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TCR Dynamics in Human Mature T Lymphocytes Lacking CD3γ[1]

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The contribution of CD3γ to the surface expression, internalization, and intracellular trafficking of the TCR/CD3 complex (TCR) has not been completely defined. However, CD3γ is believed to be crucial for constitutive as well as for phorbol ester-induced internalization. We have explored TCR dynamics in resting and stimulated mature T lymphocytes derived from two unrelated human congenital CD3γ-deficient (γ^-) individuals. In contrast to γ^- mutants of the human T cell line Jurkat, which were selected for their lack of membrane TCR and are therefore constitutively surface TCR negative, these natural γ^- T cells constitutively expressed surface TCR, mainly through biosynthesis of new chains other than CD3γ. However, surface (but not intracellular) TCR expression in these cells was less than wild-type cells, and normal surface expression was clearly CD3γ-dependent, as it was restored by retroviral transduction of CD3γ. The reduced surface TCR expression was likely caused by an impaired assembly or membrane transport step during recycling, whereas constitutive internalization and degradation were apparently normal. Ab binding to the mutant TCR, but not phorbol ester treatment, caused its down-modulation from the cell surface, albeit at a slower rate than in normal controls. Kinetic confocal analysis indicated that early ligand-induced endocytosis was impaired. After its complete down-modulation, TCR re-expression was also delayed. The results suggest that CD3γ contributes to, but is not absolutely required for, the regulation of TCR trafficking in resting and Ag-stimulated mature T lymphocytes. The results also indicate that TCR internalization is regulated differently in each case. ***The Journal of Immunology,*** **2003, 170: 5947–5955.**

T cells detect and respond to Ags by way of the T cell Ag receptor, a cell surface protein complex of the following four different dimers: TCR$\alpha\beta$, CD3$\gamma\epsilon$, CD3$\delta\epsilon$, and $\zeta\zeta$ (1). The TCR is expressed on T cells as the result of an equilibrium between synthesis and transport to the cell surface of new chains and their internalization and recycling or degradation (2). In resting T cells, cell surface-expressed TCR complexes are long lived. That is, they are relatively independent of synthesis, surface transport, and degradation for several hours. However, they are constitutively and rapidly internalized and recycled back to the cell surface (2, 3). Constitutive TCR internalization and recycling is believed to be controlled, in turn, by a phosphorylation-dephosphorylation equilibrium promoted by serine kinases and phosphatases that operate constitutively on a CD3γ internalization motif (4, 5), although participation of ζ has also been reported (3).

Upon Ag binding TCR recycling is reduced, and degradation is strongly increased. As the internalization rate is also (modestly) increased, a net long-term drop of surface TCR levels (termed down-modulation) ensues (3, 6). TCR down-modulation is believed to be a means of attenuating further T cell stimulation after Ag recognition (7, 8). Several CD3 subunits including CD3γ (9) may be involved in this complex process, which apparently does not require CD3γ phosphorylation (10, 11, 12). Indeed, a recent report indicated that ligand-induced TCR down-modulation is independent of constitutive TCR cycling (5). Artificial transmembrane activation of T cells in the absence of ligand, for example with phorbol esters, also causes a rapid, albeit partial, TCR down-modulation. However, in this case a different mechanism is involved. This includes increased internalization but not degradation, and leads to intracellular accumulation of internalized complexes (13–16). A critical role has been ascribed to CD3γ in this process, and in particular to the phosphoserine-dependent di-leucine internalization motif borne by this chain (17, 18), suggesting shared features with constitutive TCR internalization and recycling.

Lack of CD3γ completely prevents surface TCR expression on certain human and mouse T cell lines (11, 14, 19). Mature T cells from CD3γ-deficient (γ^-)[4] humans (20) or knockout mice (21), in contrast, express abundant TCR levels, although less than normal controls. Nevertheless, a structural role for CD3γ in TCR conformation, and thus Ab binding, cannot be formally excluded (22). γ^- T cells are strictly resistant to phorbol ester-induced TCR down-regulation (23, 24), confirming that CD3γ is important for TCR regulation in resting and phorbol ester-activated T lymphocytes. However, ligand-induced TCR down-modulation is preserved when CD3γ is lacking (25), although conflicting results have been

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[4] Abbreviations used in this paper: γ^-, CD3γ deficient; JGN, Jurkat γ negative; BFA, brefeldin A; CHX, cycloheximide; HVS, *Herpesvirus saimiri;* γ^+, CD3γ sufficient; SEB, *Staphylococcus* enterotoxin B; HTLV-I, human T cell leukemia virus type I; Endo H, Endo-β-*N*-acetylglucosaminidase H; EGFP, enhanced green fluorescence protein; DPBS, Dulbecco's PBS.

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reported in the mouse model (24). Thus, TCR surface levels are carefully regulated by different mechanisms in resting T cells and in T cells activated by different agents. The precise contribution of CD3γ to TCR output, input, and intracellular traffic in each case has not been completely defined. Therefore, we have explored constitutive and ligand (or PMA)-induced TCR dynamics in mature T cells derived from two unrelated human γ^- individuals.

Materials and Methods

T cell lines

Herpesvirus saimiri (HVS)- and human T cell leukemia virus type I (HTLV-I)-transformed T cell lines were derived from PBL of two healthy congenital γ^- individuals carrying different mutations in CD3G (D.S.F. and F.K., Ref. 26) or normal, CD3γ-sufficient (γ^+) donors, as previously described (23, 27). D.S.F. is a compound heterozygote (g.38 A>G; g.IVS2-1 G>C) born to unrelated Spanish parents. F.K. is a homozygote (g.242 A>T) born to consanguineous Turkish parents. Both are currently healthy and well into their teens. The HVS-transformed T cell lines used in the experiments had been cultured for 6 years (DSF4, CD4$^+$ γ^-; DSF8, CD8$^+$ γ^-; CTO, CD8$^+$ γ^+), 4 years (AGU, CD4$^+$ γ^+), or 2 years (FK2, CD8$^+$ γ^-; YK2, CD8$^+$ $\gamma^{+/-}$). The HTLV-I-transformed T cell lines had been cultured for 2 years (DSF, CD4$^+$ γ^-, BS1.1 CD4$^+$ γ^+). HVS-transformed cells were always grown in parallel in 1:1 RPMI:Panserin medium from Life Technologies (Paisley, UK) and PAN Biotech (Aidenbach, Germany), respectively. HTLV-I cells were grown in RPMI alone. In all cases media were supplemented with 40 IU/ml human rIL-2 (Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD), 10% FCS (Flow Laboratories, Rockville, MD), and 1% glutamine (BioWhittaker, Berkshire, UK). Media were replaced every 3–4 days. Except where indicated, all experiments were performed on resting HVS-transformed cells that were generated by starving cells overnight in RPMI/Panserin with 2% FCS but no IL-2 the day before medium replacement was due. Before use, cells were washed twice in PBS and resuspended in RPMI/Panserin 10% FCS without IL-2. The human T cell line Jurkat and a TCR surface-negative variant that synthesizes no CD3γ (Jurkat γ negative (JGN), 19) were provided by Dr. B. Rubin (Centre National de la Recherche Scientifique, Centre Hospitalier Universitaire, Purpan, Toulouse, France). In some experiments, HVS-transformed Vβ3$^+$ T cells were isolated using the MACS anti-FITC MultiSort kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, 2×10^7 cells were stained with 40 μl of anti-TCRVβ3-FITC mAb (Immunotech, Luminy, France) and 60 μl of MACS buffer (PBS with 0.5% BSA and 2 mM EDTA) for 30 min at 4°C. After two washes with MACS buffer, 20 μl of anti-FITC-coated magnetic beads (Dynal Biotech, Oslo, Norway) and 80 μl of MACS buffer were added to the cell pellet and incubated for 15 min at 6°C. Cells were washed twice, resuspended in 1 ml of MACS buffer, and run in the AutoMACS magnetic cell sorter (Miltenyi Biotec) using the Posseld D protocol. Purity was monitored by flow cytometry.

Retroviral transduction

CD3γ cDNA was cloned into the LZRS-enhanced green fluorescence protein (EGFP) bicistronic retroviral vector (28) and transfected into PG13 cells. Puromycin-resistant cells were sorted for EGFP expression, cloned, and selected for EGFP transfer capacity to Jurkat and normal or γ^- T cells as described (29).

Abs and flow cytometry

The expression of different surface or intracellular markers was studied by flow cytometry according to a standard procedure (23). The following mAbs were used: CD3 (UCHT1) as hybridoma supernatant, or from Immunotech (IOT3b); Vβ3 (CH92) from Immunotech; CD4 (Leu3a) and CD45RO (UCHL1) from BD Biosciences (Mountain View, CA); TCRβ (βF1) from Endogen (Woburn, MA); and CD4 (RPA-T4) from BD PharMingen (San Diego, CA). CD3 (Leu4) hybridoma supernatant and APA1/1 ascitic fluid were generous gifts from Dr. B. Alarcón (Centro de Biología Molecular (CBM), Consejo Superior de Investigaciones Cientificas, Madrid, Spain). All commercial Abs were FITC- or PE-conjugated, and for UCHT1 and Leu4 hybridomas, a PE-conjugated goat anti-mouse IgG (H + L) from Caltag Laboratories (Burlingame, CA) was used. Background fluorescence was defined in all cases with an isotype-matched irrelevant mAb from Caltag Laboratories. Cells were washed twice in PBS 1% FCS, and analyzed in an Epics Elite Analyzer cytofluorometer (Coulter Electronics, Hialeah, FL). For comparative stainings we used the mean fluorescence intensity, defined as the average fluorescence value of the corresponding mAb referred to the logarithmic scale of fluorescence intensity along the *x*-axis of the histograms. Brefeldin A (BFA) (10 μg/ml; Sigma-Aldrich, St. Louis, MO) or cycloheximide (CHX) (50 μg/ml; Calbiochem, La Jolla, CA) treatments were done at 37°C in flat-bottom 96-well microtiter plates (10^6 cells/ml) (3).

To study ligand-induced internalization, 10^6 cells/ml were incubated at 37°C in RPMI/HEPES 25 mM/BSA (1 mg/ml) in the presence of 20 μg/ml UCHT1-FITC or an irrelevant Ab (negative control). At several time points, cells were washed in ice-cold RPMI/BSA (1 mg/ml), split, and subsequently acid pH-treated or left untreated. For acid treatment, cells were resuspended in 300 μl of acid medium (RPMI, 25 mM sodium acetate, brought to pH 2.8 with HCl) for 2.5 min and then neutralized with 0.8 ml of RPMI brought to pH 9 with NaOH. This treatment removed 90–99% of surface-associated (but not internalized) mAb. After two washes with PBS, cells were analyzed by flow cytometry. The percentage of CD3 internalization was calculated using the following described equation (30): (specific median fluorescence intensity in acid-treated cells/untreated cells) $\times$ 100, where the specific median fluorescence intensity is that of cells incubated with anti-CD3 minus that of the negative control. The CD3 internalization rate was determined by linear regression analysis (Prophet; BBN Systems and Technologies, Yarbley, PA) and is expressed as a percentage of the total surface-labeled CD3 that was internalized per minute.

Staphylococcus enterotoxin B (SEB)-induced down-modulation was induced as described (31). Vβ3$^+$ cells at 5×10^5 cells/500 μl were incubated in growth medium containing 20 μg/ml SEB (Toxin Technology, Madison, WI) for 2 h at 37°C. Then cells were washed in cold PBS 1% FCS, and cell surface expression of CD3 was assessed by direct immunofluorescence (30 min., 4°C, Leu4; BD Biosciences), washed twice in cold PBS 1% FCS, and analyzed by flow cytometry.

To study CD3 re-expression, TCR down-regulation was first induced as described previously (8). Briefly, 10^6 resting cells/ml were incubated in the presence of a 1/2 dilution of a CD3-specific mAb (Leu4 hybridoma supernatant) or an irrelevant mAb (negative control; Caltag Laboratories) for 24 h, stained with PE-conjugated goat anti-mouse IgG for 45 min, and analyzed by flow cytometry to ascertain down-regulation. Cells were then incubated at 37°C in the presence of Leu4 (to maintain down-regulation) or the irrelevant Ab (test) for several time periods. After two washings with PBS 1% FCS, CD3 re-expression was monitored with PE-conjugated Leu4 by flow cytometry. Results are given as the mean fluorescence intensity of untreated relative to anti-CD3-treated cells.

Where appropriate, polynomial regression (Prophet; BBN Systems and Technologies) was used for expression kinetics plots.

TCR labeling and immunoprecipitation

For surface iodination at least 10^7 cells were washed twice and resuspended in 150 μl of PBS. Then cells were ^{125}I-labeled by adding 2 mCi Na^{125}I (Amersham, Arlington Heights, IL), 30 μl of a 140 IU/ml lactoperoxidase solution (Sigma-Aldrich), and 10-μl aliquots of a 0.06% H_2O_2 PBS solution five times at 5-min intervals. The labeling reaction was stopped by adding a 20-mM KI and 1-mM tyrosine PBS solution (Sigma-Aldrich). The samples were lysed in 1% Digitonin (Sigma-Aldrich) -containing lysis buffer (1% Digitonin, 150 mM NaCl, 20 mM Tris-HCl (pH 8), 10 mM iodoacetamide, 1 mM PMSF).

Surface biotinylation was done as described (32). In brief, 15×10^6 cells were washed three times in Dulbecco's PBS (DPBS) (0.14 M NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl,) (pH 7.4) and labeled with 200 μg/ml/10^7 cells of Sulfo-NHS-LC biotin (Pierce, Rockford, IL) in DPBS plus 0.25 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 1 g/L glucose for 30 min at 4°C in rotation. Excess biotin was quenched with 10 mM NH_4Cl for 15 min on ice. Cells were washed twice with DPBS, incubated at 37°C in RPMI/HEPES 25 mM/BSA 1 mg/ml for different time periods, and subsequently lysed on ice (5×10^6 cells/lane) for 45 min in 1% Digitonin-containing (pH 7.6) lysis buffer (1% Digitonin (Sigma-Aldrich), 50 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM PMSF (Sigma-Aldrich), and 8 mM iodoacetamide (Sigma-Aldrich)). Finally, cell debris were removed by centrifugation (2,000 $\times$ *g*, 14,000 $\times$ *g*; 30 min each), and supernatants, hereafter referred to as lysates, were collected and transferred to new tubes.

For immunoprecipitation, lysates were precleared twice (for 2 h and overnight, respectively) by incubation with Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) containing 1% Digitonin, followed by a 2-min centrifugation at 12,000 $\times$ *g* in an Eppendorf centrifuge at 4°C. The precleared supernatants were subsequently incubated for 1.5 h at 4°C with 250 μl of OKT3 supernatants (anti-CD3) coupled to protein G-Sepharose beads (Amersham Pharmacia Biotech). The beads were then washed five times in lysis buffer, resuspended in Laemmli sample buffer, and boiled for 5 min before a short spin at 12,000 $\times$ *g*. For deglycosylation with Endo-β-*N*-acetylglucosaminidase H (Endo H) (Boehringer Mannheim, Mannheim, Germany), immunoprecipitates were resuspended after

the last wash in 45 μl of denaturing buffer (5% SDS, 10% 2-ME) and boiled for 10 min. After a 2-min/12,000 × *g* centrifugation, supernatants were transferred to new Eppendorf tubes and deglycosylated overnight at 37°C with Endo H with 4.5 μl of 10× Endo H buffer (0.5 M sodium cytrate (pH 5.5)) and 1 μl of Endo H (1000 IU/ml).

Electrophoresis was done in 10% SDS-polyacrylamide gels under reducing or nonreducing conditions where indicated (nonreducing conditions preserve TCRαβ dimers). For Western blots, the electrophoresed proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by standard procedures. The membrane was blocked in a solution of 2.5% BSA/DPBS (Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at room temperature, rinsed once with 100 ml of Low buffer (0.1% Tween 20 in DPBS), and incubated with 1/12,500 of streptavidin-HRP (Amersham) for 1 h at room temperature. After six washes with 100 ml of Low buffer, the membrane was processed by the ECL method (Amersham Pharmacia Biotech). The samples were analyzed by autoradiography. The films were electronically scanned (Geldoc 2000 analyzer; Bio-Rad) to determine the relative intensity and m.w. of each protein.

Confocal microscopy

For intracellular stainings, 5×10^6 cells were washed twice in PBS 0.1% BSA and adjusted to a final concentration of 2.5×10^5/ml. A total of 2.5×10^4 cells were dropped onto nontreated glass slides by cytocentrifugation at 400 rpm for 3–4 min in a Cytospin 3 cytocentrifuge (Thermo-Shandon, Pittsburgh, PA). Samples were air dried and then fixed in acetone at room temperature for 5 min. Slides were again air dried, incubated with anti-TCRβ (100 μl βF1, 20 μg/ml, 40 min in a wet chamber), washed twice in PBS, dried carefully, stained with Cy3-conjugated rabbit anti-mouse IgG (H + L)-specific Ab (100 μl, 1/200 dilution) from Jackson ImmunoResearch Laboratories (West Grove, PA), washed twice, dried, and stained with CD4-specific FITC-conjugated mAb (100 μl, 1/2 dilution). After two final washes, a drop of mounting medium was added to the slides, and cells were analyzed under a Bio-Rad MRC-1024 confocal microscope.

CD3 endocytosis was monitored as previously described (31). Briefly, 2×10^6 cells/ml were preincubated in serum-free RPMI supplemented with 25 mM HEPES buffer (pH 7.2) and 1 mg/ml BSA for 30 min at 37°C to deplete transferrin from cells. Cells were washed and incubated with soluble anti-CD3 (UCHT1, IgG1) mAb for 30 min at 37°C (or 4°C, as a time 0 control). After two more washes in RPMI/HEPES, 1 mg/ml BSA, cells were further incubated at 37°C for an extra 0.5, 1, 2, 4, and 6 h, with 150 nM rhodamine-transferrin added for the last 15 min. Cells were then washed and fixed for 20 min at 4°C and 37°C in PBS containing 3.7% paraformaldehyde and 30 mM sucrose. Formaldehyde was quenched for 10 min in 50 mM NH_4Cl-PBS, and cells were permeabilized with BSA-PBS, 0.1% Triton X-100 at 37°C for 15 min, and incubated with FITC-coupled sheep anti-murine IgG1 Ab (1/100; Amersham) and anti-CD63 (1/500 1B5, IgG2b kindly donated by Dr. M. Marsh (Cell Biology Unit, MRC Laboratory for Molecular Cell Biology, University College, London, U.K.)). After three more washes in permeabilizing buffer, cells were re-stained with Alexa 488-coupled goat anti-fluorescein Ab (1/100; Molecular Probes, Eugene, OR) and Texas Red conjugated anti-mouse IgG2b (Southern Biotechnology Associates, Birmingham, AL). The anti-fluorescein Ab was required to enhance green fluorescence, which was otherwise barely detectable in γ^- cells in preliminary experiments. Finally, samples were washed three times in permeabilizing buffer and once in PBS, and mounted on microscope slides in Mowiol supplemented with 200 mg/ml DABCO (Sigma-Aldrich). The samples were examined under a Zeiss LSM 510 confocal microscope. A z-series of optical sections in 0.5-μm steps was performed. Red and green fluorescence emissions were acquired separately. No immunofluorescence staining was ever observed when second Abs were used without the first Ab or with an irrelevant first Ab. For whole-cell staining of CD3, cells were fixed, quenched, permeabilized, and stained with FITC-labeled anti-CD3 followed by Alexa 488-coupled goat anti-fluorescein Ab.

Results

TCR dynamics in nonstimulated γ^- T cells

Natural γ^- T cells from two unrelated individuals (identified as D.S.F. and F.K.) constitutively expressed abundant TCR at the cell surface, as detected both by cytofluorometry (Fig. 1*A*) and immunoprecipitation (Ref. 22 and Fig. 1*B*) with CD3- and TCR-specific monoclonals. However, these surface levels were consistently less than normal controls. The Jurkat mutant JGN (for γ negative) is shown to illustrate the fact that it lacks TCR expression completely, therefore precluding the analysis of TCR dynamics in the

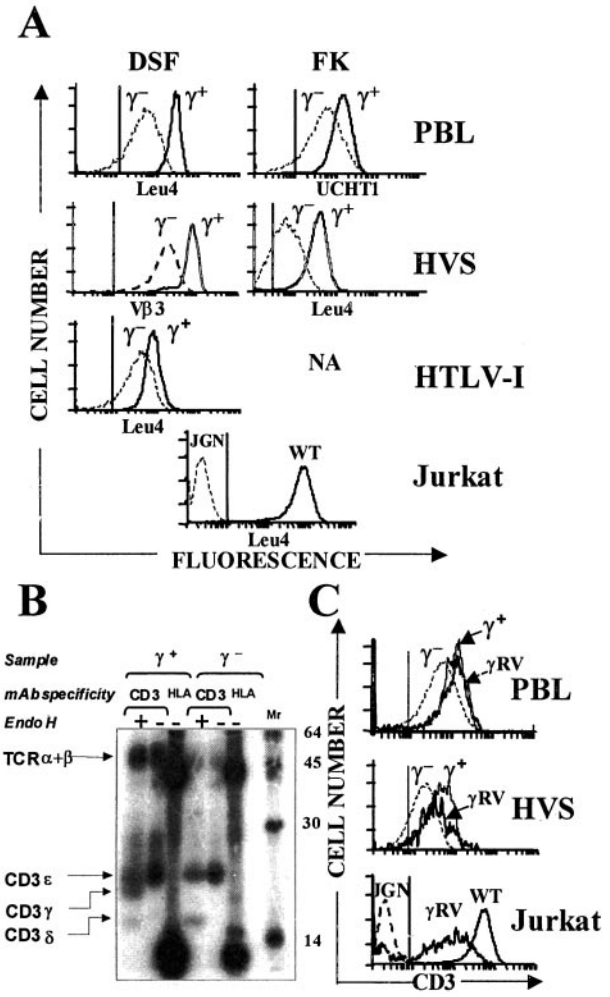

FIGURE 1. Abundant constitutive surface TCR expression in natural γ^- T cells as compared with Jurkat γ^- mutant JGN and correction by CD3γ transduction. *A*, Normal (γ^+) and γ^- natural CD4$^+$ T cells from two unrelated individuals (D.S.F., F.K.), either primary (PBL) or virus-transformed (HVS, HTLV-I), were compared with wild-type (WT) and γ^- (JGN) Jurkat T cells by flow cytometry. For Vβ3 expression, purified Vβ3$^+$ γ^- and γ^+ T cells were compared. NA, not available. *B*, TCR immunoprecipitation of radioiodinated HVS-transformed γ^- and γ^+ T cells digested, where indicated, with Endo H to reveal CD3γ. HLA class I molecules were analyzed as a control. The positions of the expected deglycosylated proteins are indicated by arrows. *C*, Comparative CD3 expression of transduced (thick lines) vs untransduced (dashed lines), natural γ^- (PBL, HVS from F.K. and D.S.F., respectively), and JGN (Jurkat) T cells exposed to a retroviral vector carrying CD3γ cDNA (γRV). CD3 expression by normal controls (γ^+, WT) is shown as thin lines, for reference.

absence of CD3γ. To demonstrate that CD3γ was the limiting factor for surface TCR expression, natural γ^- T cells from the two donors (PBL from F.K. and HVS-transformed T cells from D.S.F.) were retrovirally transduced with an intact CD3γ cDNA (γRV). The results showed that CD3γ transduction restored normal surface TCR levels in natural γ^- T cells, but not in JGN (Fig. 1*C*). This indicates that the reduced levels of TCR/CD3 cell surface expression in natural γ^- cells was caused only by the lack of CD3γ. The fact that the corrected γ^- T cells did not overexpress the TCR suggests that they did not develop intrathymically because of extraordinary receptor dynamics. Whereas the observed low TCR expression by natural γ^- T cells could be caused, at least in part, by the structural impact of the missing chain on Ab binding to the mutant complex, it could also be caused by an impaired TCR

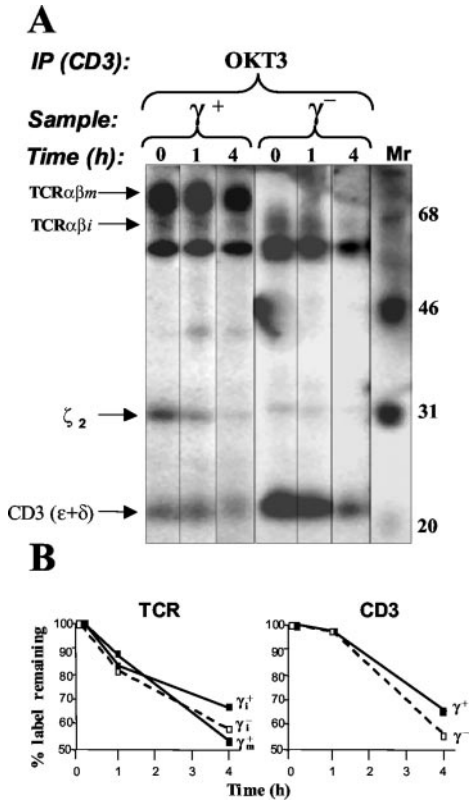

FIGURE 2. Half-life of constitutive surface TCR complexes is normal in resting γ^- T cells. *A*, γ^- and γ^+ CD4$^+$ T cells were surface biotinylated, incubated for different time periods, lysed, and immunoprecipitated with anti-CD3 (OKT3). After electrophoresis, the gel was immunoblotted with streptavidin-HRP and developed by chemiluminescence. Positions of the expected TCR and CD3 proteins are indicated by arrows (*m*, mature; *i*, immature). Note that CD3γ is poorly labeled by this procedure. *B*, Densitometry of labeled TCR and CD3 proteins relative to time 0. The impaired association of ζ to the mutant TCR and its lack of mature TCR$\alpha\beta$ dimers (22) precluded a similar analysis of these bands.

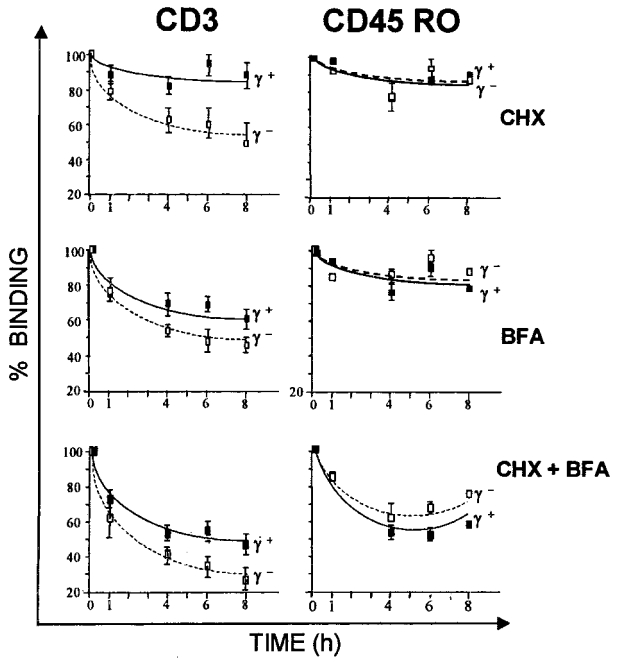

FIGURE 3. Surface TCR complexes are dependent on synthesis, normally internalized, and poorly recycled in resting γ^- T cells. The effect of CHX or BFA on the constitutive surface expression of CD3 and CD45RO in γ^- (□) and γ^+ (■) CD4$^+$ T cells was determined by flow cytometry at different times. Results are given relative to untreated cells. The mean ± SEM of six independent experiments is shown.

assembly and/or recycling, or by increased internalization or degradation of the mutant complex.

To address some of these possibilities, γ^- T cells were surface biotinylated, incubated for increasing times, and analyzed by immunoprecipitation to assess the half-life of the mutant complex (Fig. 2). The results confirmed that γ^- T cells expressed high amounts of CD3 chains (probably CD3$\delta\epsilon$ dimers; D. A. Zapata, W. Schamel, P. S. Torres, B. Alacón, and J. R. Regueiro, unpublished results) loosely associated to immature TCR$\alpha\beta$ dimers and especially to ζ dimers (Fig. 2*A* and Ref. 22). However, note that the loose association did not correlate with differential surface expression levels of CD3 and TCRβ (Fig. 1*A*), suggesting that γ^- TCR/CD3 complexes contain both TCR and CD3 chains, but that they are loosely associated and thus less resistant to the immunoprecipitation procedure. Quantification by densitometry of the biotinylated TCR and CD3 proteins, which in the case of γ^- T cells excluded the mature TCR$\alpha\beta$ and ζ dimers, showed that labeled TCR and CD3 chains were extinguished from γ^- and γ^+ T cells with similar kinetics ($t_{1/2}$ of 4.9 vs 4.2 h or 6 h for mature or immature γ^+ TCR, respectively, and 4.4 vs 5.5 h for CD3). This indicated that the half-life of the mutant receptor was essentially normal once it was expressed at the cell surface. That is, constitutive degradation of surface γ^- TCR was equivalent to that of wild-type TCR despite the lack of CD3γ.

In a second set of experiments we explored the relative contribution of synthesis and surface transport of new chains (as compared with internalization and recycling of chains expressed at the cell surface) to the constitutive TCR surface levels observed in the absence of CD3γ. This was achieved by comparative BFA treatment, which impairs both constitutive endoplasmic reticulum-Golgi transport (33, 34) and TCR recycling (but not internalization, Ref. 3), or by treatment with CHX, which selectively blocks protein synthesis. The results (Fig. 3) confirmed that, as described (3), the surface expression of normal γ^+ TCR was relatively synthesis independent. That is, surface γ^+ TCR/CD3 expression levels remained essentially stable in the presence of CHX within the studied 8-h time period. However, BFA treatment resulted in a strong, long-lasting partial reduction of TCR membrane levels. Because surface γ^+ TCR expression was independent of newly synthesized complexes, this reduction in TCR surface levels cannot be caused by the reported effect of BFA on anterograde transport from the endoplasmic reticulum to the Golgi compartment. Rather, it must be caused by the documented capacity of BFA to induce tubulation and fusion of the *trans*-Golgi network with early endosomes (33), which has more recently been shown to affect TCR endocytic transport and thus recycling (3). Therefore, normal surface TCR levels are maintained essentially through recycling of internalized molecules, with marginal contribution from newly synthesized components. In stark contrast, surface expression of the mutant γ^- TCR fell significantly upon CHX treatment, indicating that it was strongly dependent on synthesis and surface transport of new chains. Therefore, recycling of internalized complexes was insufficient to maintain membrane γ^- TCR levels in CHX-treated cells. A possible explanation for this unexpected effect could be an increased degradation of internalized complexes. However, impaired return to the cell surface could not be caused by increased γ^- TCR degradation, as it was shown previously to

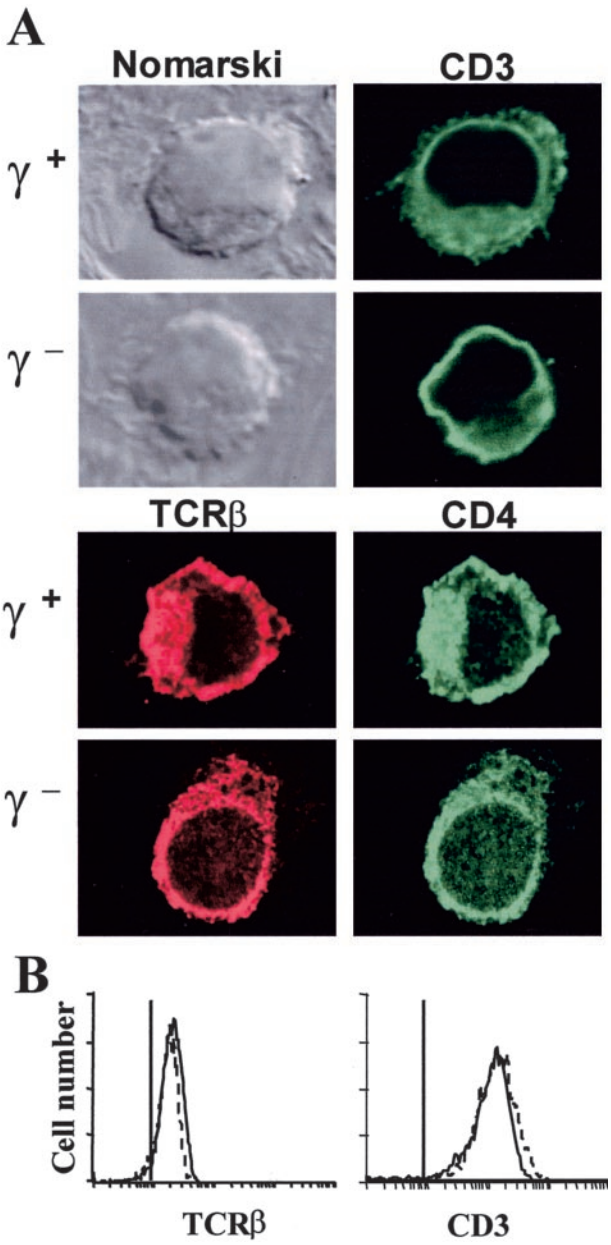

FIGURE 4. Intracellular expression of TCR in resting γ^- T cells. Permeabilized γ^- (dashed lines) and γ^+ (solid lines) T cells were stained for CD3 (UCHT1 in *A*, APA1/1 in *B*), or TCRβ and CD4 (RPA-T4) and analyzed by confocal microscopy (*A*) or flow cytometry (*B*).

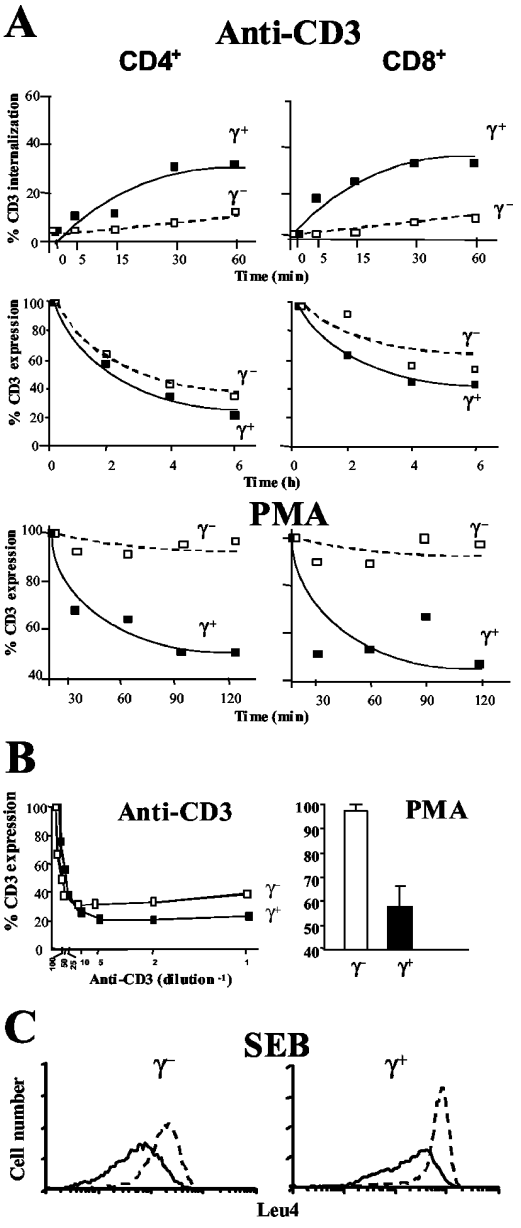

FIGURE 5. Ligand-induced TCR internalization is delayed in γ^- T cells. *A*, γ^- and γ^+ HVS-transformed T cells, either CD4$^+$ (*left*) or CD8$^+$ (*right*), were incubated with anti-CD3 (UCHT1-FITC) or PMA for different times. For CD3 internalization (*top*), cells were then washed and acid-treated to eliminate cell-surface associated (but not internalized) CD3 fluorescence. Results are given relative to untreated cells. The CD3 internalization rate was determined by linear regression analysis and is expressed as a percentage of the total surface-labeled CD3 that is internalized per minute. Data represent the mean of two experiments, and the SD was below 10% in all cases. Representative anti-CD3- (*middle*) and PMA-induced (*bottom*) CD3 down-modulation in γ^- and γ^+ T cells are included for comparison. *B*, Anti-CD3- and PMA-induced down-regulation in primary γ^- (from FK) and γ^+ T cells. *C*, SEB-induced (solid lines) TCR internalization of purified Vβ3$^+$ γ^- and γ^+ HVS-transformed T cells compared with unstimulated cells (dashed lines).

be normal (Fig. 2). Rather, re-assembly and/or surface transport of internalized γ^- TCR complexes might be affected. This suggested that constitutive membrane γ^- TCR levels were maintained mainly through biosynthesis, and only marginally through recycling, despite normal internalization. Accordingly, BFA treatment, which includes the CHX effect but in addition impairs recycling, caused only a small additional drop in surface γ^- TCR compared with CHX (Fig. 3). Expression of CD45RO, which is relatively independent of synthesis and recycling, was hardly affected by CHX or BFA treatment on γ^- and γ^+ cells. This demonstrated that the observed differences were specific for the TCR complex.

Collectively, the results indicated that the reduced constitutive expression of surface TCR in resting T cells lacking CD3γ was caused, at least in part, by an impaired assembly or surface transport of recycling complexes. In contrast, constitutive internalization and degradation were apparently normal despite the absence of CD3γ.

The reduced externalization and preserved internalization and degradation of surface TCR observed in γ^- T cells could lead to an increase in the pool of intracellular TCR and CD3 chains. This possibility was assessed qualitatively by confocal microscopy and quantitatively by flow cytometry (Fig. 4). The results showed that cytoplasmic staining was at least equivalent (TCRβ) or slightly increased (CD3) in γ^- T cells (compare Fig. 4*B* with Fig. 1*A*).

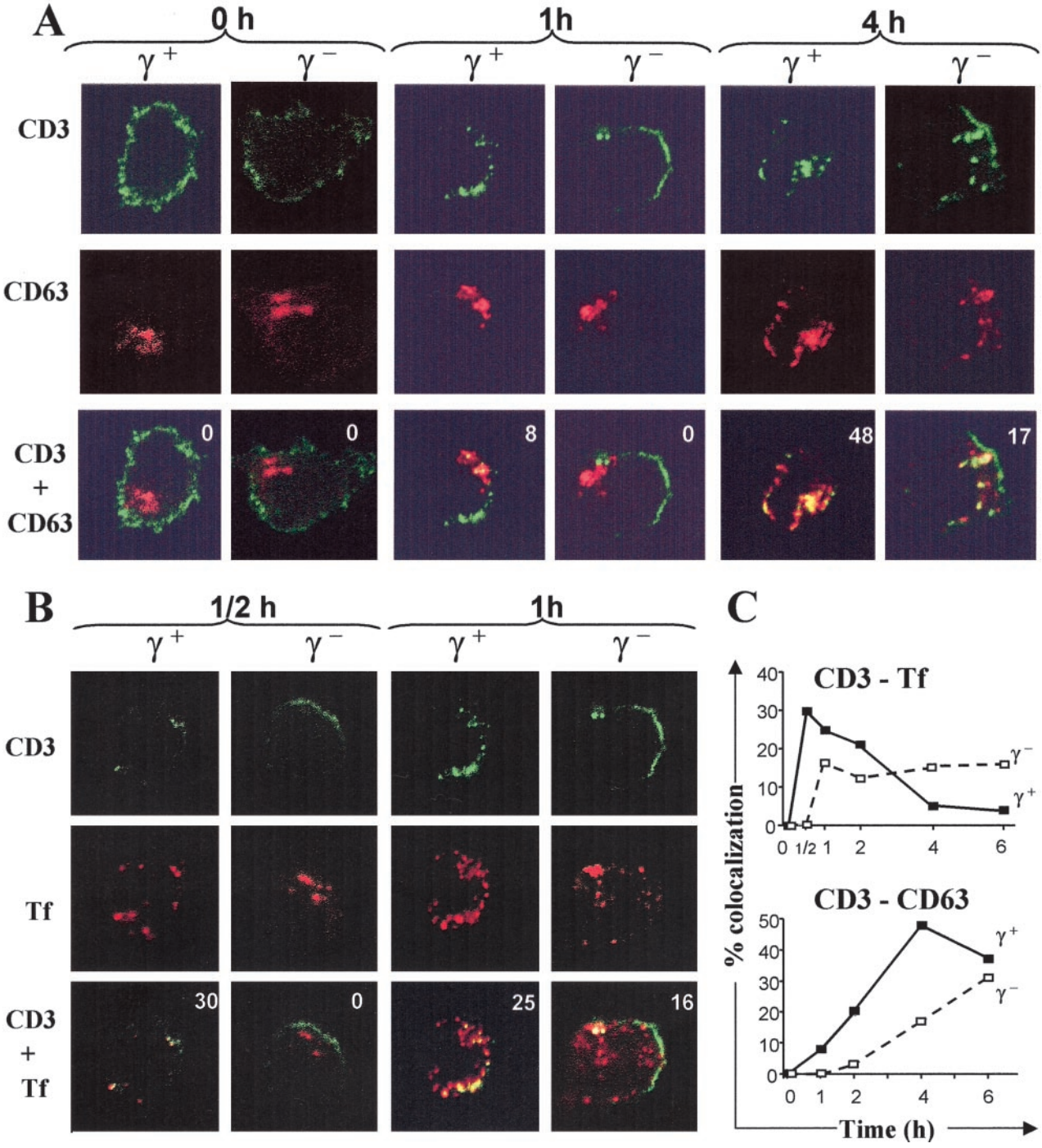

FIGURE 6. Entry of engaged TCR complexes into early endosomes is delayed in γ^- T cells. $CD4^+$ T cells were incubated for 30 min with anti-CD3 (UCHT1, IgG1) at 37°C (or 4°C for time 0), washed, and incubated in medium for different times at 37°C. Rhodamine-labeled transferrin (Tf) was added for the last 15 min. Cells were then fixed, permeabilized, stained with FITC-coupled anti-IgG1-specific second Ab, anti-CD63 (1B5, IgG2b) mAb, and anti-IgG2b-specific Texas Red-labeled second Ab, and analyzed by confocal microscopy. FITC (CD3) and rhodamine (Tf, in *B*) or Texas Red (CD63, in *A*) emissions were acquired separately. The images show a medial optical section of representative γ^- (*right*) and γ^+ (*left*) T cells at the indicated times. Areas of colocalization appear yellow in the computer-generated composite image, with the respective percentages indicated in white numbers. *C*, Colocalization kinetics of CD3 and Tf (early endosomes) or CD63 (lysosomes), respectively, in γ^- vs γ^+ T cells.

TCR dynamics in PMA- or ligand-stimulated γ^- T cells

Ligand binding dramatically alters TCR intracellular trafficking by blocking recycling and increasing degradation, resulting in down-modulation. We have previously reported that γ^- T cells from DSF down-modulated their TCR complex upon anti-CD3, but not PMA, treatment (23, 25), although less efficiently than γ^+ T cells (Fig. 5*A*, *middle* and *bottom*). This is also the case for the unrelated γ^- primary T cells from FK (Fig. 5*B*). As anti-CD3 mAb is not a physiological ligand for the TCR, it was important (with relevance to the patients) to establish whether Ag- or superantigen-induced TCR down-modulation was affected. Stimulus with SEB on purified $V\beta3^+$ T cells (Fig. 5*C*) confirmed that γ^- T cells could down-modulate their TCR in response to superantigen as well as anti-CD3. Therefore, mature T cells lacking CD3γ offered an

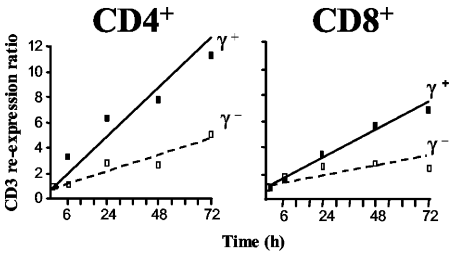

FIGURE 7. Re-expression of surface TCR/CD3 complexes is impaired in γ^- T cells. γ^- and γ^+ T cells, either $CD4^+$ (*left*) or $CD8^+$ (*right*), were incubated with anti-CD3 for 24 h to induce complete surface TCR down-regulation (see Fig. 5*A*). After washing, CD3 re-expression was monitored at different time points by flow cytometry (Leu4). The results are given relative to cells kept under anti-CD3 treatment.

opportunity to explore the relative contribution of this chain to the intracellular fate of bound TCR. The internalization rate of anti-CD3-engaged TCR was first studied by flow cytometry as described in *Materials and Methods* (Fig. 5*A*, *top*). The results indicated that stimulated γ^- T cells can internalize the mutant TCR complex, albeit at a slower rate than γ^+ T cells (0.18 vs 0.78%/minute in a $CD4^+$ T cell line, and 0.15 vs 0.8%/minute in a $CD8^+$ T cell line). Next, the intracellular fate of engaged TCR ensembles was followed intracellularly by confocal microscopy and colocalization studies, with transferrin used to mark early endosomes (Tf^+) and anti-CD63 to mark the late endosomal and lysosomal compartments (35) involved in degradation ($CD63^+$, Fig. 6). The results confirmed that engaged γ^- T cells can internalize bound TCR complexes and deliver them to early endosomes first (Fig. 6*B*), and later on to degradation compartments (Fig. 6*A*). Once again, the kinetics were delayed as compared with γ^+ T cells (see Fig. 6*C* for quantitative colocalization comparisons). It is of note that a mutant TCR cap still lingers on the cell surface of γ^- T cells 30 min or even 1 h after stimulus, times when in γ^+ T cells significant amounts of the normal complex are already internalized (Fig. 6*B*). At later time points (i.e., 4 h), the normal complex leaves transferrin-containing endosomes and colocalizes with the late endosomal/lysosomal compartment ($CD63^+$), whereas the mutant receptor is still significantly colocalized with transferrin and only partially located in late endosomes. However, finally the mutant TCR complex seems to catch up (Fig. 6*C*), suggesting that only early ligand-induced endocytosis is impaired rather than later entry into the degradative pathway. TCR degradation by γ^- T cells after ligand binding was also observed by surface biotinylation, acid treatment, and immunoprecipitation (data not shown). Therefore, constitutive (Fig. 2) as well as ligand-induced TCR degradation was independent of the CD3γ chain.

Because ligand binding finally down-regulated most TCR complexes from the γ^- T cell surface and targeted them for degradation, it offered the chance of analyzing TCR re-expression, which in normal T cells can take place initially only through new synthesis and surface transport and later on mostly through recycling. The results (Fig. 7) confirmed those obtained by CHX treatment of resting T cells (Fig. 3), namely that TCR re-expression was impaired selectively in γ^- T cells, most likely because of impaired TCR assembly or surface transport of recycling complexes.

Discussion

Our results indicate that CD3γ contributes to, but is not absolutely required for, the cell surface expression and intracellular trafficking of TCR complexes both in resting and in ligand-stimulated T lymphocytes (Fig. 8). Indeed, lack of CD3γ clearly impairs the most significant metabolic steps of constitutive TCR output, namely assembly and surface transport of internalized complexes as shown by two independent assays (CHX treatment, Fig. 3; and re-expression, Fig. 7). As a consequence, most surface γ^- TCR was constitutively provided by synthesis of new chains rather than by recycling of expressed ones. This suggests that the biosynthetic pathway is more permissive than the recycling pathway for incomplete complexes. The impaired recycling may explain the lower surface TCR levels seen in resting γ^- T cells (Figs. 1 and 6*A*) and correlates with an impaired maturation of TCR chains (Fig. 2*A*) and the intracellular accumulation of TCR chains. This was shown consistently by intracellular staining (Fig. 4*B*, Ref. 22) and metabolic labeling experiments (22). Immunoprecipitation experiments showed that γ^- CD3 complexes ($\delta\epsilon$ dimers) are poorly associated to TCR and ζ dimers as compared with γ^+ CD3 complexes (Figs. 1*B* and 2*A*). The contribution of CD3γ to TCR assembly or membrane transport, and thus to constitutive surface TCR expression, may map to the extracellular or transmembrane domains, because it has been shown that a partially truncated or even a tailless CD3γ (or δ, Ref. 36) can rescue normal TCR expression in human or murine T lineage cells (14, 17, 24).

The specific steps affected by the lack of CD3γ may not be equivalent in resting vs activated T cells. For instance, the lack of CD3γ does not seem to affect constitutive TCR internalization

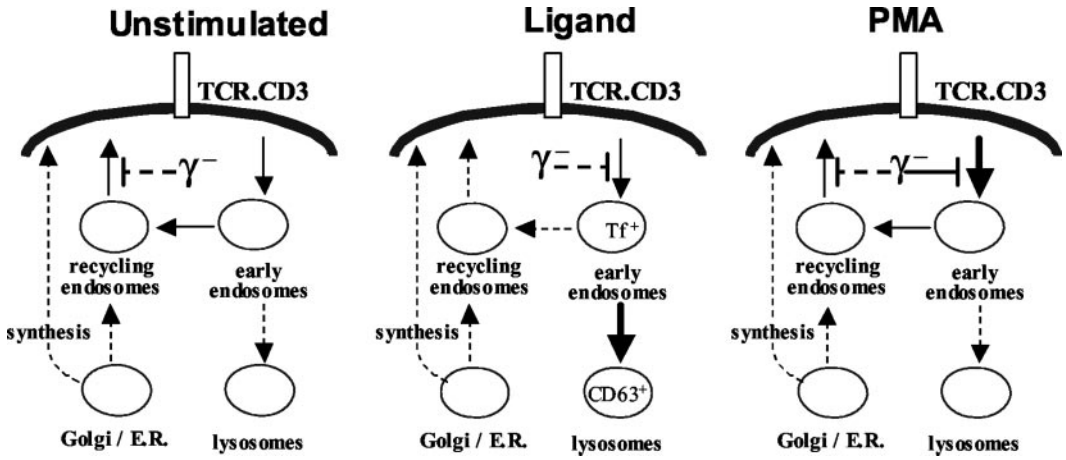

FIGURE 8. Proposed contribution of CD3γ to TCR dynamics. The impaired (- - -|) or blocked (—|) steps affected in γ^- T cells are indicated in this simplified model of TCR dynamics in resting or activated T cells (adapted from Ref. 2).

(Figs. 2 and 3), whereas ligand-induced (Figs. 5 and 6) or PMA-induced (Fig. 5*A*) TCR internalization are delayed or absent, respectively (Fig. 8). Constitutive or ligand-induced entry into degradative compartments, however, seem to be CD3γ independent (Figs. 2 and 6). These results are consistent with the notion that TCR internalization is regulated differently in resting vs Ag-stimulated T lymphocytes, as reported in a very recent study (5). These results are also consistent with the requirement of a CD3γ leucine-based internalization motif for efficient ligand-induced TCR down-regulation (9), as well as with the participation of invariant chains other than CD3γ in this process (3, 10–12, 14, 37). Ligand-induced TCR down-regulation is believed to involve both protein kinase C/CD3γ-dependent and protein kinase C-independent activation pathways (9). Our data indicate that the latter are sufficient to induce TCR down-regulation, although the former are required for full down-regulation.

Our results seem to be in conflict with the proposed critical role of a CD3γ motif in constitutive TCR internalization (4, 5). However, those experiments were performed with γ^- Jurkat mutant cells (JGN) that were originally selected for nonexpression of the TCR (Fig. 1*A*). The role of CD3γ is difficult to address before transfection in these cells. Because human (20) and murine (21) γ^- mature T cells show abundant surface TCR expression, JGN may potentially harbor additional mutations affecting TCR surface transport dynamics. Indeed, JGN-derived transfectants with or without an intact CD3γ leucine-based internalization motif, but not a newly derived mutant termed E3, showed equivalent ligand-induced TCR down-regulation, which the authors interpreted as proof that JGN carries an additional defect in TCR regulation (9). Also, in contrast with natural γ^- T cells (Fig. 1*C*, *top* and *middle*), reconstitution of JGN or E3 with expression vectors encoding CD3γ did not recover full surface TCR expression, suggesting that additional transport defects were present in the latter (Refs. 9 and 38 and Fig. 1*C*, *bottom*). It would be interesting to study mutants selected for low rather than absent TCR surface expression.

Ag-induced TCR down-modulation may be an important mechanism to attenuate TCR-mediated signaling after recognition, as promoting it impairs T cell restimulation (4, 8, 39) and blocking it enhances T cell activation (40, 41). γ^- T cells show a slightly impaired ligand-induced down-modulation, probably because of a delayed internalization step (Figs. 5 and 6). However, TCR-mediated signaling was not enhanced. Rather, it was either normal or impaired, depending on the response (20–24, 42). This discrepancy may be caused by the signaling role of CD3γ itself, and therefore requires further clarification by transfection of truncated CD3γ chains that restore normal TCR dynamics (24). It could be argued that γ^- T cells may express the TCR because of extraordinary receptor dynamics imposed by intrathymic selection constraints. However, CD3γ transduction in natural γ^- T cells from two unrelated individuals resulted in the restoration of normal constitutive surface TCR, not overexpression (Fig. 1*C*). The fact that murine γ^- or δ-deficient mature T cells can also express significant surface TCR$\alpha\beta$ levels supports a certain redundancy between these homologous CD3 chains, as shown in previous phylogenetic studies (43). Also, the recent demonstration of the unexpected absence of CD3δ from TCR$\gamma\delta$ complexes (44) further suggests that TCR ensembles are very flexible structures.

In summary, using different γ^- T cell lines, we have shown in this study that the TCR can be constitutively synthesized, exported, internalized, and degraded, but not recycled, in the absence of CD3γ, probably causing its reduced surface levels. Once engaged, early endocytosis was delayed, although down-modulation and degradation finally took place (Fig. 8). TCR re-expression after down-modulation was also reduced, pointing again to a defect in assembly and surface transport during recycling. Therefore, CD3γ improves but is not absolutely required for TCR regulation. Such improved regulation may be crucial for survival, as dramatically illustrated by the case of a γ^- patient who died before his third birthday with severe combined immunodeficiency syndrome (45). However, his γ^- brother (DSF) and an unrelated γ^- individual (FK) are presently healthy and in their teens (26), suggesting a certain redundancy among CD3 chains (46). The reasons for this clinical variability are not understood at present. They are independent of TCR expression levels, because all three γ^- individuals showed a similar surface TCR phenotype (Fig. 1*A*, and Ref. 47). But they could be caused by the existence of undefined modifying genes in these individuals, as shown in other immunodeficiencies (48).

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