An orderly inactivation of intracellular retention signals controls surface expression of the T cell antigen receptor

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Exit from the endoplasmic reticulum (ER) is an important checkpoint for proper assembly of multimeric plasma membrane receptors. The six subunits of the T cell receptor (TCR; TCRα, TCRβ, CD3γ, CD3δ, CD3ε, and CD3ζ) are each endowed with ER retention/retrieval signals, and regulation of its targeting to the plasma membrane is therefore especially intriguing. We have studied the importance of the distinct ER retention signals at different stages of TCR intracellular assembly. To this end, we have characterized first the presence of ER retention signals in CD3ε. Despite the presence of multiple ER retention signals in CD3γ, εγ dimers reach the cell surface when the single CD3ε ER retention signal is deleted. Furthermore, inclusion of this CD3ε mutant promoted plasma membrane expression of incomplete αβγε and αβδε complexes without CD3ζ. It therefore appears that the CD3ε ER retention signal is dominant and that it is only overridden upon the incorporation of CD3ζ. We propose that the stepwise assembly of the TCR complex guarantees that all assembly intermediates have at least one functional ER retention signal and that only a full signaling–competent TCR complex is expressed on the cell surface.

Multimeric plasma membrane protein complexes typically assemble early in the secretory pathway, in parallel with the synthesis and folding of individual subunits in the ER. Exit from the ER is an important checkpoint for many such complexes, as only fully assembled complexes are allowed to pass this control step (1). ER retention/retrieval signals (herein referred to as ER retention signals) are present in some receptor subunits, and the assembly of complexes is thought to mask the ER retention signal, permitting targeting to the membrane of the fully assembled complexes (2). The TCR is an especially complex model because it is formed by six different subunits that all contain ER retention determinants.

In the TCR, the TCRα and TCRβ subunits (or TCRγ and TCRδ in γδ T cells) are responsible for the recognition of the MHC/antigen ligand. These are noncovalently bound to the signal-transducing subunits CD3γ, CD3ε, CD3δ, and CD3ζ (the CD3ζ subunit is CD247). During assembly, CD3ζ first dimerizes with either CD3γ or CD3δ, and the resulting εγ and δζ dimers associate with the TCRα and TCRβ subunits (3–5). The resulting αβγε or αβδε complexes are either retained in the ER or degraded in lysosomes (6). Only when CD3ζ is incorporated into the complex is the TCR transported to the plasma membrane (6–8). In this way, expression of signal-transducing subunits independent of ligand-binding subunits or vice versa is tightly controlled. Indeed, in T cell mutants and knockout mice lacking TCRβ, CD3ε, or CD3ζ, TCR expression is severely impaired (9–12). In addition, T cell precursors in RAG-deficient and SCID mice that are unable to express the TCR gene subunits have a very low level of CD3ε, and the CD3ε, δζ dimer expression at the cell surface (13, 14), and their development is arrested at the most immature CD4−CD8− stage.

Removal of the ER retention signal in the cytoplasmic tail of CD3ζ permits this subunit to reach the cell surface by itself (15, 16). This signal consists of an elongated α helix followed by a βI’ turn and contains three important, closely spaced residues: tyrosine, leucine, and arginine. The presence of arginine in the ER retention signal is characteristic of type II proteins despite
the fact that CD3ε is a type I membrane protein (17–19). Other ER retention signals in the TCR have not been analyzed in detail, although TCRα contains an ER retention signal in its transmembrane region (20) and TCRβ in both its extracellular and transmembrane domains (21). With regard to the other CD3 subunits, CD3γ has a conserved arginine residue in position −3 from the COOH terminus, and CD3β has either an arginine or a lysine residue at the same position. Their removal from Tacγ and Tacδ chimeras disrupts ER retention (22).

In addition to ER retention signals, binding of incompletely folded subunits and complexes to chaperonins such as calnexin can also influence ER retention (23). Moreover, endocytosis signals in several subunits of the TCR complex offer a further level of regulation (22, 24, 25). The CD3γ subunit contains an important double leucine signal for endocytosis that is hidden in the complete TCR complex but unmasked by PKC-mediated phosphorylation of an upstream serine (26). In partial complexes, this double leucine signal is constitutively exposed and only masked upon integration of CD3ξ into the TCR complex (27, 28).

During assembly, all individual ER retention determinants in TCR subunits must be annulled before the TCR complex can be transported to the plasma membrane. The ER retention determinants may become progressively overridden as the TCR complex assembles or alternatively, all determinants might become inoperative at once, when all the TCR subunits are assembled. To study this process, we have characterized the ER retention signals in CD3γ and analyzed the predominance of CD3γ and CD3ε signals in the εγ dimer. All the determinants in CD3γ are overridden when it assembles with CD3ε. However, the single ER retention signal in CD3ε remains active in the εγ dimer and only becomes inoperative upon completion of the last assembly step, i.e., the incorporation of CD3ξ. These results support a model of sequential inactivation of ER retention signals during stepwise assembly.

RESULTS
The cytoplasmic tail of CD3γ contains multiple intracellular trafficking signals
To identify ER retention signals in the cytoplasmic tail of CD3γ, a chimeric protein containing the CD3γ cytoplasmic tail appended to the human CD4 extracellular and transmembrane domains was generated (chimera 44γ; Fig. 1 A). The chimera was transfected into COS cells, and cell surface expression was analyzed by flow cytometry. Expression of the 44γ chimera at the cell surface was 2.5-fold lower than that of wild-type CD4 (Fig. 1 B). Furthermore, unlike CD4, the 44γ chimera was predominantly located in the ER of transfected COS cells, with a similar distribution to CD3γ (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041133/DC1). These results indicated that the cytoplasmic tail of CD3γ contains an ER retention signal.

To characterize this putative ER retention signal, five deletion mutants of the 44γ chimera were generated and transfected into COS cells (Fig. 1 A). Deletion of the three COOH-terminal amino acids (44γΔ1) promoted a moderate increase in surface expression of the chimera (Fig. 1 B). This effect was slightly accentuated when five or nine additional amino acids were deleted from the COOH-terminal end (44γΔ2 and 44γΔ3). In contrast, deletion of seven more amino acids (44γΔ4) provoked an important reduction in 44γ expression (Fig. 1 B), suggesting that a signal that facilitates transport to the plasma membrane might reside between amino acids 142 and 148. Finally, deletion of most of the cytoplasmic tail, including the double leucine endocytotic signal, enhanced surface expression to above the levels of wild-type CD4. This could be due to the 44γΔ5 mutant lacking not only the double leucine endocytotic signal of CD3γ, but also that of the cytoplasmic tail of CD4 (29). The increase in surface expression of the mutant chimeras was coincident with a redistribution of the chimera to the Golgi apparatus, plasma membrane, and vesicular structures (Fig. S1).

The fact that some 44γ chimera was detected at the plasma membrane might be due to excessive protein expression in transfected COS cells, thereby overriding the ER retention machinery. Hence, we studied the surface expression of the 44γ chimera in stable transfectants of the human NK cell line YT. Several cell clones with each construct were studied to exclude clonal effects. Only the mutant 44γΔ5 was expressed at the cell surface at a level that matched that of wild-type CD4 (Fig. 1 C). There was only a slight increase in surface expression of chimeras 44γΔ1 through 44γΔ3 and a marginal reduction of 44γΔ4.

We determined how the rate of exit from the ER was affected in these 44γ mutants. Human CD4 has two N-glycosylation sites, one of which is converted to the complex type in the mature protein (30). Hence, we assessed the acquisition of partial endo-H resistance of the 44γ mutants after metabolic labeling of the COS cells. Export from the ER of the 44γΔ1 and 44γΔ2 chimeras was accelerated twofold. These mutants required 50 min to acquire 50% endo-H resistance (t1/2 = 50) compared with the 95 min for wild-type 44γ (Fig. 1, D and E). Deletion of further amino acids reduced the exit rate to t1/2 = 115 min for 44γΔ3, whereas the ER export of 44γΔ4 and 44γΔ5 was dramatically reduced (t1/2 >> 120 min). Thus, it seemed clear that signals other than those for ER retention also regulate the level of 44γ chimera surface expression.

The cytoplasmic tail of CD3γ contains a di-leucine endocytotic motif (Fig. 1 A; reference 25) and a putative DxE ER export signal (31). The combination of ER retention, export, and endocytosis is ultimately responsible for the surface expression of the 44γ mutants. Deletion of the ER retention signal in the COOH-terminal end of the chimera might explain the accelerated rate of ER export for 44γΔ1 and 44γΔ2, whereas removal of the putative DxE ER export signal would explain the decrease in ER export of 44γΔ4 and 44γΔ5. The diminished ER exit of 44γΔ3 compared with 44γΔ2 might be due to a positional effect on the
DxE signal. Nevertheless, it seems that the di-leucine endocytotic signal is mainly responsible for regulating the surface levels of the 44γ/H9253 chimera. This could explain why only 44γ/H9004 is highly expressed at the surface despite the reduced rate of ER export (Fig. 1, B, C, and E).

**CD3γ contains ER retention determinants in its extracellular, transmembrane, and cytoplasmic domains**

To further characterize this ER retention signal and to determine the impact this signal has on 44γ chimera surface expression, point mutations of the last three amino acids of the 44γ were introduced. To avoid interference from the di-leucine internalization signal, leucine 131 was replaced by alanine. Expression of the double mutants at the cell surface was analyzed in transfected COS cells and in stable YT transfectants. In both cell types, mutation of the di-leucine motif alone (44γL131A mutant) caused a two- to sixfold increase in surface expression of the 44γ chimera (Fig. 2 A). Replacement of arginine 158 with alanine (44γL131A/R158A mutant) resulted in an additional two- to sixfold increase in surface expression of the 44γ chimera (Fig. 2 A).
cells) to fourfold (YT cells) increase in surface expression. In contrast, mutation of the other two COOH-terminal amino acids (44Y_{L131A/N160A} and 44γ_{L131A/R159A} mutants) did not have a major impact on cell surface expression. The effect of arginine 158 mutation was also reflected in the cellular redistribution of this 44γ mutant (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20041133/DC1). Thus, of the three amino acid residues deleted in mutant 44γΔ1, only arginine 158 seems to be important for ER retention of 44γ.

Once an ER retention signal had been identified in the cytoplasmic tail of CD3γ, we evaluated the role of this signal in the context of the whole CD3γ molecule. Surprisingly, mutation of arginine 158 to alanine in CD3γ did not prevent ER retention, because the mutant was exclusively located in the ER when examined by confocal microscopy (Fig. 2 B). Nor was CD3γ redistributed to the plasma membrane when an additional mutation in the di-leucine motif was introduced (Fig. 2 B, γ_{L131A/R158A}). These results suggested that CD3γ contains other ER retention determinants in addition to the COOH-terminal signal. Furthermore, a truncated CD3γ with only the first two cytoplasmic amino acids of CD3γ was still retained in the ER in COS cells, suggesting that ER retention determinants were present in the extracellular and/or transmembrane domains (Fig. 2 B, γTr). Indeed, two new domain shuffle chimeras were both retained in the ER: γ44, with the extracellular domain of CD3γ, and 4γ4, with the CD3γ transmembrane domain (Fig. 2, C and D).

Expression of the CD4/CD3γ chimeras and of the CD3γ point mutants was also evaluated by flow cytometry. Surface expression of the single di-leucine motif mutant of CD3γ (γ_{L131A}) was slightly higher than the wild-type CD3γ but lower than the mutant in the cytoplasmic ER retention signal (Fig. 2 E, γ_{R158A}). The double mutation of the cytoplasmic ER and endocytotic signals (γ_{L131A/R158A}) acted synergistically to increase the surface expression of CD3γ. Nevertheless, all CD4-CD3γ chimeras and CD3γ mutants were expressed at lower levels than CD4 (Fig. 2 E), further indicating that ER retention determinants reside not only in the cytoplasmic tail of CD3γ, but also in the transmembrane and extracellular domains. These extracellular and transmembrane domain retention determinants in CD3γ could represent distinct sequence motifs or an unfolded state of the protein. In any case, it appears that in contrast to CD3ε, which contains a single ER retention signal (15), retention of free CD3γ is regulated by multiple signals. It therefore seems that the expression of CD3γ on the cell surface is tightly regulated.

Dimerization with CD3ε abolishes all ER retention determinants in CD3γ

One of the earliest steps in TCR assembly is the dimerization of CD3ε with either CD3γ or CD3δ (3, 4, 6). The resulting εγ and εδ dimers are retained in the ER unless they assemble with TCRα, TCRβ, and CD3ζ. Because both
CD3γ and CD3ε contain ER retention signals, we examined the relative contribution of each signal to dimer retention. COS cells were cotransfected with the CD3γ mutants and CD4 chimeras (refer to Fig. 2) and either wild-type CD3ε or a deletion mutant of CD3ε lacking its single ER retention signal (εmut; reference 15). The 4γ4 chimera was excluded from this study because it lacks the extracellular domain of CD3γ necessary for assembly with CD3ε (32). Cellular distribution of the dimers was distinguished from that of the single chains by immunostaining with a CD3 mutant-specific antibody, UCHT1 (33). When associated with wild-type CD3ε, all CD3γ chimeras and mutants were located in the ER, independent of the presence of CD3γ ER retention signals (εmut; reference 15). The 4γ4 chimera was excluded from this study because it lacks the extracellular domain of CD3γ necessary for assembly with CD3ε (32). Cellular distribution of the dimers was distinguished from that of the single chains by immunostaining with a CD3γ mutant-specific antibody, UCHT1 (33). However, transfection of εmut resulted in export of the 4γ dimers from the ER and targeting to the plasma membrane (Fig. 3, A and B). The same effect was seen when wild-type CD3δ and εmut were expressed in COS cells (not depicted).

In the absence of the CD3ε ER retention signal, the 4γ dimer was transported to the cell surface regardless of the CD3γ cytoplasmic ER retention signal (compare εR158A with γ in Fig. 3 B). However, mutation of the CD3γ di-leucine endocytosis signal increased cell surface expression of the 4γ dimer fourfold (Fig. 3 B, γL131A). Expression did not increase further when the cytoplasmic ER retention signal of CD3γ was mutated as well as the endocytotic signal (γL131A/R158A), nor in the absence of the whole cytoplasmic tail (γtr), nor in the absence of both the transmembrane and cytoplasmic domains (γ44).

These results suggest that the only functional ER retention signal in the 4γ dimer is that in CD3γ and all ER retention determinants in CD3γ are abrogated upon dimerization with CD3ε. Therefore, the ER retention determinants in CD3γ do not seem to play a major role in regulating 4γ expression at the cell surface. However, CD3γ does contribute to this task by mediating the endocytosis of the 4γ dimer via its di-leucine signal.

The single ER retention signal of CD3ε is only overridden during the last assembly step

Once 4γ (or 8δ) dimers are assembled in the ER, they associate with the TCRα and TCRβ chains to form αβγε and
αβδε complexes. These incomplete TCR–CD3 complexes remain in the ER or are degraded in lysosomes. The TCR complex can only reach the plasma membrane when the ε subunit is incorporated (6–8, 34). Indeed, reconstruction of the TCR complex in HeLa cells showed that transfection of the ε subunit was sufficient to drive transport of the TCR complex to the cell surface (35). Bearing this in mind, we studied whether the CD3ε ER retention mutant promoted surface expression of the ε subunit. COS cells were transfected with plasmids encoding these subunits, but the γL131A mutant was used to avoid internalization of the αβγε complex. When transfected with the CD3ε ER retention mutant, both the CD3γε dimer and TCRβ were transported to the plasma membrane, even in the absence of ε (Fig. 4 A). Two-color flow cytometry with anti-TCRβ and anti-CD3ε antibodies was used to quantify the αβγε complex on the cell surface. This showed that the αβγε complex is expressed at a high level independent of ε (Fig. 4 B). In contrast, when wild-type CD3ε was transfected, TCRβ and the CD3γε dimer were predominantly found in the ER and were consequently practically undetectable at the cell surface (Fig. 4, A and B). Similar results were obtained when wild-type CD3γ instead of the di-leucine mutant was used, although surface expression of the αβγε complex reached lower levels (Fig. S3, available at http://www.jem.org/cgi/
Deletion of the ER retention signal in CD3ε is sufficient to allow surface expression of the TCR complex in ζ-deficient T cells. (A) Surface expression of TCR complexes in ζ-expressing and ζ-deficient T cells. The parental murine T cell hybridoma 2B4 and its ζ-deficient MA5.8 mutant were stably transfected with either wild-type human CD3ε or with human εmut. Cells were double stained with the anti-murine TCRβ antibody H57-597 and the anti-human CD3 antibody Leu4 and analyzed by two-color flow cytometry (top). Representative clones for each condition are shown. The result of single staining with anti–human CD3 antibody. Transfection of human CD3ε-expressing cells with murine TCR and CD3 chains are indicated.

To confirm that the CD3ε ER retention signal is also dominant in the complexes lacking CD3ζ (αβγε and αβδε) in ηmut cells, we transfected human wild-type CD3ε and human εmut into the CD3ζ-deficient mutant MA5.8 of the murine hybridoma 2B4 (36). Human CD3ε can assemble with murine TCR chains, and its cell surface expression can be followed by flow cytometry with anti-human CD3 antibodies (37). Stable transfecteds were analyzed by two-color flow cytometry using an anti-human CD3 dimer antibody and an anti-murine TCRβ antibody. Transfection of human wild-type CD3ε in ζ-sufficient 2B4 cells led to its incorporation into a sizeable population that was not observed in ζ-deficient MA5.8 cells (Fig. 5A). However, transfection of εmut led to its expression in both the ζ-expressing and ζ-deficient cell lines, although it was expressed less in the absence of CD3ζ. The level of human CD3ε expression on the cell surface was also examined with an anti-CD3ε antibody in flow cytometry and compared with its intracellular expression in detergent-permeabilized cells. Wild-type CD3ε clearly failed to reach the cell surface in ζ-deficient cells despite the intracellular accumulation of the protein (Fig. 5A). Finally, all the transfecteds were surface biotinylated and the human CD3ε-containing complexes were recovered with an anti-human CD3 antibody. The immunoprecipitates were resolved by two-dimensional SDS-PAGE under non-reducing/reducing conditions and showed that εmut was indeed expressed at the cell surface and was associated with the CD3ζ into the αβγε complex.

Expression of TCR complexes containing the human CD3ε ER retention mutant was clearly lower in ζ-deficient cells (Fig. 5B). Wild-type human CD3ε was again not detected in ζ-deficient cells. Further, the results confirm that the CD3ε ER retention signal prevents cell surface expression of the incomplete αβγε and αβδε complexes and suggest that...
this signal becomes inoperative only upon completion of the last assembly step with the incorporation of CD3ζ.

Masking of the CD3ε ER retention signal upon CD3ζ assembly is position dependent

A prediction of the stepwise model of ER retention signal annulment is that silencing of a given ER retention signal must be position specific; e.g., if CD3ζ assembly overrides the ER retention signal in CD3ε, this must occur in the context of the specific topological position of CD3ε in the TCR complex. To evaluate this hypothesis, we constructed two new CD3γ-ε chimeras. In one of the chimeras, the cytoplasmic tail of CD3γ was substituted by the tail of CD3ε (Fig. 6 A, γγε chimera). The other chimera was generated by appending the ER retention signal of CD3ε at the COOH-terminal end of CD3γ (Fig. 6 A, γret). Next, we examined whether assembly of the CD3γε chimeras with the ER retention mutant of CD3ε resulted in expression of the γε dimer at the cell surface. As previously demonstrated (Fig. 3), assembly of wild-type CD3γ with εmut but not with wild-type CD3ε allowed transport of the εγ dimer to the cell surface (Fig. 6 B). In contrast, assembly of the CD3γε chimeras γγε and γret with εmut prevented export of the εγ dimer to the plasma membrane (Fig. 6 B) and retained the dimer in the ER (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20041133/DC1). These results therefore show that abrogation of the ER retention signals during assembly of the εγ dimer is dependent on both the sequence of the cytoplasmic tail and the position of the ER retention signals within the dimer.

To extend these results to the full TCR complex, a CD3γε− mutant of Jurkat was transfected with wild-type CD3γ or with the γγε or γret chimeras, and stable clones were selected. Transfection of wild-type CD3γ but not the γγε or γret chimeras reconstituted the surface expression of the TCR complex at high levels (Fig. 6 C). These results show that if the CD3ε ER retention signal is misplaced (either at the position of the CD3γ tail or at the tip of CD3γ), CD3ζ assembly can no longer silence it.

DISCUSSION

In this study, we have tried to better understand the mechanisms that regulate expression of TCR–CD3 complexes at the cell surface and prevent nonassembled subunits and incomplete complexes from reaching or remaining in the plasma membrane. We found that removal of the CD3ε ER retention signal is sufficient to permit expression of εγ and εδ dimers at the surface of transfected COS cells. Indeed, even though CD3γ contains multiple ER retention signals, these signals all become inoperative upon assembly with seven clones with the highest TCR expression for each construct are represented at the bottom. Two-color flow cytometry histograms for the clones with highest TCR expression are shown at the top. The total number of clones analyzed was 42 for the wild-type CD3γε construct, 14 for the γret construct, and 48 for the γγε construct. NT, nontransfected.
Figure 7. Model of sequential inactivation of TCR retention signals. Surface expression of free CD3ε is prevented by a single ER retention signal (triangle), whereas expression of free CD3γ is prevented by several ER retention signals (triangles) and a di-leucine endocytosis signal (circle). CD3δ may contain at least one cytoplasmic ER retention signal similar to those of CD3γ and CD3ε (triangle) and a di-leucine endocytosis signal (circle). Upon assembly of εγ and εδ dimers, the ER retention determinants of CD3γ and CD3δ are overridden, but surface expression of the εγ and εδ dimers is prevented by the CD3ε ER retention signal that remains functional. The endocytosis signals in CD3γ (and probably CD3δ) remain active in the εγ and εδ dimers and are responsible for the internalization and removal of the small amounts of dimer that somehow reach the cell membrane on their own. TCRα and TCRβ contain additional ER retention determinants, but these do not seem to be operative in the εγεδ (and probably εδεζ) complex. Only after assembly of CD3ζ does the CD3ε ER retention signal become nonfunctional, thus allowing the full TCR–CD3 complex to reach the plasma membrane. The stability of the full complex on the cell surface is also increased because the CD3γ and CD3δ endocytic signals are inactive. Removing both the CD3ε ER retention signal and the CD3γ endocytic signal artificially increases surface expression of free εγ dimers and incomplete εγεδ complexes.

Studies in COS cells, because efficient cell surface expression of the εγεζζ complex with wild-type CD3ε was not observed. This might indicate that CD3δ is mandatory for surface expression. However, our transfection experiments (not depicted) and the results of Kappes and Tonegawa (35) suggest that this is not the case. Moreover, transfection of all TCR subunits in non–T cells is sufficient to detect the full TCR complex at the cell surface (35, 40). This discrepancy might be explained by the greater sensitivity of radioiodination (40) compared with the flow cytometry used here, or by the fact that the transfected population underwent selection for high TCR expression (35). However, the poor efficiency of ER export and the weak surface expression of the wild-type CD3ε-containing εγεζζ complex might be better explained by the inefficient assembly of CD3ζ into the complex. Nevertheless, the results in COS cells and the model (Fig. 7) have been validated in ζ-expressing and ζ-deficient murine T cells transfected with human CD3ε (Fig. 5). Human wild-type CD3ε is targeted to the plasma membrane within the TCR complex only after assembly of CD3ζ, whereas the human CD3ε ER retention mutant can reach the membrane with and without CD3ζ. Studies in CD3ζ-deficient mice and cell lines show that TCR–CD3 surface
expression is extremely low in the absence of this subunit (34, 41). Accordingly, our results show that the CD3ε ER retention signal remains functional in the incomplete αβγε complex, preventing cell surface expression. These results also indicate that the orderly inactivation of intracellular retention signals serves to not only prevent surface expression of the CD3 dimer (or TCR dimer), but also that of TCR–CD3 complexes lacking CD3ζ.

What then is the mechanism that overrides CD3ε’s ER retention signal upon assembly of ζ? It has been proposed that the cytoplasmic tail of CD3ζ hides the otherwise exposed di-leucine endocytosis motif in CD3γ by steric hindrance (28). Indeed, in ζ-deficient cells it has recently been demonstrated that the TCR is more rapidly internalized and that expression of CD3ζ, or a CD3ζ chimera with its cytoplasmic tail partially replaced by a foreign sequence, restores normal TCR internalization (27). Steric masking of the di-lysine ER retention motif in the α chain of the heterodimeric high affinity receptor for immunoglobulin E (FcεRI) upon assembly with the γ chain has been proposed to regulate plasma membrane targeting (2). Similarly, steric hindrance by the cytoplasmic tail of CD3ζ could be responsible for annihilating the CD3ε ER retention signal, although other mechanisms involving CD3ζ-dependent rearrangements of the TCR complex cannot be excluded. Interestingly, the γ chain of FcεRI and CD3ζ are structural and functionally related, and indeed, the FcεRI γ chain can take over the role of CD3ζ in TCR assembly in ζ−/− mice (42–44). These results suggest a common mechanism underlying ER retention of immune receptor complexes by components of the CD3ζ family. In any case, masking of the CD3ε ER retention signal by CD3ζ assembly is position dependent. Thus, either replacement of the cytoplasmic tail of CD3γ by CD3ε or apposition of an extra CD3ε ER retention signal to the COOH-terminal end of CD3γ prevents surface expression of the full TCR complex (Fig. 6).

We have shown that the di-leucine endocytosis signal of CD3γ, together with the ER retention signal in CD3ε, is also important to reduce the expression of the γγ dimer on the cell surface. Thus, high level expression of the γγ (and by extension εδ) dimer on the cell surface is prevented by impairing its export from the ER–Golgi and by stimulating the rapid endocytosis of dimers from the cell surface. This regulation of CD3 dimer and free CD3 subunit expression is required to prevent ligand-independent triggering of signaling cascades. It has been shown that small amounts of γγ and εδ dimers expressed on the surface of immature thymocytes, also known as clonotypic-independent complexes, can promote thymic differentiation and proliferation upon cross-linking with anti-CD3 antibodies (45). One might ask what would be the consequence of augmenting the expression of CD3 dimers at the cell surface on thymic maturation. Studies in which a TCRβ transgene that lacks the variable region (46), or even all extracellular domains (47), was expressed in MHC class I– and II–deficient mice indicate that the pre-TCR function is independent of ligand recognition. The pre-TCR could therefore serve merely as a platform to express sufficiently high levels of the CD3 dimers at the cell surface to initiate ligand-independent signaling. Assembly of the CD3 dimers with TCRβ, pTα, and CD3ζ must override the CD3ε ER retention signal as well as those in TCRβ (21) and pTα (48). In this regard, it should be noted that the addition of an extra ER retention signal to TCRβ abolishes pre-TCR function (49). These results suggest that the pre-TCR must be expressed on the cell surface to carry out its signaling role. Furthermore, unlike the natural ER retention signals present in the TCR and CD3 subunits, the artificial signal introduced in TCRβ does not appear to be annulled during assembly.

We have made an initial attempt to characterize the signals that regulate the intracellular retention of the CD3γ chain. In accordance with previous observations (22), we found that the cytoplasmic tail of CD3γ contains an ER retention signal at the COOH terminus. Although this sequence is reminiscent of the double arginine ER retention signal in type II membrane proteins (18, 19), we have discovered that only one of the two arginines in CD3γ is important for ER retention. Thus, the cytoplasmic CD3γ ER retention signal better resembles that of CD3ε, which contains only one important basic residue (arginine −3; references 15 and 16). In addition to the ER retention signal, the cytoplasmic tail of CD3γ contains a putative ER export sequence of the DxE type (31) and a di-leucine endocytosis signal (22, 50). However, CD3γ also contains ER retention determinants in its extracellular and transmembrane domains that have yet to be characterized. These extracellular and transmembrane retention determinants in CD3γ could represent distinct sequence motifs or an unfolded state of the protein. In any case, it appears that in contrast to CD3ε, the retention of free CD3γ is regulated by multiple signals.

In conclusion, the results presented here suggest that the TCR–CD3 complex is endowed with a complicated system of intracellular retention signals that become overridden in a stepwise fashion as assembly proceeds. Assembly is regulated in such a way that all intermediates have at least one functional retention signal. This system guarantees that only a full signaling-competent TCR–CD3 complex is expressed at the cell surface.

MATERIALS AND METHODS

Cells. COS-7 cells were obtained from American Type Culture Collection (ATCC) and grown in DMEM plus 5% FBS (Sigma-Aldrich). The human NK cell line YT was provided by M. López-Botet (Universidad Pompeu Fabra, Barcelona, Spain), the murine B24 T cell hybridoma was from the ATCC, the CD3ε-deficient mutant MA5.8 of B24 was provided by J. Aswell (National Institutes of Health, Bethesda, MD), and the CD3γ-deficient mutant R3.25 of Jurkat was provided by B. Rubin (CNRS, Toulouse, France). All cells were grown in RPMI medium plus 5% FBS.

Constructs. All constructs were generated by PCR using human cDNAs as templates. PCR products were cloned into the pSRα or pSRα-HA (unpublished data) vectors. The 4γ chimera is composed of the extracellular and the transmembrane domains of CD4 (finishing in position Vγ3 of the mature polypeptide) fused to the complete intracellular domain of CD3γ.
(from position A115 of the mature polypeptide). 44γΔ1 to 44γΔ5 constructs have a stop codon at positions 157, 152, 148, 141, and 121, respectively, of the mature human CD3γ protein. Truncated CD3γ (γt) contains only the first two amino acids of the cytoplasmic tail. Wild-type human CD3ε and the CD3ε mutant lacking the last five COOH-terminal amino acids (enmut) have been described (15, 51). Point mutants were generated by introducing the mutation encoding for alanine in the positions 131, 158, 159, and 160 as template. The 44γ, γ4, and γγ4 constructs were created as isolated fragments by PCR, cloned into the intermediate vector pGEM-7Zf(+), and the chimeric cDNAs were subcloned into the pSRα-HA vector. The γtet construct was created by PCR using a 3’ primer encoding for the last COOH-terminal 15 amino acids of human CD3ε (KGQRLY5GSLQQRRL) appended to the last amino acid of CD3γ, and the PCR product was cloned into the pSRα-HA vector.

Antibodies. The mAb anti-human SP34 that recognizes the CD3ε extraacellular domain, the mAb anti-human CD3 UCHT1, the mAb anti-human CD4 H1P2/6, and the mAb anti-CD8 Jov1.1 were provided by C. Terhorst (Beth Israel Deaconess Hospital, Boston, MA), P. Beverley (The Edward Jenner Institute for Vaccine Research, Berkshire, UK), F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain), and M. Owen (CRUK, London, UK), respectively. The anti-CD3γ antiserum 448 has been described (5). The following mAbs were purchased as indicated: anti-HA epitope 12CA5 (Roche Diagnostics), anti-murine TCRβ H57-597 and anti-human CD3 Leu4 (BD Biosciences), and anti-human CD3ε SK7 (StemCell Technologies Inc.). All secondary fluorochrome-labeled antibodies were purchased from BD Biosciences.

Cell transfections. COS cells were transiently transfected as described previously (32), and stable transfectants of YT, 2B4, MAS.8, and R3.25 cells were generated by electroporation and selection in geneticin.

Flow cytometry. 24 h after transfection, COS cells were detached from the plate with 0.02% EDTA in PBS and divided into two aliquots for surface- and intracellular staining. For intracellular staining, cells were first fixed with 2% paraformaldehyde in PBS for 20 min at 4°C and then permeabilized with 0.1% saponin in PBS at 4°C for 1 h. Permeabilized and nonpermeabilized cells were incubated with 4 μg/ml of the appropriate mAb for 30 min at 4°C and then with a secondary FITC- or PE-labeled antibody. For two-color staining, directly labeled antibodies were used. Surface expression is indicated as a percentage, as mean fluorescence intensity (MFI), or by multiplying both parameters.

Confocal microscopy. Upon transfection, COS cells were plated on glass coverslips, fixed in paraformaldehyde at room temperature 24 h later, and then permeabilized with saponin as described above. The coverslips were mounted in Mowiol and examined with a confocal microscope (Radiance 2000; BioRad Laboratories).

Cell labeling and immunoprecipitation. 48 h after transfection, COS cells were labeled with 35S-methionine for 15 min and then chased for different times in standard medium before lysing with 1% NP-40 lysis buffer (150 mM NaCl, 20 mM Tris–HCl, pH 7.8, 10 mM iodoacetamide, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Postnuclear lysates were immunoprecipitated with an anti-CD4 antibody, and the immunoprecipitates were resuspended in endo–H buffer (50 mM sodium citrate, pH 5.5, 0.1% SDS, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) before digesting half of the sample with endo–H. The samples were resolved by SDS-PAGE and analyzed by autoradiography.

For surface biotinylation, 50 × 10⁶ MAS.8 and 2B4 were incubated with 0.5 mg/ml sulfo-NHS-biotin (Pierce Chemical Co.) in PBS supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ for 45 min on ice. After washing, surface complexes were recovered by incubating the labeled intact cells with human anti-CD3 antibody Leu4 before lysis. Protein G was added and immunoprecipitates were subjected to two-dimensional SDS-PAGE (first dimension under nonreducing and second dimension under reducing conditions), immunotransferred to a nitrocellulose membrane, hibridized with streptavdin horseradish peroxidase (Southern Biotechnology Associates, Inc.), and developed by ECL (Bio-Rad Laboratories).

Online supplemental material. Fig. S1 shows intracellular distribution of 44γ deletional mutants. Fig. S2 shows intracellular distribution of the 44γ point mutants. In Fig. S3, the ER retention signal in CD3ε and the di-leucine endocytosis signal in CD3γ both regulate surface expression of the incomplete αβεγε complex. Fig. S4 illustrates the intracellular distribution of γγ4 dimers. Figs. S1–S4 are available at http://www.jem.org/cgi/content/full/jem.20041133/D1C1.

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REFERENCES


