigen during the stimulation of T cell clones (18). T cell clones MS-S2 ($I\text{-}A^k$ autoreactive) and 24-2 (specific for $I\text{-}A^b$ plus KLH) were established in our laboratory (Department of Immunology, Faculty of Medicine, University of Tokyo, Tokyo, Japan) (19, 20).

The mAbs used for stimulation and staining of TCR were H57-597 (anti–TCR-β) (21), F23.1 (anti–TCR Vβ8) (22), 145-2C11 (anti-CD3ε) (23), 500.A2 (anti-CD3ε) (24), and RM-2.1 (anti-CD2) (25). HMT3-1 (anti-CD3ε) (26), H28-710 (anti–TCR-α) (27), H146-968A (anti–TCR-ζ) (28), and 4G10 (antiphosphotyrosine; Upstate Biotechnology, Inc., Lake Placid, NY) were used for immunoprecipitation and immunoblotting analysis. Rat mAb against mouse Thy1 (30H-12) (Becton Dickinson & Co., Mountain View, CA) was used for two-color staining.

Cell Stimulation and Flow Cytometry (FCM) Analysis. Freshly isolated spleen cells were incubated with 10 μg/ml of anti–TCR-β (H57-597), 10 μg/ml of anti-CD3ε (145-2C11, 500.A2), 10 μg/ml of anti-Vβ8 (F23.1), 10 μg/ml of anti-CD2 (RM-2.1), 10 μM of ionomycin (Sigma Chemical Co., St. Louis, MO), or 10 μg/ml of SEB (Sigma Chemical Co.). For stimulation of T cell clones, cells were incubated with mAbs against the TCR–CD3 complex in the presence of B cell hybridoma, LK35.2. Stimulation of cells was stopped by the addition of ice-cold PBS containing kinase and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 25 mM *p*-nitrophenyl *p*-guanidinobenzoate). After stimulation, cells were incubated with anti–TCR-β or anti-CD3ε mAbs, followed by FITC-conjugated goat anti–hamster Ig for indirect staining. When the stimulating antibody and the staining antibody were the same, indirect staining method was used. For direct staining, cells were stained with FITC-conjugated anti–TCR-β (H57-597-FITC) or anti-CD3ε (145-2C11-FITC). Anti–Thy1-biotin (30H-12-biotin) followed by streptavidin PE was used for spleen cells analyzed by two-color FCM analysis to track $Thy1^+$ cells. FCM analysis was performed on the FACStar® Plus (Becton Dickinson & Co.).

Immunoprecipitation and Immunoblotting Analysis. Cells were harvested, washed in PBS, and lysed in 1% digitonin or 1% NP-40, 10 mM Tris, pH 7.5, and 150 mM NaCl with the phosphatase inhibitors indicated above. Postnuclear supernatants of digitonin lysates from 3×10^7 cells were subjected to immunoprecipitation for 1 h with hamster mAbs, as described previously (20). The immune complexes were washed in PBS containing 0.5% digitonin and the phosphatase inhibitors indicated above, solubilized in SDS sample buffer containing 5% 2-ME, and separated by SDS-PAGE, as described by Laemmli (29) and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% BSA (or 5% skim milk) in PBS and were incubated with the appropriate primary antibody followed by ^{125}iodine-labeled protein A. The amount of protein detected was estimated by densitometric analysis of the autoradiographs.

Immunoelectron Microscopy. After stimulation of cells with anti-CD3ε mAb, cells were fixed with 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 30 min at room temperature. They were infused with a graded series of sucrose solution up to 2.3 M in 0.1 M PBS and were then quickly frozen with liquid Freon 22. Ultrathin sections were cut with a cryo-ultramicrotome (Ultracut Om-U4 equipped with FC-4; Leica, Wetzler, Germany). Ultrathin frozen sections were collected on Formvar-coated grids and were double-stained with anti–TCR-β and CD3ε mAbs by using the following procedure (30). Sections were washed with 50 mM glycine in PBS, blocked with 2% gelatin, 2% BSA in PBS, and then stained with 10 μg/ml of anti-CD3ε (145-2C11), followed by colloidal gold–labeled protein A (Amersham Corp., Arlington Heights, IL). After washing, the sections were blocked with ×10-diluted normal mouse serum and incubated with 10 μg/ml of biotin-labeled anti–TCR-β (H57-597) mAb followed by colloidal gold–labeled streptavidin (Amersham Corp.). After further washing, they were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, stained with 0.3% uranyl acetate oxalate, and transferred to the surface of a uranyl acetate-methylcellulose mixture. After standing for 10 min, the sections were embedded in the same mixture and examined under an electron microscope (model JEM 1200 EX; JEOL, Tokyo, Japan).

Results

Differential Modulation of TCR by mAbs to the TCR–CD3 Complex. In a previous study, we reported that an allo–anti–I-J mAb inhibited signal transduction induced by anti-TCR but not by anti-CD3 (16). Transfection of various TCR-ζ chain constructs into T cell hybridomas that lack TCR-ζ chains indicated the presence of two parallel signal-transducing units in the TCR–CD3 complex (4, 5). The results of these studies suggest that the TCR undergoes dis-

[1] *Abbreviations used in this paper:* DN, double negative; FCM, flow cytometry; SEB, staphylococcal enterotoxin B.

sociation from CD3 when the TCR complex is ligated. Thus, we undertook this study to define the molecular mechanism of the dissociation of the TCR–CD3 complex.

Whole C3H spleen cell suspensions were incubated with either anti-TCR or anti-CD3 mAb or medium for 0, 3, 15, and 45 min at 37°C. The samples were harvested and washed, and the cells were subjected to immunofluorescence staining with anti-TCR or anti-CD3 mAbs. In the cultures treated with medium as a control or with anti-TCR mAb, there were no significant changes in the level of cell surface expression of either TCR or CD3 over the 45-min time course of the experiment (Fig. 1). In contrast, after 15 min of incubation in the presence of anti-CD3, the level of surface TCR-β expression was reduced (down-modulated) >50%, whereas the level of CD3 expression was not affected. Even after 45 min of incubation with anti-CD3, cell surface expression of CD3 remained at control levels, although the TCR surface expression had been down-modulated by 90%. Treatment with ionomycin did not induce the down-modulation of the TCR–CD3 complex. Treatment with anti-CD2 mAb also did not affect the cell surface expression of the TCR–CD3 complex (data not shown). These results suggested that, after treatment with anti-CD3 mAb, the TCR-β is uncoupled from CD3 and is selectively down-modulated.

Immunoelectron Microscopy of Ultrathin Frozen Sections Doubly Stained with Anti-TCR and Anti-CD3. We wished to determine whether the down-modulation of TCR molecules that was observed after stimulation of the T cells with the anti-CD3 mAb was due to the capping and subsequent shedding with mAb or to the internalization of TCR molecules. For this purpose, double-immunolabeled ultrathin sections were analyzed by electron microscopy. Cells were fixed with 3% paraformaldehyde and 0.1% glutaraldehyde 30 min after treatment with medium, anti-TCR, or anti-CD3 mAbs. Quick-frozen Ultrathin frozen sections were stained sequentially with biotin–anti-CD3 and anti-TCR mAbs, and each was followed by colloidal gold–labeled protein A or streptavidin.

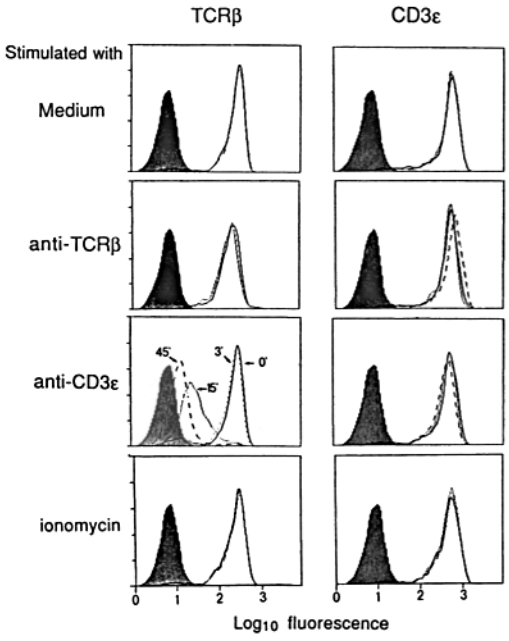

Figure 1. Cell surface expression of TCR and CD3 after stimulation with anti–TCR-β, anti-CD3ε mAbs, or ionomycin. Spleen cells were stimulated with anti–TCR-β (H57-597), anti-CD3ε (145-2C11) mAbs, or ionomycin for 3, 15, and 45 min. After stimulation of the cells, two-color staining was performed, and Thy1$^+$ cells were analyzed using FCM. Each panel contains expression of TCR-β or CD3ε of Thy1$^+$ cells at times 0, 3, 15, and 45 min after stimulation and is compared with that of the Thy1$^-$ fraction at time 0 min as a negative control. Down-modulation of TCR was only observed when cells were stimulated with anti-CD3 mAb.

Fig. 2 shows immunoelectron micrographs of spleen cells before and after treatment with medium (Fig. 2, *a* and *d*), anti-TCR (Fig. 2 *b*) or anti-CD3 mAbs (Fig. 2, *c* and *e*). The labeling of TCR (by 10 nm of gold particles) and CD3 (by 15 nm of gold particles) appeared uniformly on the surface of cells incubated with medium (Fig. 2, *a* and *d*). There was no significant change in cell surface expression of TCR or CD3 after treatment of the spleen cells with anti-TCR mAb (Fig. 2 *b*). These results are consistent with the observations above, obtained by FCM analysis, which indicate that no significant changes in the levels of surface TCR or CD3 expression were seen. In the case of the anti-CD3–treated cells, the labeling of the TCR was found to be localized to the cytoplasm of the cells, whereas the labeling of CD3 was seen at the cell surface (Fig. 2, *c* and *e*). These results indicated that, after stimulation of T cells with anti-CD3 mAb, the TCR dissociated from the TCR–CD3 complex and was down-modulated by internalization. In contrast, the CD3 molecules remained at the cell surface. Whether the TCR also dissociated from the TCR–CD3 complex after treatment with anti-TCR mAb could not be established from these results.

Dissociation of the TCR-α/β with ζ Chain from the TCR–CD3 Complex after Stimulation. To investigate further the effects of anti-TCR and anti-CD3 treatment on the dissociation of the TCR–CD3 complex, we attempted to quantify the total amount of TCR protein before and after incubation of T cells with anti-TCR, anti-CD3 mAbs, or medium by using immunoblot analysis. Spleen cells were treated with the various agents for 30 min and then were harvested and lysed with 1% NP-40. Whole-cell lysates were subjected to SDS-PAGE, and immunoblot analysis was performed with an anti–TCR-α mAb. The total amounts of TCR-α protein measured at different cell concentrations from the three different treatments were essentially similar (Fig. 3 *a*). This result supports the notion that, after treatment of the T cells with anti-CD3, TCR dissociates from the receptor complex and appears to be internalized such that the overall amount of TCR protein does not differ from the cells treated with medium or anti-TCR, a condition in which the TCR is not down-modulated.

We were curious whether the TCR–CD3 complex also underwent a dissociative event when T cells were treated with anti–TCR-β, even though the TCR did not appear to undergo the down-modulation seen with the anti-CD3 treatment. Spleen cells were treated with anti-TCR or anti-

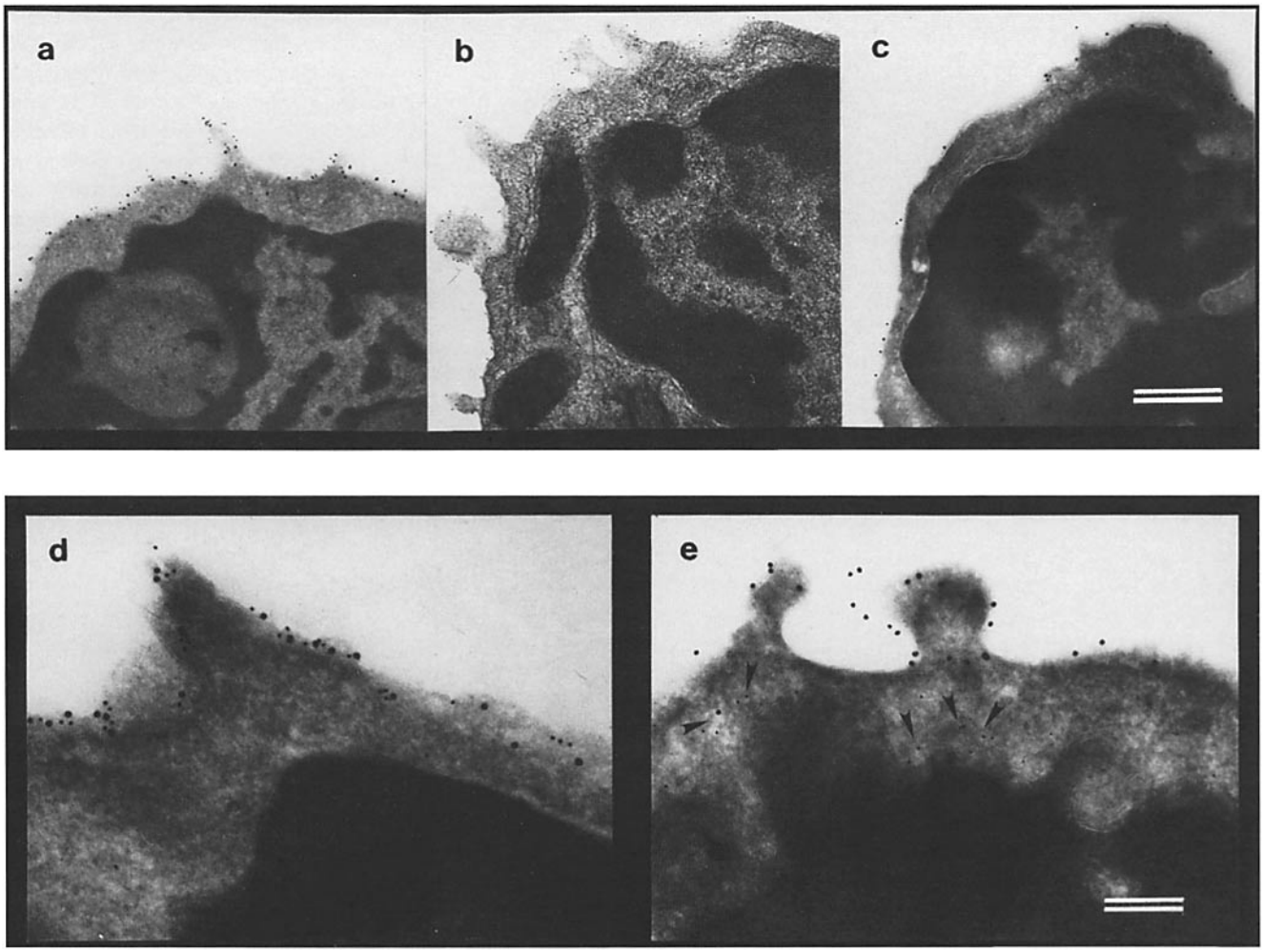

Figure 2. Immunoelectron micrographs of spleen cells labeled with anti-TCR and anti-CD3 mAbs. (*a* and *d*) Spleen cells were cultured for 30 min with medium, (*b*) stimulated with anti–TCR-β mAb (H57-597), or (*c* and *e*) stimulated with anti-CD3ε mAb (500.A2). Colloidal gold–labeled streptavidin, 10 or 15 nm for biotin–anti-CD3ε mAb, and 5 or 10 nm colloidal gold–labeled protein A for anti-TCR mAb were used. In *a–c*, ×33,000; bar, 500 nm; in *d* and *e*, ×132,000; bar, 200 nm. Arrowheads (*e*) indicate cytoplasmic location of TCR-β chain.

CD3 mAbs for 3 and 30 min. Cells were harvested and lysed with 1% digitonin. The lysates were then subjected to immunoprecipitation with either anti-CD3 (HMT3-1) (Fig. 3, *b* and *c*), anti–TCR-ζ (H146-968) (Fig. 3 *d*), or anti–TCR-β (H57-597) (Fig. 3 *e*) mAbs. The immunoprecipitates were subjected to SDS-PAGE and subsequent immunoblot analysis with the anti–TCR-α and anti–TCR-ζ mAbs (Fig. 3, *b* and *d*), antiphosphotyrosine (4G10) mAb (Fig. 3 *c*), or anti–TCR-α and anti-CD3ε mAbs (Fig. 3 *e*) as probes.

As shown in Fig. 3 *b*, the anti-CD3 immunoprecipitates from both anti-TCR– and anti-CD3–treated spleen cells after 30 min contain much less TCR (two- to threefold) than that of the control (treatment with medium for 30 min). The amount of TCR-ζ chains coprecipitated with anti-CD3ε mAb was also decreased in a ratio similar to that of TCR obtained from both treatments. These results indicated that a substantial amount of TCR-α chain was dissociated from CD3ε when T cells were stimulated with either anti-TCR or anti-CD3. Given that the cell surface expression of both TCR and CD3 was not affected by treatment with anti-TCR mAb, these results indicate that, as seen with the anti-CD3 treatment, dissociation of the TCR and CD3 results from the treatment of the cells with anti-TCR. However, the latter situation is not accompanied with the down-modulation of the TCR.

An antiphosphotyrosine blot of anti-CD3 immunoprecipitates from the spleen cells treated with anti-TCR or anti-CD3 mAbs is shown in Fig. 3 *c*. The amount of phosphorylated ζ chains precipitated with anti-CD3ε from both anti-TCR and anti-CD3 treatment after 30 min was increased much more (two- to fourfold) than that from the control. A 70-kD phosphorylated protein appeared after 3 min of treatment with anti-TCR, and anti-CD3, and remained after 30 min of treatment with anti-TCR, but it was not detected from the cell treated with anti-CD3 after 30 min. These results suggested that the signal transduction events induced by anti-CD3 may differ from those induced by anti-TCR.

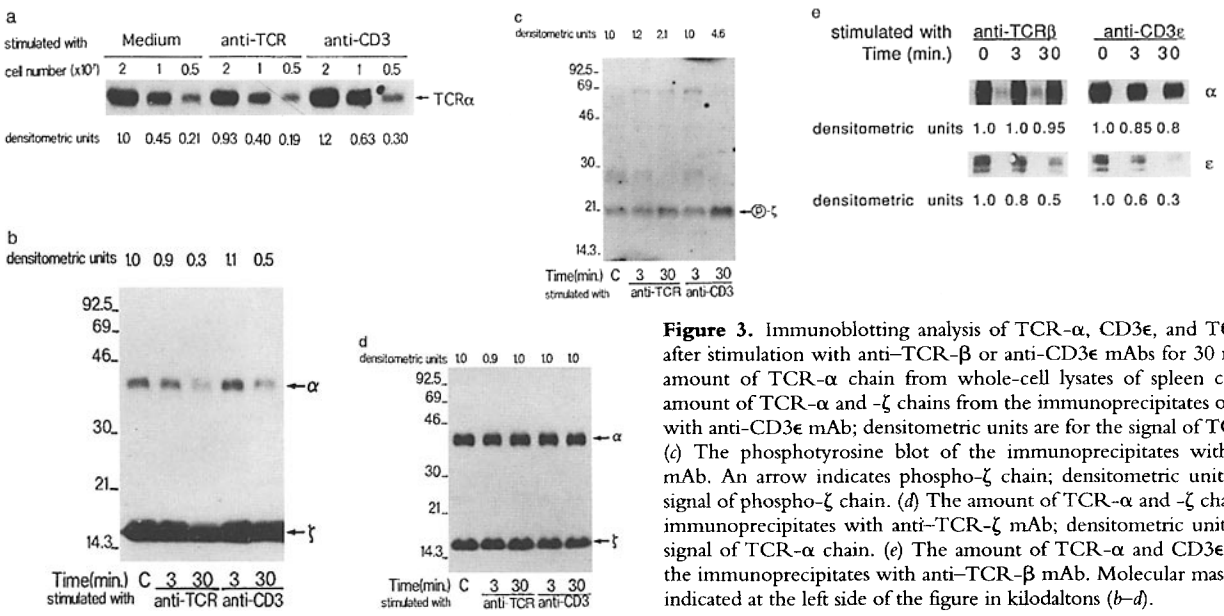

Figure 3. Immunoblotting analysis of TCR-α, CD3ε, and TCR-ζ chains after stimulation with anti–TCR-β or anti-CD3ε mAbs for 30 min. (*a*) The amount of TCR-α chain from whole-cell lysates of spleen cells. (*b*) The amount of TCR-α and -ζ chains from the immunoprecipitates of spleen cells with anti-CD3ε mAb; densitometric units are for the signal of TCR-α chain. (*c*) The phosphotyrosine blot of the immunoprecipitates with anti-CD3ε mAb. An arrow indicates phospho-ζ chain; densitometric units are for the signal of phospho-ζ chain. (*d*) The amount of TCR-α and -ζ chains from the immunoprecipitates with anti–TCR-ζ mAb; densitometric units are for the signal of TCR-α chain. (*e*) The amount of TCR-α and CD3ε chains from the immunoprecipitates with anti–TCR-β mAb. Molecular mass markers are indicated at the left side of the figure in kilodaltons (*b–d*).

The anti–TCR-ζ immunoprecipitates were analyzed by immunoblot with the anti–TCR-α and anti–TCR-ζ mAbs as probes (Fig. 3 *d*) to study the association between TCR and ζ chain during treatment with either anti-TCR or anti-CD3. The amount of TCR-α coimmunoprecipitated with the TCR-ζ chain was essentially similar to that from cells treated with anti-TCR or anti-CD3 for 30 min. These results suggested that TCR and ζ remain tightly associated and that these two components dissociate together from the antigen receptor complex upon ligation by either anti-TCR or anti-CD3.

To confirm dissociation between TCR and CD3 after stimulation of cells with anti-TCR or anti-CD3, analysis of the anti–TCR-β immunoprecipitates was performed by immunoblot, with anti–TCR-α and anti-CD3ε as probes (Fig. 3 *e*). In this case, it was found that the amount of CD3ε coimmunoprecipitated by anti–TCR-β decreased after treatment with either anti-TCR or anti-CD3. The total amount of TCR-α protein immunoprecipitated was not affected by the mAb treatments, however. This result would be anticipated if the TCR dissociates from CD3 upon ligation of the TCR–CD3 complex by either anti-TCR or anti-CD3.

In summary, the results of these immunoprecipitation experiments indicate that ligation of the TCR–CD3 complex with mAb is accompanied by the dissociation of the complex in such a manner that the TCR and ζ chains separate from the CD3ε. In addition, a differential consequence results from anti-CD3 ligation as compared with anti-TCR ligation: The former situation leads to downmodulation of the TCR, whereas the latter does not.

Effect of TCR Ligation with Superantigen or an anti-Vβ8 mAb. We wish to determine whether the dissociation of the TCR and CD3 mediated by ligation of the TCR–CD3 complex by anti-TCR could also be demonstrated when the TCR–CD3 complex is ligated with anti-Vβ mAb or a superantigen. For this purpose, spleen cells from transgenic mice bearing a TCR Vβ8 specific for the HY antigen were stimulated with either an anti-Vβ8 mAb or a superantigen, SEB (17). Murine TCR Vβ8 is known to be reactive to SEB. Immunofluorescence staining analysis with anti–TCR-β or anti-CD3ε mAbs was performed after the stimulation of spleen cells. As shown in Fig. 4 *a*, no downmodulation of the TCR–CD3 complex was observed even after 45 min of incubation with either anti-Vβ8 mAb or SEB. This result is consistent with the lack of TCR downmodulation that was seen in the spleen cells treated with anti-TCR mAb, as described in Fig. 1.

Evidence for the dissociation was obtained from the analysis of immunoprecipitates with the anti-CD3 mAb of lysates of cells treated with either anti-Vβ8 mAb or SEB. The results of immunoblot analysis of these immunoprecipitates, using the anti–TCR-α mAb as the probe, showed that the amount of TCR coimmunoprecipitated with anti-CD3ε was reduced similarly, either with the anti-Vβ8 or SEB. These results indicate that the dissociation of the TCR from the TCR–CD3 complex is also induced when the receptor complex is ligated by SEB as well as another anti-TCR reagent, anti-Vβ8 mAb. Thus, it appears that the dissociation of the TCR–CD3 complex is a general response to ligation of the TCR–CD3 complex either by anti-TCR mAb or superantigen.

TCR–CD3 Dissociation in T Cell Clones. Finally, we sought to determine whether the anti-TCR– or anti-CD3–induced uncoupling of TCR and CD3 would occur in T cell clones, which were maintained in vitro for >1 yr by antigen stimulation and IL-2 supplementation. Two T cell clones, MS-S2 (I-A^k autoreactive) and 24-2 (specific for KLH in the context of I-A^b), were stimulated with anti-TCR or anti-CD3 mAbs for 0, 3, 15, and 45 min in the

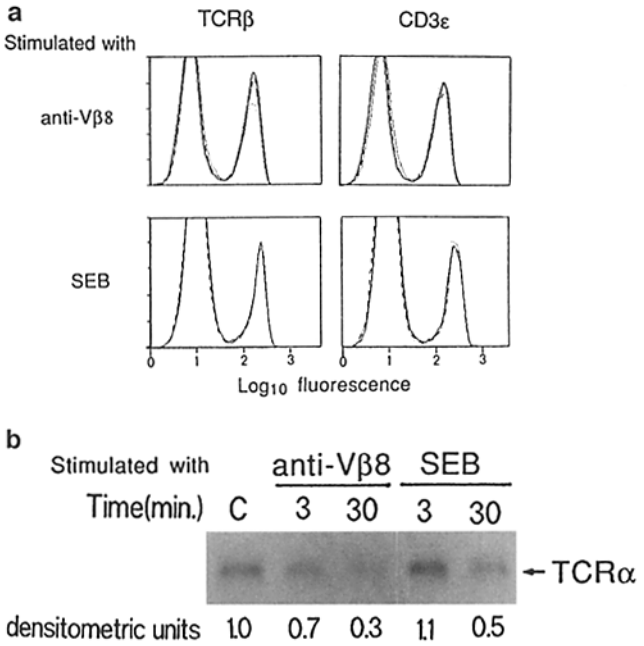

Figure 4. Uncoupling between TCR and CD3 complex after stimulation of spleen cells from Vβ8 transgenic mice with anti-Vβ8 mAb or SEB. (*a*) Cell surface expression of TCR-β and CD3ε after stimulation with anti-Vβ8 mAb (F23.1) or SEB. Spleen cells from Vβ8 transgenic mice were stimulated with anti-Vβ8 mAb (F23.1) or SEB for 3, 15, and 45 min. After stimulation, cells were stained with anti–TCR-β and anti-CD3ε mAbs and analyzed by FCM. (*b*) Immunoblot analysis of TCR-α proteins from the immunoprecipitates with the anti-CD3ε mAb after stimulation of spleen cells from Vβ8 transgenic mice with anti-Vβ8 mAb or SEB for different times, as indicated in the figure. Densitometric units indicate the signal of TCR-α chain.

presence of the B cell hybridoma, LK35.2, which is a Fcγ receptor–positive cell line. In both T cell clones, no down-modulation of TCR-β or CD3ε expression was observed upon stimulation with anti-TCR over the time course of the experiment (Fig. 5). Thus, like normal resting cells, ligation of the TCR–CD3 complex by anti-TCR does not result in the down-modulation of the surface-expressed TCR. In the case of the treatment with anti-CD3, however, cell surface expression of TCR was rapidly down-modulated in both T cell clones (Fig. 5). In fact, within 3 min the down-modulation of the TCR reached maximal level. Furthermore, some down-modulation of the CD3 complex was detected after 45 min of treatment with anti-CD3, and by 45 min as much as 30% reduction in the CD3 level was measured. Thus, T cell clones also showed modulation of TCR from the cell surface upon stimulation with anti-CD3. The dissociation response of T cell clones upon ligation of the TCR–CD3 complex appeared to be more rapid than that of resting spleen cells.

Discussion

Previous studies demonstrate that there is functional uncoupling between TCR and CD3 complexes in either immature or mature T cells after ligation of the TCR–CD3 complex (12–16). In the case of immature $CD4^+8^+$ thymocytes, cells that are resistant to anti-TCR mAb injection are unable to mobilize intracellular Ca^{2+} upon further stimulation with the anti-TCR mAb. However, these cells are eliminated when mice are treated with anti-CD3 mAb (12). This result led us to hypothesize that there is a physical dissociation of the TCR–CD3 complex upon receptor ligation.

In this study, we demonstrate two major observations. First, from the analysis of cell surface expression of TCR-β or CD3ε before and after stimulation, as detected by flow cytometry (Fig. 1), anti-CD3 treatment of the T cells resulted in the down-modulation of the TCR-α/β, whereas surface expression level of CD3ε was not affected. In contrast, anti-TCR treatment failed to modulate the surface expression of either TCR-α/β or CD3ε. As shown by immunoelectron micrography (Fig. 2), this specific down-modulation of the TCR-α/β by anti-CD3ε treatment appeared to be due to internalization. Second, the amount of TCR-α/β coimmunoprecipitated with ζ chain was not changed by treatment with either anti-CD3 or anti-TCR; however, the amount of TCR-α/β coimmunoprecipitated with anti-CD3ε significantly decreased after 30 min of treatment with anti-TCR and anti-CD3 (Fig. 3). From an analysis of a shorter exposure of the immunoblot in Fig. 3 *b*, the amount of ζ chains precipitated with anti-CD3ε was decreased in a ratio similar to that of TCR-α/β obtained

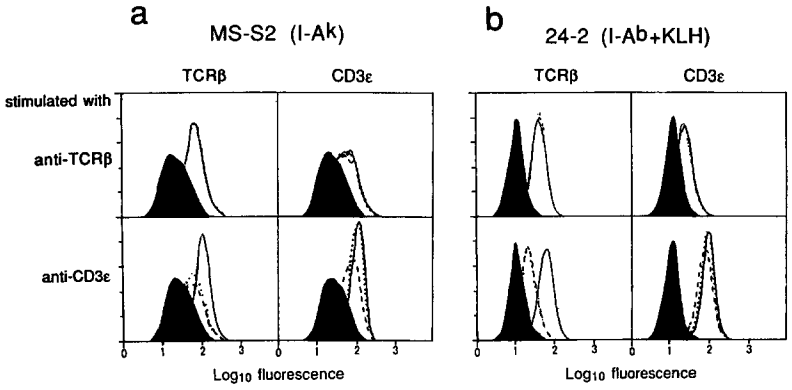

Figure 5. FCM analysis of TCR-β and CD3ε chain of T cell clones after stimulation. (*a*) $I\text{-}A^k$ autoreactive T cell clone, MS-S2. (*b*) $I\text{-}A^b$–restricted KLH-specific T cell clone, 24-2. T cell clones mixed with LK35.2, a B cell hybridoma, were stimulated with anti–TCR-β or anti-CD3ε mAbs for 3, 15, and 45 min. At each time point, cells were harvested and stained with anti-Thy1 and anti–TCR-β or anti-CD3ε mAbs. $Thy1^+$ cells were analyzed.

from both treatments. These results suggested that the TCR dissociation from CD3 complex occurred in such a manner that the TCR-α/β and associated ζ chains are released as a unit.

In Fig. 6, we present a view of the physical dissociation of TCR and CD3 that results after treatment with anti-TCR or anti-CD3 mAbs. Both anti-TCR and anti-CD3 mAb's ligation of the TCR–CD3 complex mediates dissociation of the complex in such a way that TCR and ζ chains remain associated. In the case of anti-CD3ε stimulation, the dissociated TCR is down-modulated by internalization, whereas the CD3 complex remains at the cell surface. TCR–CD3 ligation through the TCR by anti–TCR-β, anti-Vβ8, or superantigen (SEB) does not affect the surface expression of either TCR or CD3, whereas the TCR–CD3 complex undergoes dissociation.

Although we have not presented all of our data, two different anti-CD3 mAbs (145-2C11 and 500.A2) and five different anti-TCR reagents (H57-597; anti–TCR-β constant region, F23.1; anti–TCR Vβ8, A2B4; anti–TCR-α of 2B4TCRαβ Tg mice, 1B2; anti–TCR-α of 2C Tg mice, T3.70; and anti–TCR-α of HY Tg mice) were tested, and results basically similar to those shown in Fig. 1 were obtained with all of the reagents. The TCR–CD3 dissociation upon stimulation of TCR Vβ8 Tg T cells with SEB was investigated (Fig. 4), and evidence for the dissociation of TCR from CD3 was obtained; however, no down-modulation of TCR was observed. In addition, TCR appeared to be dissociated from CD3 in the stimulation with specific peptides and MHC in the 2C Tg system (data not shown).

Several groups have reported the co-down-modulation of TCR-α/β and CD3γδε when the TCR–CD3 complex was stimulated with multivalent cross-linking of anti-TCR mAb or anti-CD3 mAb with anti–mouse antibodies (31, 32). In these reports, TCR-α/β was down-modulated coincident with CD3γδε. Two major differences in the experimental system might serve to explain the differences between their experiments and ours. First, we stimulated TCR or CD3 molecules by using cell–cell interactions. When anti-TCR or anti-CD3 mAbs were added to the spleen single-cell suspensions, splenic FcR^+ cells played the role of a cross-linker, because anti-FcR mAb (2.4G2) abrogated the effect of these antibodies. In physiological conditions, the TCR ligation with antigenic peptide on MHC molecules must be mediated by direct cell–cell interactions. During the interactions of T cells and APC, so-called coreceptor molecules, including various adhesion molecules, are expected to be stimulated. In our system, sufficient coreceptor signals should be operative in the stimulation using antibodies. In addition, the observations obtained as a result of stimulation with SEB and MHC molecules were similar to those obtained by stimulation with anti-TCR mAbs in our system. Second, we analyzed only very early (up to 1 h) molecular events after TCR ligation. Intracellular signal transduction events induced directly by TCR ligation should be examined within a few minutes to 1 h. For instance, tyrosine kinases are known to be activated within 1 min after TCR ligation, and their activities decrease quickly and return to baseline levels after 1 h (33). Changes in the intracellular signaling molecules after several hours might be the result of multiple cellular events, including the activation and inactivation of various genes (34). In fact, in our system, cell surface expression of CD3ε was substantially decreased after overnight stimulation with either anti-TCR or anti-CD3 mAbs.

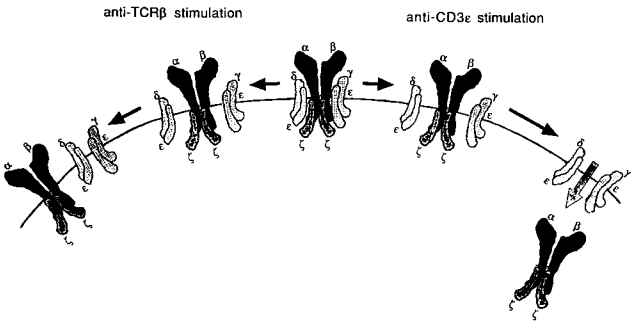

Figure 6. Dissociation and down-modulation of TCR-α/β accompanied with TCR-ζ from the CD3 complex. This model illustrates the dissociation of TCR-α/β and CD3 components that accompanies the ligation of the TCR–CD3 complex by anti-TCR and anti-CD3 mAbs. Both anti-TCR and anti-CD3 mAb's ligation of the TCR–CD3 complex mediates dissociation of the complex in such a way that TCR and ζ chains remain associated. Anti-CD3ε stimulation causes dissociation of TCR-α/β, and the dissociated TCR is down-regulated by internalization with TCR-ζ chain, whereas CD3 components still express at the cell surface. TCR–CD3 ligation through the TCR by anti–TCR-β, anti-Vβ8, or superantigen (SEB) does not affect the surface expression of either TCR-α/β or CD3.

In the immature T cell, signal transduction via the CD3 complex plays an important role in the differentiation of the $CD4^-8^-$ (double-negative [DN]) thymocytes to the $CD4^+8^+$ (double-positive) stage (35, 36). Thymocytes in RAG-$1^{-/-}$ or RAG-$2^{-/-}$ mice are stacked at the DN stage because of the lack of TCR-β rearrangements (37–40). Injection of anti-CD3 mAb into RAG-$2^{-/-}$ mice or treatment of fetal thymus organ cultures of RAG-$1^{-/-}$ mice with anti-CD3 not only induced differentiation of DN thymocytes to double-positive thymocytes but also resulted in a dramatic increase in the cell numbers in the thymus (35, 36). These studies suggest that signal transduction via the CD3 complex may compensate for the requirement of TCR-β gene rearrangement in DN thymocytes and may differ from the signal transduction via the TCR-α/β. In our studies, we found a qualitative difference in the responses to the two treatments (anti-TCR versus anti-CD3) in that neither anti-TCR mAbs nor SEB treatment resulted in down-modulation of TCR–CD3. These results indicate that early events in T cell activation, such as Ca^{2+} influx or activation of tyrosine kinases itself, may not play an important role in the process of TCR down-modulation. Although TCR-α/β linked to the TCR-ζ chain dissociated from the CD3 complex (Fig. 3 *c*), there is the possibility that phosphorylation of tyrosine kinases or TCR-ζ chain is involved in the dissociation of the TCR and the CD3 complex.

Julius and his colleagues observed that pretreatment with anti-CD4 mAb inhibited anti-TCR mAb–mediated activation but failed to inhibit activation with anti-CD3 mAb (14). They also observed, using T cell clones, that association of the tyrosine kinase $p56^{lck}$ with CD4 inhibited stimulation with anti-TCR mAb but did not alter the effect of anti-CD3 mAb treatment (15). In Fig. 3 *c*, the amount of phosphorylated ζ chains induced by anti-CD3 treatment was twofold greater than that induced by anti-TCR treatment; also, a 70-kD phosphorylated protein (ZAP-70 or Syk?) was still detected 30 min after treatment with anti-TCR mAb, whereas the 70-kD phosphoprotein induced by anti-CD3 treatment was already dephosphorylated or dissociated from the TCR–CD3 complex under similar conditions. These results could be interpreted as suggesting that signal transduction events induced by anti-TCR mAb are distinguishable from those induced by anti-CD3 mAb. These observations raise the possibility that tyrosine kinases such as $p56^{lck}$ and $p59^{fyn}$ play a key role distinguishing signal transduction through TCR-α/β from that through the CD3 complex. Different combinations of tyrosine kinases may provide the dissociation, followed by down-modulation of TCR, although natural ligation of TCR–CD3 complex resulted in dissociation on the cell surface without down-modulation of TCR-α/β (see Fig. 4).

TCR–CD3 complex is known to be a unit composed of at least eight polypeptides, i.e., αβγδεεζζ in normal T cells (1–4) and immature thymocytes (11). Recently, reports have demonstrated atypical TCR–CD3 complexes in tumor-bearing mice (41, 42). Spleen T cells in tumor-bearing mice expressed low amounts of CD3γ and undetectable TCR-ζ, but expressed the FcεRI-γ chain (41). Aoe et al. also reported the structural changes of CD3γδε and the loss of TCR-ζ in TCR complex on spleen T cells in tumor-bearing mice (42). These atypical receptor complexes were found to be poor in function as assessed by calcium mobilization (41), which might account for the diminished immune responses in tumor-bearing hosts.

We suspect, from the results presented here, that the dissociation of the TCR and the CD3 complex could help explain the unresponsiveness of T cells after stimulation and may regulate the strength of signal transduction via the TCR–CD3 complex, because we have observed in preliminary studies that anergic T cells induced by stimulation with Mls antigen did not show down-modulation of TCR by anti-CD3 treatment (data not shown). This observation suggests that Mls stimulation results in dissociation of the TCR–CD3 complex, so subsequent treatment with anti-CD3 would not be expected to cause down-modulation of the TCR.

Recent studies on TCR-altered peptide antagonists and partial agonists have suggested that TCR peptide antagonists and partial agonists delivered an insufficient signal to T cells. This caused partial phosphorylation of TCR-ζ chains, resulting in the lack of association of ZAP-70 with the TCR–CD3 complex (43, 44). When the T cell clone was stimulated with APC plus TCR–altered peptide antagonists or partial agonists, TCR-ζ chains were never fully phosphorylated. However, with antigenic peptides, fully phosphorylated TCR-ζ chains (21 kD) were detected. The partially phosphorylated TCR-ζ chains failed to associate with ZAP-70. Thus, the antagonists or partial agonists induce an insufficient signal that nevertheless could block the subsequent signaling induced by the immunogenic ligand (sufficient signal). The inhibitory effect of TCR peptide antagonists on T cell stimulation could be explained mechanistically as a result of the physical dissociation of TCR–CD3 complexes. The relationship of the phenomena induced by altered peptide ligation and the dissociation of TCR–CD3 complexes requires further examination.

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