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TCR internalization takes place both in resting T cells as part of constitutive TCR cycling, after PKC activation, and during TCR triggering. It is still a matter of debate whether these pathways represent distinct pathways. Thus, some studies have indicated that ligand-induced TCR internalization is regulated by mechanisms distinct from those involved in constitutive internalization, whereas other studies have suggested that the ligand-induced TCR internalization pathway is identical with the constitutive pathway. To resolve this question, we first identified requirements for constitutive TCR cycling. We found that in contrast to PKC-induced TCR internalization where both CD3-ζ-S126 and the CD3-ζ leucine-based internalization motif are required, constitutive TCR cycling required neither PKC nor CD3-ζ-S126 but only the CD3-ζ leucine-based motif. Having identified these requirements, we next studied ligand-induced internalization in cells with abolished constitutive TCR cycling. We found that ligand-induced TCR internalization was not dependent on constitutive TCR internalization. Likewise, constitutive internalization and recycling of the TCR were independent of an intact ligand-induced internalization of the TCR. In conclusion, ligand-induced TCR internalization and constitutive cycling of the TCR represents two independent pathways regulated by different mechanisms. The Journal of Immunology, 2002, 168: 5434–5440.

Several studies have demonstrated that the surface expression level of the TCR is involved in a dynamic and tightly regulated interplay with the activation and developmental status of the T cell. Double-positive thymocytes express low levels of TCR compared with more mature thymocytes and T cells (1, 2), and in mature T cells the number of TCR on the cell surface determines whether the T cell is activated (3) and the biological response elicited by the T cell (4–6). TCR expression is itself influenced by the activation status of the T cell. Thus, after stimulation the TCR is quickly internalized from the cell surface (7, 8). Therefore, regulation of TCR surface expression is probably an important event affecting the function of the T cells.

In resting T cells, the TCR constitutively cycles between the plasma membrane and intracellular compartments (9–12); 70–85% of the TCR in the cycling pool is expressed on the cell surface and 15–30% is found inside the cell (9–12). The constitutive TCR internalization rate constant is ~0.012 min⁻¹, meaning that ~1.2% of surface-expressed TCR is internalized per minute. Likewise, the exocytic rate constant is 0.055 min⁻¹, meaning that ~5.5% of the intracellular pool of recycling receptors is transported to the plasma membrane per minute (12). The TCR amino acid motifs involved in constitutive TCR cycling and how the cycling is regulated still remain to be determined.

Activation of protein kinase C (PKC) induces TCR down-regulation from the cell surface by increasing the TCR internalization rate constant ~10 times without affecting the exocytic rate constant (12). PKC-induced phosphorylation of CD3-ζ-S126 most probably induces a conformational change, which leads to exposure of the CD3-ζ leucine (L)-based receptor-sorting motif. This motif consists of the DxxxL sequence that binds the AP-2 at the plasma membrane (13, 14). AP-2, in turn, links the TCR to the clathrin-dependent internalization machinery. Some studies have indicated that PKC plays a role in the constitutive cycling of the TCR and that the PKC-induced pathway is identical with the constitutive pathway (10, 15). However, in these studies cells were stimulated with phorbol esters and the role of PKC in the constitutive cycling of the TCR in unstimulated cells is still not known.

Finally, TCR surface down-regulation is seen after ligand stimulation. In several studies, TCR surface down-regulation is observed within minutes of ligand stimulation, implying that ligand stimulation increases the TCR internalization rate (7, 16, 17). However, a recent study showed that ligand-induced TCR down-regulation did not increase the TCR internalization rate and that the observed surface down-regulation was due to lack of recycling of ligated TCR (11). From this, it was suggested that the ligand-induced internalization pathway was in fact identical with the constitutive TCR internalization pathway and therefore absolutely dependent on a functional constitutive cycling of the TCR.

Thus, whether the TCR can be sorted via one or more different pathways is still a matter of debate. In this work, we focused on the mechanisms regulating the constitutive and the ligand-induced pathways. We speculated that if the ligand-induced internalization pathway is indeed identical with the constitutive pathway then a...
block in the ligand-induced pathway should parallel a block in the constitutive pathway. Given that the mechanisms regulating the constitutive cycling of the TCR were unknown, the aim of this study was first to identify these mechanisms and thereafter to examine whether the same mechanisms were involved in ligand-induced internalization of the TCR.

**Materials and Methods**

**Cells, Abs, and chemicals**

JGN and E3 TCR cell surface-negative variants of the human T cell line Jurkat that synthesizes no CD3ε were produced in our laboratory (18). JGN- and E3-WT cells express wild-type (WT) CD3γ, JGN- and E3-L131I/132A cells express CD3γ with leucine 131 and 132 to alanine mutations, and JGN- and E3-S320V cells express CD3γ with serine 126 to alanine mutation (19). J76 is a WT clone of Jurkat (20). J.CaM1.6, a p56<sup>Lck</sup>-deficient variant of the Jurkat clone E6-1 (21), J45.01 (J45), a CD45-deficient variant of E6-1 (22), and the Burkitt’s lymphoma cell line Raji were from American Type Culture Collection (Manassas, VA). J.CaM1.6 and J45 cells were transfected as previously described with p56<sup>Lck</sup> and CD45, respectively (17). DO11.10 is a mouse T cell hybridoma (23). In some experiments, CD3<sub>γ</sub>-L131I/132A or WT was cloned into pEGFP-N1 (Clontech Laboratories) to produce these constructs were subsequently transfected into JGN cells (JGN-L131I/132A-GFP and JGN-WT-GFP) as previously described (19). Cells were cultured in RPMI 1640 supplemented with penicillin (2 × 10<sup>5</sup> U/L; Leo Pharmaceutical Products, Ballerup, Denmark), streptomycin (50 mg/L; Merck, Darmstadt, Germany), and 10% (v/v) FCS (Life Technologies, Paisley, U.K.) at 37 °C in 5% CO<sub>2</sub>. PE-conjugated and unconjugated UCHT1 anti-human CD3ε mAb were obtained from Dako-patts (Glostrup, Denmark). PE-conjugated and unconjugated anti-murine CD3ε (145-2C11) and anti-murine TcR Vβ8 (F23.1) mAbs were obtained from BD Pharmingen (San Diego, CA). The anti-TCR mAb F101.01 was produced in our laboratory (24). Cy5-conjugated F(ab)2 fragments of donkey anti-mouse IgG H<sub>µ</sub> L and PE-conjugated F(ab)2 fragments of goat anti-mouse IgG H L were obtained from Jackson ImmunoResearch (West Grove, PA). The phorbol ester phorbol 12,13-dibutyrate (PDB) and anti-mouse IgG H<sub>µ</sub> L-based motif is required for constitutive TCR internalization. To stop constitutive exocytosis, cells were treated with BFA for the indicated time and TCR expression was subsequently determined by FACS analyses. C and D, Analyses of constitutive TCR recycling. Cells were analyzed in the recycling assay using the anti-CD3ε mAb UCHT1 as described in Materials and Methods. Newly expressed TCR is given as the percentage of total TCR cell surface expression of untreated cells.

**Conconf microscopy**

Cells were washed in PBS and fixed for 10 min with 1% paraformaldehyde at room temperature. The cells were permeabilized for 10 min at room temperature with washing buffer (HEPES-buffered PBS containing 0.1% saponin) and incubated with primary Abs at room temperature for 10 min. Cells were washed three times in washing buffer and stained with Cy5-conjugated secondary Abs at room temperature for 10 min. Confocal microscopy was performed on a Zeiss LSM510 connected to a Zeiss Axiovert 100 M microscope (Carl Zeiss, Jena, Germany). Green-fluorescent protein (GFP) and Cy5 fluorescence were detected using band pass filter BP 505–550 and long pass filter LP 650, respectively.

**Recycling assay, TCR down-regulation, and phosphotyrosine blots**

TCR recycling was analyzed by determining the size of the intracellular pool of recycling receptors as follows. First, all TCR expressed at the cell surface was blocked by incubating the cells with saturating amount of unconjugated anti-CD3ε or anti-TCR mAb (1 μg/ml) at 4°C for 30 min. After washing at 4°C, the cells were transferred to a 37°C water bath for the indicated time to allow TCR cycling. The cells were subsequently transferred to 4°C, stained with PE-conjugated mAb of the same type as used for TCR blockade, and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). The recycling pool of TCR was calculated as the percentage of TCR expressed at the cell surface using the equation \( \frac{\text{H} - \text{C}}{\text{H}} \times 100\% \), where \( \text{H} \) is the mean fluorescence intensity (MFI) of cells incubated with unconjugated mAb at 4°C followed by incubation at 37°C and staining with PE-conjugated mAb, \( \text{C} \) is the MFI of cells incubated with unconjugated mAb followed by incubation at 4°C and staining with PE-conjugated mAb, and \( \text{T} \) is the MFI of cells directly stained by incubation with PE-conjugated mAb. The initial slope of the curves reflects the exocytic rate constant.

**Results**

The CD3γ L-based motif is required for constitutive TCR cycling

It has been shown that the CD3γ L-based motif is required for PKC-induced internalization of the TCR (19). However, it is not known whether the CD3γ L-based motif is also required for constitutive TCR internalization and recycling. To examine this we used two CD3γ-deficient cell lines (JGN and E3) transfected with wild-type CD3γ (CD3γ-WT) or CD3γ with a nonfunctional L-based motif due to a CD3γ-Leu131/132A mutation (CD3γ-L131I/132A). Three different methods were used to study the constitutive cycling of the TCR in these cells. 1) Blocking exocytosis while leaving endocytosis intact with the fungal metabolite BFA should lead to a decrease in surface-expressed TCR in cells with functional cycling of the TCR. Accordingly, a decrease in TCR surface expression was observed in JGN- or E3-WT cells treated with BFA (Fig. 1, A and B). In contrast, BFA treatment did not decrease TCR surface expression in JGN- and E3-L131I/132A cells. This indicated that constitutive TCR internalization was abolished in these cells (Fig. 1, A and B). 2) To analyze TCR exocytosis (recycling), cells were incubated with unconjugated anti-CD3ε mAb UCHT1 at 4°C to block cell surface expressed TCR. After extensive washing the cells were incubated at 37°C for different time periods and subsequently analyzed by FACS using PE-conjugated UCHT1. This procedure allowed us to

FIGURE 1. The CD3γ L-based motif is required for constitutive TCR cycling. A and B, Analyses of constitutive TCR internalization. To stop constitutive exocytosis, cells were treated with BFA for the indicated time and TCR expression was subsequently determined by FACS analyses. C and D, Analyses of constitutive TCR recycling. Cells were analyzed in the recycling assay using the anti-CD3ε mAb UCHT1 as described in Materials and Methods. Newly expressed TCR is given as the percentage of total TCR cell surface expression of untreated cells.
specifically label and analyze only newly expressed TCR. The results showed an almost complete lack of TCR recycling in the mutant T cells JGN- and E3-L131/132A (Fig. 1, C and D). As expected, treatment of control cells with BFA inhibited TCR recycling (Fig. 1C, 3) As a final approach, WT and L131/132 cells were analyzed by confocal microscopy. According to the previous experiment demonstrating an almost complete lack of an intracellular pool of recycling TCR, it could be speculated that intracellular TCR staining would be reduced in L131/132A cells compared with WT cells. JGN cells (and E3, data not shown) transfected with CD3γ-WT stained positive for CD3 at the cell surface and in intracellular vesicles (Fig. 2A). The same staining pattern was observed for PBL (Fig. 2B). In contrast, although JGN-L131/132A showed a clear surface staining, a significant decrease in intracellular staining was observed (Fig. 2C). Likewise, T cells expressing the CD3γ-WT chain linked to GFP (JGN-WT-GFP) showed a significantly higher staining of intracellular vesicles compared with JGN-L131/132A-GFP cells (Fig. 2, D and E). The reduced occupancy of TCR in intracellular vesicles supported that constitutive TCR cycling in JGN- and E3-L131/132A cells was abolished.

![Image](http://www.jimmunol.org/)

**FIGURE 2.** Mutation of the CD3γ L-based motif reduces occupancy of TCR in intracellular vesicles. Confocal microscopy analysis of JGN-WT (A), PBL (B), JGN-L131/132A (C), JGN-WT-GFP (D), and JGN-L131/132A-GFP (E) cells. The TCR was visualized by staining with anti-TCR mAb F101.01 followed by Cy5-conjugated F(ab)_2 fragments of donkey anti-mouse IgG H + L (A–C) or directly by GFP fluorescence (D and E).

Taken together, these results demonstrated that the CD3γ L-based motif is required for constitutive internalization and recycling of the TCR.

*The vast majority of newly expressed TCR represents recycling TCR*

In the analyses used in this study, we measured exocytosis of unlabeled TCR. These TCR could come either from the recycling pool of TCR, from newly synthesized TCR, or from a combination of the two. The half-life of fully assembled TCR has been determined to be ~15 h (25, 26). This means that ~0.08% (ln(2/1.2)) of the TCR at the cell surface is exchanged with newly synthesized TCR per minute. At the same time, ~1.2% of the TCR at the cell surface is exchanged with recycling TCR per minute. Thus, in theory the vast majority ((1.2/1.2 + 0.08) × 100% = 94%) of newly expressed TCR should come from the recycling pool. To address this question experimentally, cells were preincubated with or without the protein synthesis inhibitor cycloheximide and then analyzed in the recycling assay. As seen in Fig. 3A, cycloheximide did not affect the appearance of newly expressed TCR in agreement with the theoretical considerations. Similar results were obtained for cycloheximide-treated JGN- and E3-WT cells (data not shown).

Based on our recent determinations of the distribution of the cycling pool of TCR and the constitutive endo- and exocytic rate constants, theoretical curves for re-expression of unlabeled TCR was calculated (12). As demonstrated in Fig. 3B, the theoretical curve for appearance of unlabeled TCR at the cell surface reflected the experimentally obtained curves.

Finally, another important point to address was whether mAb-induced TCR ligation, signaling, and internalization affected the appearance of newly expressed TCR as measured by the recycling assay. To study this point, we took advantage of the existence of both activating and nonactivating mAb against the TCR expressed by the mouse T cell hybridoma DO11.10. Thus, triggering the TCR with the activating anti-CD3ε mAb 145-2C11 induced both TCR internalization and tyrosine phosphorylation of intracellular substrates (Fig. 3, C and D). In contrast, incubation of the cells with the anti-Vβ8 mAb F23.1 induces neither TCR internalization nor tyrosine phosphorylation (Fig. 3, C and D). However, both mAb produced very similar results in the recycling assay, strongly indicating that mAb-induced TCR internalization and signaling did not affect the appearance of newly expressed TCR (Fig. 3E).

*Constitutive TCR cycling is independent of CD3γ-S126 and PKC activity*

The results presented thus far showed that the CD3γ L-based motif is required for constitutive TCR internalization and recycling. Because PKC-induced TCR internalization in addition to the CD3γ L-based motif is absolutely dependent on CD3γ-S126, we next analyzed the role of CD3γ-S126 in constitutive TCR cycling. We used JGN and E3 cells expressing a CD3γ chain with a CD3γ-S126 to valine mutation (CD3γ-S126V). In these transfectants PKC-induced internalization is completely inhibited (Fig. 4A and Ref. 17). Surprisingly, BFA treatment produced the same degree of TCR surface down-regulation as observed for JGN- and E3-WT cells (Fig. 4B). If constitutive internalization was indeed intact in JGN- and E3-S126V cells, it would be expected that constitutive recycling should also be intact in these cells. Accordingly, analyses of TCR recycling showed no significant difference between WT and S126V cells (Fig. 4C). Taken together, these experiments showed that constitutive TCR internalization and recycling is independent of CD3γ-S126.

CD3γ-S126 is a substrate for PKC (27, 28). If constitutive internalization were independent of PKC, this would explain why
CD3-γ-S\textsuperscript{126} was not required. We therefore examined the role of PKC in constitutive cycling of the TCR. Cells were treated with BAPTA-AM and EGTA that bind intracellular and extracellular Ca\textsuperscript{2+}. This treatment completely inhibited PKC-induced TCR internalization (Fig. 5A). Thereafter, we used two approaches: 1) TCR recycling after treatment with BAPTA-AM-EGTA for 30 min at 37°C was directly analyzed as described above. These experiments showed that recycling of the TCR was unaffected by the lack of PKC activity (Fig. 5B). 2) Cells were pretreated with BAPTA-AM-EGTA and subsequently treated with BFA to block spontaneous exocytosis. Despite the lack of PKC activity, BFA treatment produced the same degree of TCR down-regulation as observed for untreated cells (Fig. 5C). Thus, these experiments demonstrated that constitutive internalization and recycling of the TCR do not require CD3-γ-S\textsuperscript{126} and PKC activity.

**Ligand-induced TCR internalization is independent of constitutive TCR cycling**

Recent studies have indicated that the ligand-induced TCR internalization pathway is identical with the constitutive TCR internalization pathway (11). JGN- and E3-L131/132A cells exhibited a block in constitutive internalization and recycling, which allowed us directly to study whether ligand-induced TCR internalization was dependent on intact constitutive TCR internalization. WT and L131/132A cells were stimulated with different concentrations of the anti-TCR mAb F101.01 or incubated with APC pulsed with different concentrations of the superantigen SEE. Efficient ligand-induced TCR internalization was observed for all
Regulation of TCR surface expression levels during T cell ontogeny and T cell activation has received much attention (2, 8, 15, 19, 30–34). Although it is not fully understood how TCR surface expression levels are regulated, it seems to involve protein kinases (2, 9, 10, 19), phosphorylation status of the TCR chains (9, 19), and adaptor proteins such as TCR-interacting molecule (35, 36)–like adaptor protein (30). The present study was performed to broaden our knowledge of the mechanisms regulating constitutive TCR cycling and ligand-induced TCR internalization. In particular, we aimed to resolve the question of whether constitutive TCR internalization is required for ligand-induced internalization of the TCR.

Although constitutive TCR cycling has been observed in several studies (9, 10, 15), the mechanisms controlling this process have remained unknown. In the present study, we showed that constitutive TCR internalization and recycling of the TCR is dependent on the CD3γ L-based motif. Mutation of the CD3γ L-based motif abrogated the internalization step in the constitutive cycling of the TCR and thus impaired TCR cycling. Previous studies have suggested that a basic PKC activity resulting in low rate phosphorylation of CD3γ-S126 might be required for constitutive TCR internalization (10, 15). Surprisingly, we found that constitutive TCR cycling did not require PKC activity or CD3γ-S126.

Thus, although treatment of cells with BAPTA-AM-EGTA completely inhibited PKC-induced TCR internalization, constitutive TCR cycling was unaffected by this treatment. In addition to PKC activity, BAPTA-AM-EGTA treatment most likely inhibited other calcium-dependent activities in the cell; however, this did not affect constitutive TCR cycling. The independence of constitutive TCR cycling for PKC activity was further substantiated by the independence of constitutive TCR cycling for the PKC TCR substate CD3γ-S126. It could be suggested that the CD3γ L-based

**FIGURE 6.** Constitutive TCR cycling is not a requirement for ligand-induced TCR internalization. Cells were stimulated for 1 h with the indicated concentrations of the anti-TCR mAb F101.01 (A) or with APC loaded with the indicated concentrations of SEE (B) and TCR down-regulation was subsequently determined by FACS analyses. Cells were stimulated with a fixed concentration of the anti-TCR mAb F101.01 (5 μg/ml) (C) or with SEE (300 ng/ml)-pulsed APC (D) for the time indicated, and TCR surface expression was subsequently determined by FACS analyses.

**Discussion**

Because constitutive TCR cycling was not required for ligand-induced TCR internalization, we next examined whether a functional ligand-induced TCR internalization was required for TCR cycling. Given that lack of tyrosine kinase p56Lck or phosphatase CD45 significantly reduces ligand-induced TCR internalization, we analyzed TCR cycling in J.CaM1.6 cells deficient of p56Lck (21, 29) and J45 cells deficient of CD45 (22). As expected, anti-TCR mAb or superantigen-induced TCR internalization in J45 and J.CaM1.6 cells was significantly reduced and could be restored by transfection with CD45 and p56Lck, respectively (Fig. 7A and data not shown). Importantly, TCR recycling was similar in cells deficient of CD45 or p56Lck when compared with J45 cells transfected with CD45 or J.CaM1.6 transfected with p56Lck, respectively (Fig. 7, B and C). Likewise, lack of p56Lck or CD45 did not significantly affect TCR surface down-regulation after BFA treatment (Fig. 7, D and E). This indicated that constitutive TCR cycling does not require a functional ligand-induced TCR internalization pathway.
constitutive TCR recycling. Cells were analyzed in the recycling assay using the anti-CD3ε mAb UCHT1 as described in Materials and Methods. Newly expressed TCR is given as the percent of total TCR cell surface expression of untreated cells. Analyses of constitutive TCR internalization were subsequently determined by FACS analyses.

By identification of some of the mechanisms required for constitutive TCR cycling, we had the opportunity to examine ligand-induced TCR internalization in cells with abrogated constitutive TCR cycling. We found that ligand-induced internalization of the TCR was intact in two independently derived cells with abolished constitutive TCR cycling due to a disrupted CD3ɛ L-based motif. This was observed by using both anti-TCR mAb and superantigen as ligands and is in agreement with previous studies, which showed that ligand-induced internalization of the TCR is functional despite of a mutated or truncated CD3ɛ L-based motif. Ligands of the lck tyrosine kinase targets cell surface T cell antigen receptors for lysosomal degradation. 

The physiological role of constitutive TCR cycling is unknown. It has been suggested that constitutive cycling of the TCR might serve as a quality check of the receptor as only fully functional and assembled TCR can proceed through the entire cycling event. Receptors that are not properly assembled may have lost the ability to mask the CD3γ L-based motif and consequently are sorted to the lysosomes. Such a task would require a motif that could function both as an internalization signal as well as a lysosomal sorting signal, which is in fact the case of the CD3γ L-based motif (26, 39, 40). Another role for constitutive cycling might be to ensure a constant internal store of TCR. By generating an internal store of TCR that can be rerouted to the site of stimulation, constitutive TCR cycling may play a role in T cell activation. However, even though several studies have shown that TCR are recruited to the site of stimulation (41–46), it is not known to which degree these TCR originate from internal stores. Further clarification of these issues awaits the analyses of the CD3γ dileucine-mutated knock in mice (C. Menné, M. C. Haks, A.M. Kruisbeek, and C. Geisler, manuscript in preparation).

In conclusion, in this study we showed that constitutive TCR cycling is not a requirement for ligand-induced TCR internalization and vice versa. Furthermore, constitutive TCR internalization is dependent on the CD3γ L-based motif but independent of CD3γ S126 and PKC activity.

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References


