Immunity, Vol. 13, 665–675, November, 2000, Copyright ©2000 by Cell Press

On the Dynamics of TCR:CD3 Complex Cell Surface Expression and Downmodulation

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Summary

TCR downmodulation following ligation by MHC:peptide complexes is considered to be a pivotal event in T cell activation. Here, we analyzed the dynamics of TCR:CD3 cell surface expression on resting and antigen-activated T cells. We show that the TCR:CD3 complex is very stable and is rapidly internalized and recycled in resting T cells. Surprisingly, the internalization rate is not increased following TCR ligation by MHC:peptide complexes, despite significant TCR downmodulation, suggesting that constitutive internalization rather than ligation-induced downmodulation serves as the force that drives serial ligation. Furthermore, TCR downmodulation is mediated by the intracellular retention of ligated complexes and degradation by lysosomes and proteasomes. Thus, our data demonstrate that ligation induces TCR downmodulation by preventing recycling rather than inducing internalization.

Introduction

A common feature of many cell surface receptors is their constitutive or ligand-induced endocytosis, via clathrin-coated pits, and subsequent recycling back to the cell surface (Brown et al., 1983; Mellman, 1996). The transferrin receptor (TfR, CD71), for instance, is rapidly and constitutively recycled through both peripheral/perinuclear and perinuclear recycling pathways (Hopkins, 1983; Klausner et al., 1983). This process is mediated by a tyrosine (Y)-based motif, YXXØ (X, any amino acid; Ø, a bulky hydrophobic amino acid), in the cytoplasmic tail of TfR that binds to the μ chain of the AP2 complex, which subsequently associates with clathrin-coated pits for internalization (Ohno et al., 1995; Mellman, 1996). Conversely, Thy-1 (CD90), a phospholipid (PI)-anchored protein, recycles very slowly (Bretscher et al., 1980).

The T cell receptor (TCR):CD3 complex is a large multisubunit complex composed of at least eight polypeptide subunits (TCRαβ, CD3εγ, εδ, and ζζ) (Clevers et al., 1988; Klausner et al., 1990). The cytoplasmic tails of CD3 contain immune-receptor tyrosine-based activation motifs [ITAM; $(D/E)X_2YX_2(L/I)X_7YX_2(L/I)$], which are phosphorylated upon TCR ligation (Reth, 1987). These act as docking sites for SH2 domain–containing proteins, which mediate signal transduction and T cell activation. However, these ITAM motifs also resemble the AP2 binding motif, which is responsible for internalization. Clearly, not all YXXØ motifs can bind AP2 and mediate endocytosis, and there is no direct evidence that the ITAMs can mediate AP2 binding. However, it has been shown that AP2 can bind to the TCR:CD3 complex via a dileucine motif in the CD3γδ chains (Dietrich et al., 1994, 1997). How can the TCR:CD3 complex, with at least some AP2 binding motifs, remain stably expressed on the cell surface while still providing access to ITAMs for tyrosine phosphorylation?

This issue prompted us to reevaluate the extent of TCR cell surface stability, internalization, and recycling. Some early studies suggested that the TCR:CD3 complex constitutively recycles (Krangel, 1987; Minami et al., 1987), while others found evidence for internalization but no recycling (Tax et al., 1987). Cell surface expression of the TCR:CD3 complex is downmodulated upon ligation with MHC:peptide complexes (Zanders et al., 1983; Schonrich et al., 1991; Valitutti et al., 1995). Seminal studies have shown that a single MHC:peptide complex can serially ligate ~200 TCR, leading to an amplified and sustained signal (Valitutti et al., 1995). This serial engagement model has led some investigators to explore the correlation between TCR downmodulation and T cell responses. While some have shown a clear correlation (Bachmann et al., 1997; Hemmer et al., 1998; Itoh et al., 1999), others have suggested that there is no direct relationship (Blichfeldt et al., 1996; Cai et al., 1997). Indeed, recent studies imply that TCR ligation induces the downregulation of nonengaged receptors (San Jose et al., 2000). Thus, it is still unclear whether TCR internalization/downmodulation is a prerequisite for T cell signal transduction and function and whether ligation-induced downmodulation is required to "drive" serial engagement.

In this study, we have examined the dynamics of TCR:CD3 expression and downmodulation in resting and antigen-stimulated T cell hybridomas and $CD4^+$ T cells from normal and TCR transgenic mice. The following questions were addressed. (1) What is the extent of TCR recycling on resting cells? (2) How is this affected by TCR ligation? (3) What is the intracellular location of TCR in resting and activated T cells? (4) What is the intracellular fate of ligated TCR?

Results

Long-Lived TCR:CD3 Complexes Rapidly Recycle in Resting T Cells

Brefeldin A (BFA) blocks anterograde transport from the endoplasmic reticulum (ER) to the Golgi complex and causes tubulation and fusion of early endosomes with the trans-Golgi network (TGN) (Lippincott-Schwartz et al., 1991; Wood et al., 1991). This latter property was exploited to study the dynamics of TCR:CD3 cell surface

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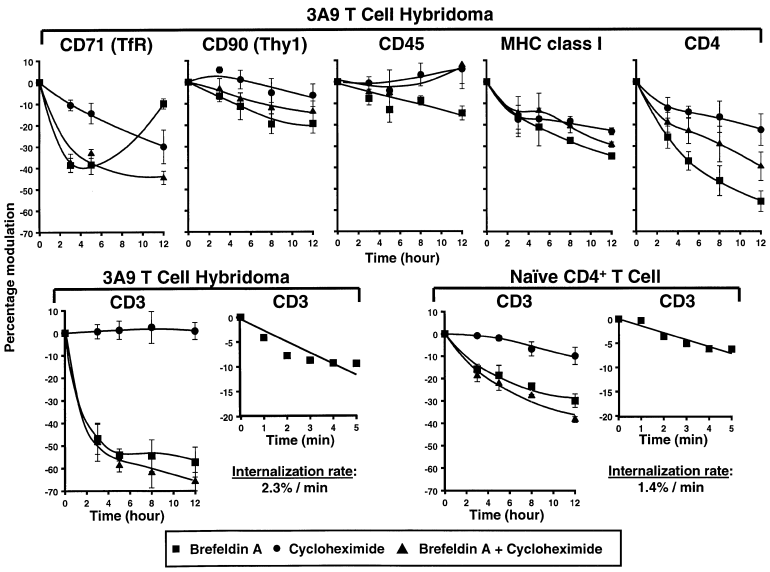


Figure 1. TCR:CD3 Complexes Are Long Lived and Rapidly Recycle on T Cells

The effect of BFA (10 μg/ml) and/or cycloheximide (50 μg/ml) on the surface expression of CD71, CD90, CD45, MHC class I, CD4, and CD3 on the 3A9 T cell hybridoma and naive $CD4^+$ T cells from B10.BR mice (CD3 only) was determined by flow cytometry at the time points indicated. The TCR internalization rate was determined by linear regression analysis following rapid exposure to BFA and is expressed as a percentage of the total surface-labeled TCR that is internalized per minute. Data represent the mean of three experiments.

expression on the HEL 48-62–specific, H-$2A^k$-restricted T cell hybridoma 3A9. We compared the effect of BFA on the expression of TCR:CD3 with TfR (CD71), Thy-1 (CD90), CD4, CD45, and MHC class I by flow cytometry. In order to distinguish between the effect of BFA on the prevention of endocytic versus ER:Golgi transport, cells were also treated with cycloheximide to block protein synthesis.

Cell surface expression of TfR, which is known to be rapidly recycled (Klausner et al., 1983), was significantly reduced by BFA (Figure 1). Some of this reduction was due to the prevention of newly synthesized molecules being transported from the ER to the Golgi, as shown by cycloheximide treatment. The return of TfR expression, following BFA treatment, to normal levels is likely due to an increase in TfR synthesis as a consequence of iron starvation. In contrast to TfR, Thy-1, a PI-anchored protein that is known to recycle slowly (Bretscher et al., 1980), and CD45 were only slightly affected by BFA and cycloheximide, suggesting that these molecules have a very slow recycling and turnover rate. The loss of surface MHC class I was due to degradation and replacement by newly synthesized molecules rather than any significant level of recycling, as the effects of BFA and cycloheximide were comparable. In contrast, CD4 expression was reduced far more by BFA than cycloheximide (2.5-fold), suggesting that CD4 is both recycled and degraded in resting T cells. We were surprised to find that, in several instances, the effect of BFA alone was greater than BFA plus cycloheximide. This may suggest that the action of BFA is dependent on a highly labile intermediate that requires protein synthesis. Alternatively, this treatment may affect proteins required for the surface stability of CD4 or CD45, such as $p56^{lck}$ for the former (Pelchen-Matthews et al., 1992).

In stark contrast to the cell surface molecules discussed above, the effect of BFA on the surface expression of TCR:CD3 complexes in the 3A9 T cell hybridoma was very different from the effect of cycloheximide. The latter had no effect on TCR:CD3 cell surface expression, which remained stable with no loss after 12 hr, suggesting that TCR:CD3 complexes are very long lived on resting T cells (Figure 1). Conversely, surface expression fell rapidly following treatment with BFA and remained low for 12 hr. This BFA-induced reduction in TCR cell surface expression cannot be due to a block in the transport of newly synthesized complexes migrating to the cell surface or the rapid degradation of internalized TCR followed by their replacement by newly synthesized complexes; otherwise, a similar effect would have been seen with cycloheximide treatment. These data suggest that TCR:CD3 complexes are continually internalized and recycled back to the cell surface. Similar but less dramatic observations were also made with naive, activated (72 hr postactivation), and memory (10 days postactivation) $CD4^+$ T cells from 3A9 TCR transgenic mice (Figure 1; data not shown). Previous studies have shown that the effect of BFA on endocytic transport is greatest soon after treatment (Lippincott-Schwartz et al., 1991; Wood et al., 1991); thus, the rate of TCR:CD3 complex internalization was determined within the first 5 min. Despite the size and complexity of the TCR, it is internalized reasonably fast at 2.3%/min in T cell hybridomas and 1.4%/min in naive splenic T cells (Figure 1). Given the incomplete effect of BFA on endocytic transport (Lippincott-Schwartz et al., 1991; H. L. and D. A. A. V., unpublished data), it seems reasonable to propose that the actual internalization rate may be faster.

Is the constitutive internalization of the TCR an active process? This possibility is supported by the dramatic effect of BFA on CD3 expression versus Thy-1 and CD45. We addressed this issue further by expressing the TCR:CD3 complex on 293T cells to assess the effect of BFA on mutant receptors. Wild-type TCR:CD3 complexes were downmodulated on 293T cells, with slightly faster kinetics than seen with T cell hybridomas (Figure 2). Interestingly, removal of the CD3εγδ cytoplasmic domains completely abrogated TCR internalization, while mutation of the CD3ζ ITAMs (Y to F) leads to a 33% reduction. These data suggest that motifs in the CD3 cytoplasmic domains, including the ITAMs in CD3ζ, actively mediate TCR internalization.

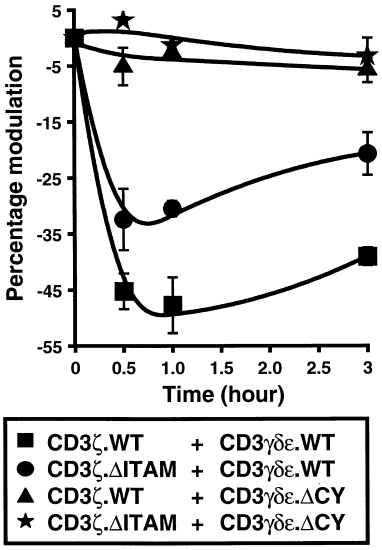


Figure 2. The CD3 Cytoplasmic Domains Mediate Active Internalization of the TCR Complex

293T cells were transiently transfected with plasmids carrying the 3A9 TCRαβ chains, wild-type or tailless CD3εγδ, and wild-type or ITAM-mutated (Y to F) CD3ζ. The CD3ζ plasmids also contain green fluorescent protein (GFP). After 3 days, $TCR^+CD3^+GFP^+$ cells were sorted, treated with BFA (10 μg/ml) for the times indicated, and TCR downmodulation determined. Data represent the mean of two experiments.

In order to confirm that the TCR:CD3 complex is rapidly internalized on resting T cells, we also monitored TCR internalization biochemically (Smythe et al., 1992). T cells were treated with NHS-SS-biotin so that only surface TCR were labeled. Cells were placed in culture, and, at various time points, aliquots were removed and treated with MESNA, a cell-impermeable reducing agent. This cleaves the biotin label of all surface TCR by reduction of the SS bond, while any internalized TCR retain their biotin, as they are refractory to the cell-impermeable MESNA. Subsequent TCR immunoprecipitation and Western blotting were then used to determine the amount of internalized, MESNA-resistant TCR. Linear TCR internalization was observed up to 45 min with an internalization rate of 0.6% of surface TCR per minute (Figure 3). Given that there is likely to be some loss of signal due to the multiple steps involved and some cell death, this rate is probably an underestimate. Subsequently, the amount of biotinylated TCR plateaus, which probably indicates that the intracellular compartments are saturated and TCR are returning to the cell surface. From this, one can conclude that it takes ~60 min to traverse the intracellular compartments (recycling time), and ~29% of the total TCR is intracellular. Taken together, these data demonstrate that the TCR:CD3 complex is very stable and long lived and that it is rapidly internalized and recycled on resting T cells compared with other cell surface molecules.

TCR:CD3 Ligation Prevents TCR Return to the Cell Surface

Previous studies have shown that ligation of the TCR by MHC:peptide complexes leads to its downmodulation from the cell surface (Valitutti et al., 1995). This implied that the ligation event triggered the internalization of the TCR:CD3 complex. Given the high rate of TCR internalization on resting T cells, we questioned whether TCR downmodulation was due to the prevention of TCR recycling rather than induction of TCR internalization.

We compared the rate of intracellular accumulation of biotinylated TCR in naive T cells from 3A9 TCR transgenic mice cultured either with HEL-pulsed B cells (LK35.2 B cell lymphoma) or B cells alone. The initial rate of internalization in the presence or absence of antigen was identical at 1.5%/min (0–45 min; Figure 4). This is consistent with the BFA experiments but somewhat faster than observed previously. This could be due to the addition of MHC class II^+ B cells; however, further studies would be required to confirm this notion. Intracellular compartments were saturated with biotinylated TCRαβ after 20 min, which plateaued and remained constant up to 45 min. With ~28% of the total surface TCR internalized in 20 min, this would suggest that the surface TCR pool would be replaced every hour. However, after 45 min, the fate of TCRαβ in the two T cell populations diverged. In unstimulated T cells, the amount of intracellular biotinylated TCRαβ decreased. Conversely, in antigen-stimulated T cells, biotinylated TCRαβ accumulated to a peak after 1 hr. While this was a modest increase (20%), it was reproducibly observed in five separate experiments (Figure 4B). Furthermore, the rate of intracellular accumulation is probably offset by the rate of TCR degradation. Densitometric analysis showed that there was ~33% more biotinylated TCRαβ in antigen-stimulated versus unstimulated T cells after 90 min, compared with ~45% downmodulation of surface TCR observed by flow cytometry. These data suggest that, under physiological conditions, ligation by MHC:peptide complexes does not significantly accelerate the rate of TCR internalization. This implies that TCR downmodulation is mediated by preventing the TCR from recycling and returning to the cell surface, rather than by inducing its internalization.

Ligation by High-Density MHC:Peptide Complexes Results in Very Rapid Downmodulation but Only Marginal Acceleration of TCR Internalization

Given the rapid rate of constitutive TCR internalization and the relatively low percentage of MHC class II molecules that are loaded with the specific peptide under physiological conditions (<1%; Vignali et al., 1993), it is likely that only a small fraction of the TCR that is internalized has been ligated. Thus, it is conceivable that ligation does increase the TCR internalization rate, but this is not visible using physiological antigen densities. To address this issue, T cells were stimulated with an APC that expresses a single, defined MHC:peptide complex, resulting in a substantially higher percentage of the MHC class II molecules loaded with the HEL 48-63 peptide (Kozono et al., 1994; Carson et al., 1997). The M12.C3 B cell line (Glimcher et al., 1985) was transfected with constructs encoding the wild-type H-$2A^k\alpha$ chain and the H-$2A^k\beta$ chain recombinantly attached to HEL 48-63 (designated HEL.48-63:H-$2A^k$.M12). In the 3A9 T cell hybridoma, the rate of cell surface TCR downmodulation was approximately twice as fast with this transfec-

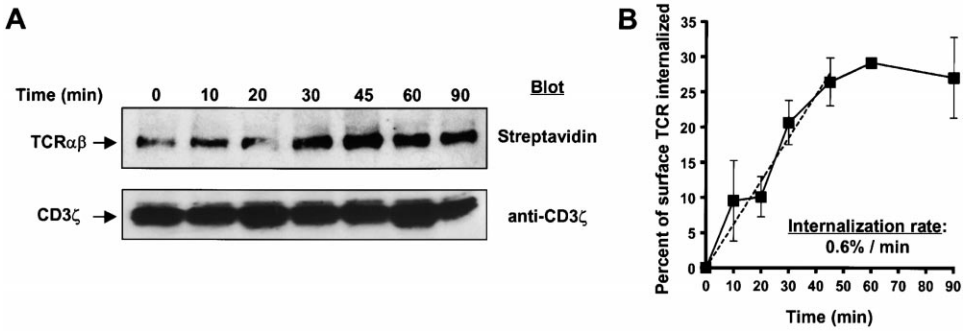


Figure 3. The TCR:CD3 Complex Is Rapidly Internalized on Resting T Cells

(A) The rate of TCR:CD3 internalization was determined by accumulation of intracellular biotinylated TCR. B10.BR splenocytes were surface biotinylated with NHS-SS-biotin (1 mg/ml) and, at the time points indicated, were treated with MESNA (200 mM), quenched with iodoacetamide, and lysed in 1% Brij 97. TCRαβ was immunoprecipitated with anti-TCR-Cβ (H57-157), immunoblotted with streptavidin-HRP, and developed by chemiluminescence. The small amount of background signal at time 0 is due to the incomplete stripping of surface biotin. Blots were subsequently stripped and reprobed with an anti-CD3ζ mAb (H146-968), followed by protein A-HRP. Data are representative of five experiments.
(B) Densitometric analysis was performed to determine the internalization rate. The background value at time 0 was subtracted, and the data were normalized to the CD3ζ loading control. The internalization rate is expressed as a percentage of the total surface-labeled TCR that is internalized per minute. Data represent the mean of five experiments.

tant compared with HEL-pulsed M12 cells expressing native H-2A^k (H-A^k.M12 + HEL) (Figure 5A). However, the internalization rate of biotinylated TCR was no faster in the presence of HEL.48-63:H-2A^k.M12 (Figure 5B). This suggested that, in T cell hybridomas, maximal ligation has little effect on the TCR internalization rate.

In contrast to hybridomas, cell surface downmodulation of TCR in naive CD4$^+$ T cells is dramatically increased in the presence of the HEL.48-63:H-2A^k.M12 transfectant, relative to HEL-pulsed B cells, with almost all surface TCR lost after only 1 hr (95%; Figure 5C). In contrast, stimulation with HEL-pulsed B cells mediates ~35% downmodulation after 1 hr. Despite this clear distinction in cell surface TCR downmodulation rates, only a very small difference is observed in the internalization rate of biotinylated TCRαβ with the two APCs (Figures 5D and 5E). This becomes evident if one compares the time required for the two stimuli to mediate 50% downmodulation (HEL.48-63:H-2A^k.M12 , ~8 min; H-2A^k.M12 + HEL , ~100 min; a 12.5-fold difference) versus 50% maximal TCR internalization (HEL.48-63:H-2A^k.M12, ~13 min; H-2A^k.M12 + HEL, ~18 min; a 1.4-fold difference). Taken together, these data suggest that even in the presence of extremely high ligand densities, which mediate substantially accelerated TCR downmodulation, the rate of TCR internalization is only slightly increased. Importantly, under physiologically stimulatory conditions, the bulk of the internalized TCR has not been ligated and is thus destined for recycling back to the cell surface.

Multiple Processes Mediate the Fate of Ligated TCR:CD3 Complexes

If the TCR is rapidly internalized and only a small proportion is ligated, how does cell surface downmodulation occur? Our data suggest that all surface TCR:CD3 complexes will be recycled in 1 hr, while surface expression is reduced less than 20% 1 hr poststimulation. One could

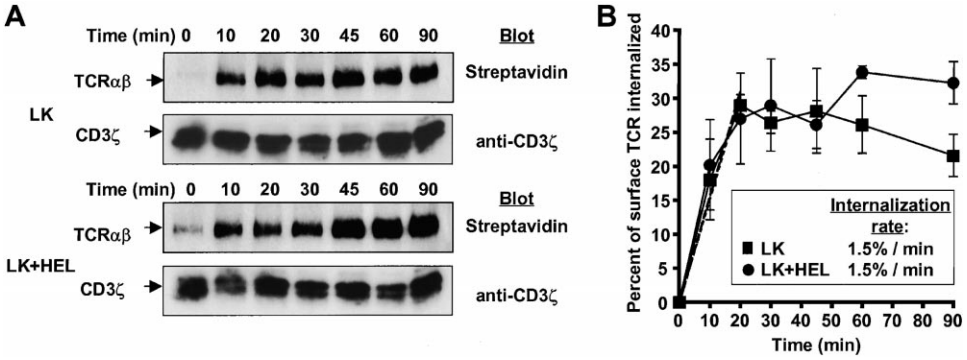


Figure 4. Ligation of MHC:Peptide Complexes Induces Intracellular Retention of TCR:CD3 and Blocks Recycling

(A) TCR:CD3 internalization was determined as described in Figure 3, except that naive splenic CD4$^+$ T cells from 3A9 TCR transgenic mice were used and incubated with either LK35.2 alone or LK35.2 prepulsed with 10 μM HEL. TCR downmodulation in untreated and biotinylated cells was identical, suggesting that biotinylation does not affect TCR ligation of MHC:peptide complexes (data not shown). Data are representative of five experiments.
(B) Densitometric analysis represents the mean of five experiments. The data and internalization rates are calculated as described in Figure 3.

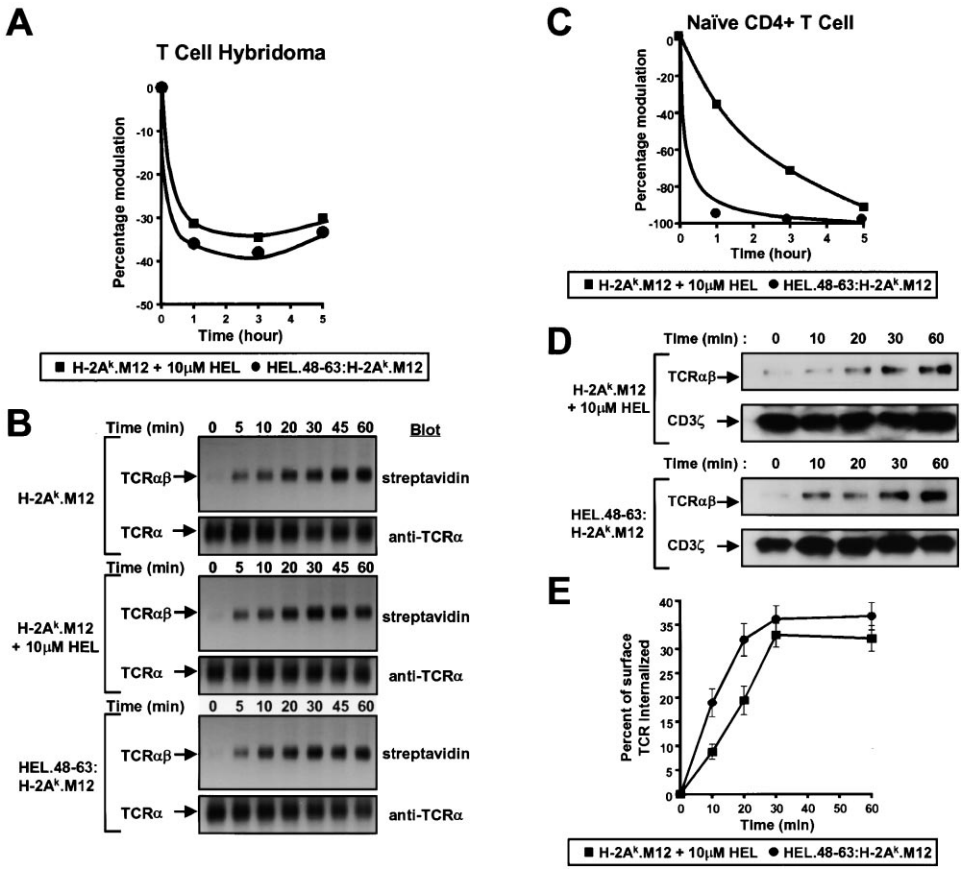


Figure 5. Rapid Downmodulation but Only Slightly Accelerated Internalization of TCR:CD3 Complexes following Stimulation with High MHC:HEL Peptide Complex Densities

(A) Downmodulation of surface TCR:CD3 on the 3A9 T cell hybridoma following stimulation with either H-2A^k.M12 prepulsed with 10 μM HEL or HEL.48-63:H-2A^k.M12 was determined by flow cytometry. Data represent the mean of two experiments.

(B) TCR:CD3 internalization was determined by the accumulation of intracellular biotinylated TCR as described in Figure 3. T cells were cultured with H-2A^k.M12, H-2A^k.M12 prepulsed with 10 μM HEL, or HEL.48-63:H-2A^k.M12. Data are representative of three experiments.

(C) Downmodulation of surface TCR:CD3 on naive T cells from 3A9 TCR transgenic mice following stimulation with either H-2A^k.M12 prepulsed with 10 μM HEL or HEL.48-63:H-2A^k.M12 was determined by flow cytometry. Data represent the mean of two experiments.

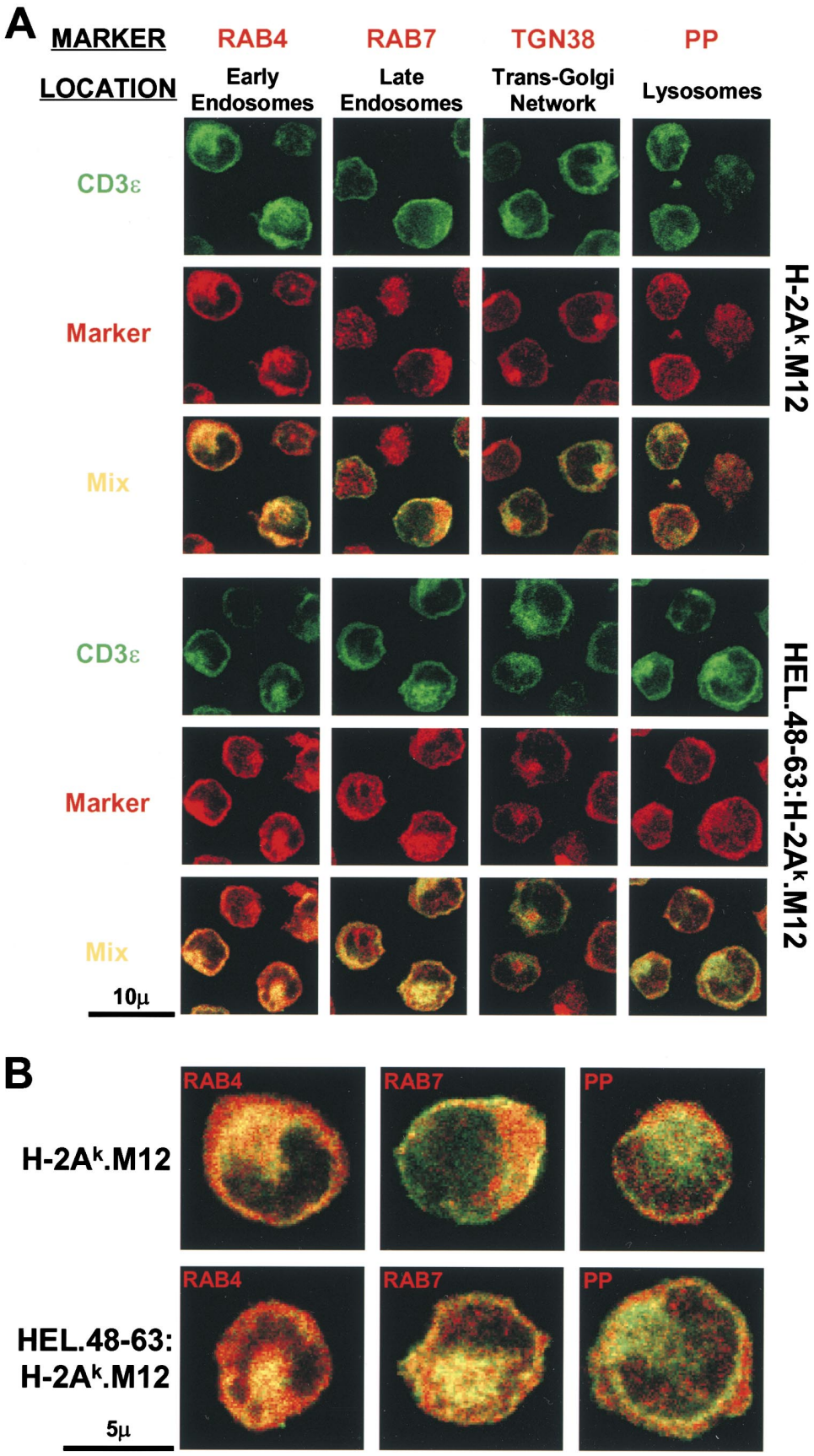
(D) TCR:CD3 internalization was determined by accumulation of intracellular biotinylated TCR as described in Figure 3. T cells were incubated with either H-2A^k.M12 pulsed with 10 μM HEL or HEL.48-63:H-2A^k.M12. Data are representative of three experiments.

(E) Densitometric analysis is the mean of three experiments. The data are calculated as described in Figure 3.

hypothesize that the intracellular sorting machinery can distinguish between ligated and un-ligated TCR complexes, such that the former are retained intracellularly, while the latter recycle back to the cell surface. Previous studies in human T cell clones have suggested that internalized CD3ζ is targeted to lysozomes for degradation (Valitutti et al., 1997). However, it is unclear how much of the intracellular TCR is actually degraded in lysozomes and whether other mechanisms exist.

To investigate this, we first analyzed the intracellular location of TCR:CD3 complexes by confocal microscopy, using an anti-CD3ϵ mAb and polyclonal Abs against markers for early endosomes (Rab4), late endosomes (Rab7), the trans-Golgi network (TGN38), and lysozomes (PP, protective protein). In unstimulated T cells, a significant proportion of the intracellular CD3ϵ appears to colocalizes with Rab4$^+$ early endosomes (Figure 6). Essentially, no CD3ϵ was seen in either Rab7$^+$ late endosomes, the TGN, or lysozomes. After antigen stimulation, virtually all of the CD3ϵ colocalized with Rab4$^+$ early endosomes. Although some CD3ϵ was observed in Rab7$^+$ late endosomes, none was seen in the TGN or lysosomes. The absence of CD3ϵ in the latter was surprising, given that a previous study with human T cells had shown that CD3ζ colocalizes with Lamp-1 vesicles 2 hr after antigen stimulation (Valitutti et al., 1997). In their study, T cells were pretreated with bafilomycin A1, which inhibits lysosomal function. Thus, it is possible that, in the absence of such inhibitors, the TCR is degraded too rapidly to be visualized. Alternatively, given that the intensity of CD3ϵ staining is relatively high compared with unstimulated cells, a significant proportion of the TCR complexes may not be degraded or recycled back to the cell surface but instead may be retained in Rab4$^+$ vesicles. It should be noted that a precise determination of how the intracellular transport routes of ligated and unligated TCR differ will require the use of higher resolution techniques that more effectively resolve the subcompartments of the endocytic pathway.

The confocal data raised the question of where TCR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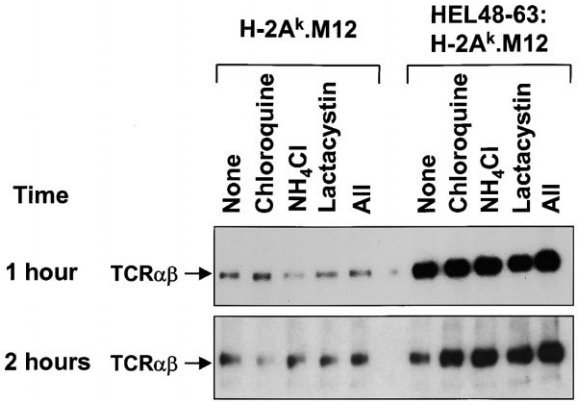

Figure 7. Ligated TCR:CD3 Complexes Are Degraded in Both Late Endosomes/Lysosomes and Proteasomes

TCR:CD3 internalization in splenocytes from 3A9 TCR transgenic mice was determined, as described in Figure 4, in the presence or absence of chloroquine (200 μM), NH_4Cl (10 mM), and/or lactacystin (20 μM). Note that the difference observed between the untreated, antigen-stimulated, and unstimulated controls is consistent with data in Figure 4. Data are representative of three experiments.

is degraded. This was addressed by treating resting and antigen-stimulated T cells with chloroquine and/or NH_4Cl, which block endosomal/lysosomal acidification and function, and lactacystin, a 26S proteasome inhibitor (Fenteany et al., 1995). As expected, this treatment had no effect on resting cells (Figure 7), which is consistent with the long-term stability of the TCR, as revealed by experiments with cycloheximide (Figure 1). In contrast, there was a significant increase in the amount of biotinylated TCR in antigen-stimulated T cells treated with chloroquine and NH_4Cl, which is consistent with a proportion of the TCR:CD3 complexes being degraded in late endosomes/lysozomes (Figure 7). It has been shown that these reagents can also block endocytosis; however, the cocktail of protease inhibitors was also found to increase in the amount of biotinylated TCR (data not shown). Surprisingly, increased biotinylated TCR was also observed in the presence of lactacystin, implicating the cytosol-resident proteasome in TCR degradation. There did not appear to be an additive effect when all three drugs were combined. Notwithstanding technical issues, it is possible that lysosomes and proteasomes are involved in degrading different components of the same complex. For instance, it is conceivable that the TCRαβ chains are degraded by lysosomes, but this degradation cannot occur unless CD3ζ is ubiquitinated (Hou et al., 1994) and degraded by proteasomes. In this instance, the effects of the drugs would not be additive. Taken together, these data suggest that there are three processes that collectively prevent TCR recycling back to the cell surface following antigen stimulation: intracellular retention, degradation in late endosomes/lysozomes, and degradation in the cytosol by proteasomes. Further studies will need to be performed in order to quantify the relative contributions of each of these mechanisms.

Discussion

In this study, we examined the dynamics of TCR cell surface expression and downmodulation on resting and activated T cells. Using two different experimental approaches, we have shown that the TCR:CD3 complex is long lived and recycles on resting T cells, confirming previous reports (Krangel, 1987; Minami et al., 1987). Our data also imply that this is an active process that requires motifs in the CD3 cytoplasmic tails, one of which is a tyrosine-based motif in CD3ζ.

A number of studies have suggested that ligation by MHC:peptide complexes induces TCR internalization and a subsequent downmodulation of cell surface expression (Valitutti et al., 1995; Bachmann et al., 1997; Valitutti et al., 1997; Hemmer et al., 1998; Itoh et al., 1999). Given the rapid rate of TCR internalization, we questioned whether this could be increased upon ligation. Indeed, we were surprised to find that, under physiological conditions, the rate of TCR internalization was not accelerated by MHC:peptide ligation. Although a very small increase was observed with extremely high ligand densities, this could not account for the substantial loss of TCR cell surface expression. Thus, the induction of internalization cannot be the primary mechanism of mediating TCR downmodulation. Alternatively, we propose that ligation induces changes in the complex that can be interpreted by the intracellular vesicular machinery, resulting in the redirection/retention of ligated complexes, thus preventing their return to the cell surface.

How do T cells distinguish between ligated and unligated complexes? TCR ligation causes phosphorylation of tyrosine residues in the ITAMs and a serine residue in the CD3γ chain (Cantrell et al., 1987; Weiss and Littman, 1994). This could mediate the binding of molecules that cause the redirection/retention of ligated complexes. Furthermore, it has been shown that the ectopic expression of a dominant active tyrosine kinase, $p56^{lck}$, results in TCR downmodulation (D'Oro et al., 1997). However, studies with PKC inhibitors and Jurkat mutants have suggested that serine phosphorylation of CD3γ is not involved in ligand-mediated TCR downmodulation (Salio et al., 1997; Lauritsen et al., 1998).

An alternative possibility is that TCR ligation induces a conformational change in, or physical dissociation of, the TCR:CD3 complex, revealing motifs that mediate

Figure 6. TCR:CD3 Complex Is Predominantly Present in the $Rab4^+$ Early Endosomes in Resting and Stimulated T Cells

(A) 3A9 T cell hybridoma was incubated with either H-$2A^k$.M12 or HEL.48-63:H-$2A^k$.M12 at 37°C for 1 hr. Confocal microscopy was performed using an anti-CD3ε mAb (green) and polyclonal Abs against markers (all in red) for early endosomes (Rab4), late endosomes (Rab7), the trans-Golgi network (TGN38), and lysozomes (PP, protection protein). Colocalization of CD3 and the markers is yellow/orange in the "mix" panels. The panels shown are representative of all T cells examined. In some panels, the marker-positive, CD3ε-negative B cells can be seen (middle/top cell in the Rab7/H-$2A^k$.M12 panel).

(B) An enlarged view of selected "mix" panels is presented. Scale bars are also shown.

the redirection/retention of ligated receptors. Two motifs have previously been shown to act as potent degradation signals: the YXXØ motifs within the ITAMs and a dileucine motif in the CD3γ and CD3δ cytoplasmic domains (Johnson and Kornfeld, 1992; Dietrich et al., 1994). More recently, CD3ε has been found to contain an endocytosis signal (Borroto et al., 1999). While it has been shown that cells that lack the cytoplasmic domains of both CD3γ and CD3δ fail to downmodulate the TCR following anti-CD3 stimulation, downmodulation in response to MHC:peptide ligation was not blocked (Luton et al., 1997a; Legendre et al., 1999). These motifs could be revealed following a conformational change or physical dissociation of the complex upon ligation. Interestingly, we have recently found that TCR ligation induces the physical separation of the CD3ζ chain from the rest of the TCR:CD3 complex (H. L., A. Szymczak, M. R., D. L. W., and D. A. A. V., unpublished data). While the CD3ζ remains on the cell surface, the TCRαβCD3εγεδ complex is internalized. We and others have shown that CD3ζ is required to maintain stable TCR cell surface expression (Dietrich and Geisler, 1998; H. L., A. Szymczak, and D. A. A. V., unpublished data). Furthermore, data have emerged for a dynamic relationship between CD3ζ and the rest of the TCR:CD3 complex, such that CD3ζ is transported to the Golgi independently of the rest of the TCR complex and is freely exchanged with newly synthesized CD3ζ at the cell surface (Ono et al., 1995; Dietrich et al., 1999). Thus, our model for the molecular mechanism of TCR downmodulation occurs in two stages: first, TCR ligation induces the separation of CD3ζ from the rest of the complex, and, second, these partial complexes reveal motifs that are recognized by the intracellular vesicular machinery and are retained and/or redirected for degradation. Therefore, ligation causes an intracellular prevention of TCR recycling rather than an induction of TCR internalization. The relative contribution of these motifs (known and unknown) to recycling and downmodulation has yet to be determined.

In this study, we addressed two other important issues: intracellular location and the fate of ligated and unligated TCR:CD3 complexes. Rapidly recycling proteins such as TfR are typically transported through a periplasmic/peripheral recycling pathway, which predominantly consists of $Rab4^+$ vesicles (Mellman, 1996). Our studies have suggested that the TCR:CD3 complex is predominantly found in $Rab4^+$ vesicles, which is consistent with previous findings demonstrating the presence of TCR in early endosomes (Luton et al., 1997b). Although a small proportion of TCR is associated with $Rab7^+$ late endosomes, none was detected in lysosomes. However, evidence was obtained for the degradation of TCR:CD3 complexes in lysosomes, consistent with previous observations (Valitutti et al., 1997).

Surprisingly, a proportion of the internalized TCR was also degraded in the cytosol by proteasomes, as evidenced by inhibition with lactacystin. While this was an unexpected result, there is increasing support for the role of proteasomes in the degradation of transmembrane proteins (Lord, 1996; Brodsky and McCracken, 1999). Interestingly, recent studies have shown that unpaired TCRα and CD3δ chains are translocated from the ER into the cytosol where they are ubiquitinated and degraded by proteasomes (Huppa and Ploegh, 1997; Yang et al., 1998). It has also been shown that the CD3ζ and CD3δ chains are ubiquitinated in a tyrosine kinase–dependent manner following TCR ligation (Cenciarelli et al., 1992, 1996). Despite the fact that lactacystin is a remarkably selective drug (Fenteany et al., 1995), we cannot rule out the possibility that degradation in lysosomes is affected. However, it has been shown that lactacystin has no effect on the lysosomal degradation of endocytosed proteins (Craiu et al., 1997). Taken together, these data suggest that there are multiple mechanisms by which ligated TCR:CD3 complexes are prevented from returning to the cell surface. Further experiments will be required to determine precisely how and to what degree each of these mechanisms contribute to the TCR retention/degradation process.

What is the physiological role of TCR:CD3 recycling and downmodulation? As the rate of TCR internalization is similar with or without ligation, it seems unlikely that internalization per se is a prerequisite for T cell signal transduction and activation. Furthermore, the significant time lag between tyrosine phosphorylation of the TCR complex and surface downmodulation also suggests that the latter is not required for T cell activation. Indeed, several groups have failed to find a correlation between TCR downmodulation and T cell responses (Blichfeldt et al., 1996; Cai et al., 1997). Our studies have shown that APCs pulsed with antigen or transfected with MHC molecules containing recombinantly attached peptides give rise to significantly different rates of TCR downmodulation but comparable levels of IL-2 production (Carson et al., 1997; H. L. and D. A. A. V., unpublished data).

Given the possibility that the same tyrosine motifs may mediate both recycling and signal transduction, it is possible that recycling is a necessary consequence of having at least some of the tyrosine residues accessible for phosphorylation. We would suggest that the rapid rate of internalization is required to drive serial ligation and facilitate the release of MHC:peptide complexes by TCR (Valitutti et al., 1995). Finally, we believe that the most likely function of TCR downmodulation is to protect T cells from overstimulation (Cai et al., 1997). By analogy, it has been shown that TCR downmodulation is an effective means of tolerance to an extrathymically expressed MHC class I transgene (Schonrich et al., 1991). It is also conceivable that the lack of cell surface TCR may limit the extent of antigen-induced cell death (AICD) caused by T cell restimulation prior to a sufficient resting period.

Experimental Procedures

Downmodulation and Flow Cytometry

The assay for downmodulation of TCR:CD3 was performed as described previously (Vignali and Vignali, 1999). In brief, the 3A9 T cell hybridoma (Allen et al., 1985) (5×10^4; 100 μl) or splenocytes from 3A9 TCR transgenic mice (gift from Mark Davis, Stanford University, Palo Alto, CA) (Ho et al., 1994) (5×10^5; 100 μl) were cultured in flat-bottom 96-well microtiter plates (in quadruplicate for each time point) with one of the following: (1) H-$2A^k$.M12 (M12.C3.F6; Nelson et al., 1992) ± 10 μM HEL overnight prepulse; (2) HEL.48-63:H-$2A^k$.M12 (Carson et al., 1997); or (3) BFA (10 μg/ml; Epicenter Technologies, Madison, WI) and/or cycloheximide (50 μg/ml; Sigma Chemical, St. Louis, MO). At the time points indicated, cells were transferred to V-well microtiter plates for flow cytometry. The efficiency of cycloheximide treatment was verified by metabolic label-

ing with [^{35}]S-methionine and measuring the reexpression of CD4 on pronase-treated cells (Wiest et al., 1997).

Flow cytometry was performed, as detailed previously, using a FACScan or a FACSCalibur (Becton Dickinson, San Jose, CA) (Carson et al., 1997; Vignali and Vignali, 1999). Cells were stained with one of the following FITC-labeled mAbs: anti-CD3ε, anti-CD4, anti-CD45, anti-CD71/TfR, anti-CD90/Thy-1, or anti-H-2K^k (PharMingen, San Diego, CA). Cells were stained with anti-H-2Aα^k-PE to gate out B cells and with PI for live/dead cell gating (T cells were >90% viable in all experiments). In experiments with splenocytes, cells were also stained with anti-CD4.APC to gate in T cells. The percentage downmodulation of TCR:CD3 was determined from the median values, using the unstimulated controls as reference.

Transient Transfection of 293T Cells

293T cells (human embryonic kidney cells; provided by David Baltimore, Caltech, and Elio Vanin, SJCRH) were incubated in 10 cm plates at 2 × 10^6/plate overnight at 37°C. DMEM (470 μl) was mixed with 30 μl Fugene 6 transfection reagent (Roche, Indianapolis, IN) for 5 min at room temperature. The following plasmids were used: 3A9.TCRα, CD3γ, CD3δ, and CD3ε (wild-type and ΔCY) in pCI*neo* (Promega); 3A9.TCRβ in pCI*puro*; and CD3ζ (wild-type and ΔITAM) in pCI.IRES.GFP*neo*. DNA (2 μg of each chain) was mixed with Fugene/DMEM and incubated for 15 min at room temperature. The mixture was incubated with the cells overnight at 37°C and replaced with fresh medium the next day. On day 4, GFP/TCR/CD3$^+$ cells were sorted (stained with anti-TCR.Vβ8.1/2.PE and anti-CD3.APC) and cultured in 96-well flat-bottom plates at 10^4/well overnight. BFA was added at 10 μg/ml, and, at different time points, cells were stained with anti-TCR.Cβ.Cy-Chrome and TCR surface downmodulation determined as before.

Surface Biotinylation and MESNA Treatment

Cells were surface biotinylated as previously described (Smythe et al., 1992; Wiest et al., 1995). In brief, T cells (2 × 10^8/ml for splenocytes from B10.B10 or 3A9 TCR transgenic mice or 2 × 10^7/ml for hybridomas) were washed three times in HBSS and labeled with 1 mg/ml NHS-SS-biotin (Pierce Chemical, Rockford, IL) HBSS for 30 min on ice. Excess biotin was quenched and the cells washed three times with 25 mM lysine/HBSS. The T cells (2 × 10^7 per IP for splenocytes, 5 × 10^6 per IP for hybridomas) were cultured at 37°C either alone or with APC ([1] LK35.2 [murine B cell lymphoma; H-2A^{kd}] ± 10 μM HEL overnight prepulse, [2] H-2A^k.M12 ± 10 μM HEL, [3] HEL.48-63:H-2A^k.M12; all at 5 × 10^6 per IP). In certain experiments, cells were incubated with a proteasome inhibitor (lactacystin, 10 μM; Kamiya Biomedical Company, Seattle, WA) and/or lysosomal degradation inhibitors (NH_4Cl, 10 mM; and chloroquine, 200 μM; Sigma). At each time point, cells were treated three times with 2-mercaptoethanesulfonic acid (MESNA) (10 mM; Sigma) on ice for 30 min. In some experiments, cells were treated with 200 mM MESNA on ice for 5 min. Cells were then washed with iodoacetamide (IAA) (Sigma) twice before lysis in 1% Brij97 (polyoxyethylene 10 oleyl ether; Sigma).

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were performed as previously described (Vignali and Strominger, 1994; Liu and Vignali, 1999). In brief, cells were lysed with 1% Brij 97 and immunoprecipitated with anti-TCRβ H57-597 precoated protein G-sepharose. Eluted proteins were resolved by SDS-PAGE, and blots were probed with streptavidin-HRP and then developed using ECLplus (Amersham). Blots were then stripped and reprobed with either anti-CD3ζ (H146-968) or anti-TCRα (H28-710) (gift from Ralph Kubo, Cytel Corp., San Diego, CA), followed by protein A-HRP. Densitometric analysis was performed with QuantityOne (Bio-Rad, Hercules, CA).

Confocal Microscopy

The 3A9 T cell hybridoma (5 × 10^5 /ml) was incubated with either H-2A^k.M12 or HEL.48-63:H-2A^k.M12 (5 × 10^5 /ml) in a 6-well plate at 37°C for 1 hr prior to staining. Cells were washed twice with PBS, centrifuged onto slides using a Cytospin 2 (Shandon, Pittsburgh, PA), and fixed with 3.7% paraformaldehyde. The cells were permeablized with 0.2% saponin and then blocked with anti-CD16/CD32 (FcγIII/IIR, Fc Block; PharMingen) in 5% normal mouse serum in PBS for 30 min. Cells were then incubated (45 min in humidified chamber at 37°C) with a biotinylated anti-CD3ε mAb (500A2; PharMingen) and rabbit polyclonal Abs against either Rab4 (gift from Marino Zerial, EMBL, Heidelberg, Germany; Cormont et al., 1996), Rab7 (gift from Marino Zerial; Chavrier et al., 1990), TGN38 (gift from George Banting, Bristol University, England; Luzio et al., 1990), or protective protein (PP; gift from Alessandra D'Azzo, St. Jude Children's Research Hospital; Rottier et al., 1998). Cells were stained with streptavidin-Alexa 488 and anti-rabbit Ig-Alexa 594 (Molecular Probes, Eugene, OR) for 45 min at 37°C. Slides were mounted using Aqua Poly/Mount (Polysciences, Inc., Warrington, PA) and analyzed on a Leica TCS NT SP confocal microscope (Leica, Germany). Images were taken using a 100× lens with 2× zoom at a resolution of 1024 × 1024 (8× scan). The optical thickness (z section) was 0.4 μ. Data were processed using Leica TCS NT (Leica, Germany) and Adobe PhotoShop (Adobe, San Jose, CA).

Acknowledgments

We are very grateful to George Banting, Alessandra D'Azzo, Ralph Kubo, Jose Moreno, and Marino Zerial for antibodies, Mark Davis and William Walker for the 3A9 TCR transgenic mice, Elio Vanin and David Baltimore for 293T cells, and Emil Unanue for M12.C3.F6. We also wish to thank Gopal Murti and the Scientific Imaging Shared Resource for assistance with the confocal microscopy and members of the Vignali lab for constructive criticisms and comments. D. A. A. V. is supported by the National Institutes of Health (AI-39480), a Cancer Center Support CORE grant (5 P30 CA21765-17), and the American Lebanese Syrian Associated Charities (ALSAC).

Received May 16, 2000; revised September 28, 2000.

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