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Expression of the T Cell Antigen Receptor ζ Chain following Activation Is Controlled at Distinct Checkpoints

IMPLICATIONS FOR CELL SURFACE RECEPTOR DOWN-MODULATION AND RE-EXPRESSION*

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The multisubunit T cell antigen receptor (TCR) is involved in antigen recognition and signal transduction, leading to T cell activation and rapid down-modulation of the cell surface expressed TCRs. Although the levels of TCR cell surface expression are pivotal to the efficiency and duration of the immune response, the molecular mechanisms controlling TCR down-modulation and re-expression upon activation, remain obscure. Here, we provide a biochemical characterization of the regulatory mechanisms governing TCR expression following long-term T cell activation. We focused primarily on the TCR ζ chain, as this is considered the limiting factor in TCR complex formation and transport to the cell surface. We found that following TCR-mediated activation ζ mRNA is up-regulated by a transcription-dependent mechanism. Concomitantly, ζ protein levels are modified according to a biphasic pattern: rapid degradation coinciding with TCR cell surface down-regulation, followed by a rebound to normal levels 24 h subsequent to T cell activation. Even though there are adequate levels of all the TCR subunits within the cell following 24 h of activation, TCR cell surface expression remained very low, provided the activating antibody is continuously present. Correlative with the latter, we detected a previously undescribed monomeric form of the ζ chain. This form could be indicative of adverse endoplasmic reticulum conditions affecting correct protein folding, dimerization, and TCR assembly, all critical for optimal receptor surface re-expression. Cumulatively, our results indicate that the levels of TCR expression following activation, are tightly controlled at several checkpoints.

The T cell antigen receptor (TCR)[1] is a multisubunit complex composed of the clonotypic α/β heterodimer that is involved in the recognition and binding of the antigen-major histocompatibility complex, as well as of the invariant CD3 chains (γ, δ, ε) and the ζ/ζ homodimer that couple antigen recognition to intracellular signal transduction pathways. Optimal T cell activation is achieved by the delivery of two signals: one mediated via the TCR upon binding of the antigen-major histocompatibility complex presented by antigen-presenting cells, and the other is delivered through costimulatory receptors (1–3). Subsequent to TCR engagement, long lasting TCR down-regulation has been observed (4), resulting in a state of sustained desensitization and unresponsiveness to renewed antigenic stimuli (5, 6). Interestingly, it was recently shown that distinct modes of T cell activation have a differential effect on the fate of cell surface expressed TCRs: whereas TCR ligation induces rapid TCR internalization and degradation (7, 8), T cell stimulation with PMA, which by-passes the TCR, leads to TCR internalization and recycling (9). These observations suggest that activation-induced TCR down-regulation may trigger the termination of an immune response and/or induce tolerance (10). Eventually, the TCRs are re-expressed on the cell surface and the cells regain their responsiveness to antigenic stimuli. Accordingly, the mechanisms controlling the down-regulation of the various TCR subunits, their synthesis, assembly, and re-expression on the cell surface appear to play a critical role in T cell function. Several key factors govern the assembly and transport of multisubunit receptors to the cell surface. The molecular chaperons calnexin and calreticulin have been implicated in facilitating TCR assembly in the endoplasmic reticulum (ER) (11–13), and oxidizing redox conditions in the ER were found to be essential to the correct folding and disulfide bond formation of the proteins involved (14, 15). Although all TCR subunits are required for the formation of the complex, the ζ chain has a distinctive role in TCR assembly and cell surface transport: it was demonstrated that the assembly proceeds according to a certain order, in which the last component to join the complex is the disulfide-linked ζ-ζ homodimer (16). In addition, it was shown that in T cell hybridomas ζ chain is synthesized in restricted amounts compared with the reminder TCR chains (16, 17). Moreover, in ζ-deficient T cell hybridomas, partial TCR complexes devoid of the ζ chain were primarily targeted to lysosomal degradation. The few ζ-deficient TCRs (~5%) that reached the cell surface were non-functional (17). Similar results were obtained in T cells isolated from ζ-deficient mice (18–20). These observations suggest that ζ is the limiting chain for optimal receptor assembly and that it is critical to TCR cell surface expression.

In the study presented here, we address the question of how T cell activation affects ζ expression and consequently, TCR expression. Our investigation was based on our previous observations (21), demonstrating that the activity of the ζ gene 5′-flanking region is transcriptionally up-regulated following T cell stimulation. We now describe the effect of T cell activation on endogenous ζ chain expression and how the latter affects the entire TCR complex. Our results show that ζ chain expression, as opposed to that of the other TCR subunits, is tightly and

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[1] The abbreviations used are: TCR, T cell antigen receptor; ER, endoplasmic reticulum; CsA, cyclosporin A; CHX, cyclohexamide; Act-D, actinomycin D; PMA, phorbol 12-myristate 13-acetate; MES, 2-[*N*-morpholino]ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

uniquely controlled at several checkpoints. Following T cell activation, ζ protein levels are modified in a biphasic pattern as reflected by an initial rapid degradation, which is followed by a transcriptionally dependent recovery. Finally, we demonstrate that even though adequate levels of ζ chain and the other TCR subunits are expressed within the cell following 24 h of activation, virtually no TCRs appear on the T cell surface. These results indicate that there is yet another checkpoint which controls TCR assembly and/or transport to the cell surface following activation.

Taken together, our observations show that following T cell activation, TCR expression is tightly controlled as reflected by its rapid down-modulation and slow re-expression. The unique characteristics of ζ chain expression detected during these processes highlight the key role played by ζ in this cascade of events.

EXPERIMENTAL PROCEDURES

Animals—BALB/C female mice were bred in the Hebrew University SPF facility. Mice aged 2–3 months were used in all experiments.

Cells—The murine thymoma cell line EL-4 was maintained in complete RPMI 1640 medium containing 8% fetal calf serum. Splenocytes were harvested and cultured overnight in complete RPMI medium to minimize basal activation levels.

Antibodies and Reagents—The 145-2C11(2C11) monoclonal antibody is directed against the murine CD3ε chain (22) and was used as a diluted ascites fluid or hybridoma supernatant. The monoclonal anti-ζ antibody H146, was kindly provided by Dr. R. Kubo (23) and Dr. D. Weist. Anti-CD3δ polyclonal antibodies were generated in rabbits as described (24). Cyclohexamide (CHX), actinomycin D (Act-D), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Cyclosporin A (CsA) was obtained from Sandoz, Switzerland.

T Cell Activation—EL-4 cells were cultured at 5×10^5 cells/ml in the absence or presence of 2 ng/ml PMA. For activation of normal T cells, 2×10^6 splenocytes/ml were cultured in 24-well plates (Nunc, Denmark) in the presence of anti-CD3ε antibodies (ascites fluid diluted 1:2000). CsA (2 μg/ml) was added in conjunction with anti-CD3ε antibodies, as specified. In experiments using CHX (10 μg/ml) and/or Act-D (5 μg/ml), cells were first activated for 12 h as described above, and then the inhibitors were added to the cell cultures for the time periods specified.

Northern Blot Analysis—Total RNA was isolated from cell pellets ($20–25 \times 10^6$ splenocytes or 5×10^6 EL-4 cells), using an RNAzol kit (Biotex, Houston, TX). Total RNA (15–20 μg) was separated on a formaldehyde-agarose gel and transferred to a Hybond-N membrane (Amersham, United Kingdom). Specific mRNAs were detected by hybridization with ζ and δ cDNA probes labeled with [α-^{32}P]dCTP (Amersham) according to the random primer labeling method (25). Scanning densitometry was performed using a Bio-Rad imaging densitometer and Molecular Analyst software.

Cell Lysis, Immunoprecipitation, Electrophoresis, and Immunoblotting—Splenocytes (200×10^6/ml) were lysed as described previously with either Tris (26) or MES (27) buffers containing 0.5% Triton X-100, protease, and phosphatase inhibitors. Cell pellets were lysed for 15 min on ice with gentle mixing and centrifuged (15,000 rpm) for 10 min at 4 °C. After centrifugation, the supernatants were separated and designated the detergent-soluble fractions while the pellet was designated the detergent-insoluble fraction. The soluble fraction was immunoprecipitated with anti-CD3ε antibodies and samples were subjected to 13% SDS-PAGE or to two-dimensional non-reducing/reducing SDS-PAGE as described previously (26). The separated proteins were transferred to nitrocellulose filters. Filters were incubated with the desired specific antibody, washed, and incubated with protein A-horseradish peroxidase conjugate. The specific proteins were detected using the enhanced chemiluminescence system (ECL, Amersham).

Cell Surface Labeling—For labeling of cell surface-expressed proteins, cells (10×10^6/ml) were subjected to biotinylation in a buffer (pH 7.4) containing 20 mM Hepes, 150 mM Nacl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 50 μg/ml D-biotynil-ε-amidocaproicacid-*N*-hydroxysuccinimide ester (biotin-ester) (Roche Molecular Biochemicals), for 50 min at 25 °C. The reaction was terminated by the addition of 10 mM ammonium chloride and washes with phosphate-buffered saline at 4 °C.

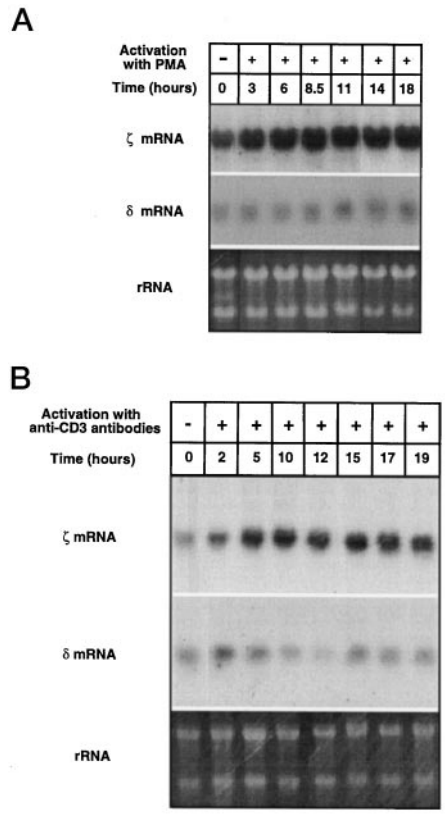

FIG. 1. **ζ mRNA is up-regulated following T cell activation.** *A,* EL-4 cells (5×10^5/ml) were incubated with PMA (2 ng/ml) for the indicated time intervals. Cells were harvested, total RNA was prepared and subjected to Northern blot analysis as described under "Experimental Procedures." Specific ζ and δ mRNA were detected by hybridization with the respective ^{32}P-labeled cDNA. *B*, normal splenocytes (2×10^6/ml) were incubated with anti-CD3ε antibodies (ascites 1:2000) for the indicated time, the cells were then harvested and specific mRNA was analyzed as described in *A*.

RESULTS

ζ mRNA Is Up-regulated following T Cell Stimulation—We previously showed that the ζ gene 5′-flanking region is responsive to PMA stimulation (21). To determine whether the endogenous ζ gene is also affected by PMA stimulation, we measured ζ mRNA levels in EL-4 cells stimulated with PMA. As shown in Fig. 1*A*, ζ mRNA levels were up-regulated early after PMA treatment and remained high during 18 h of continuous stimulation. mRNA levels of CD3δ (Fig. 1*A*), were relatively unaffected by this treatment. Although cultured EL-4 cells are frequently used as a model for various T cell activation studies, they differ significantly from normal T lymphocytes in their functional characteristics. Therefore, we used an experimental system that more faithfully mimics physiological conditions, namely a whole splenocyte population to which anti-CD3 antibodies were added as stimulators. The anti-CD3 antibodies were "presented" by splenic antigen-presenting cells bearing Fcγ receptors, allowing cell-cell interactions and T cell activation mediated via the TCR and costimulatory molecules. As shown in Fig. 1*B*, upon TCR-mediated activation, ζ mRNA was up-regulated in normal T cells similarly to what was observed in EL-4 cells (Fig. 1*A*). Maximum levels of up-regulated ζ mRNA were generally 2–3-fold higher than in untreated cells. Unlike ζ mRNA, which remained highly up-regulated up to 19–24 h following stimulation, CD3δ mRNA was relatively unchanged under the same experimental conditions. These results indicate that TCR-mediated mRNA up-regulation is not

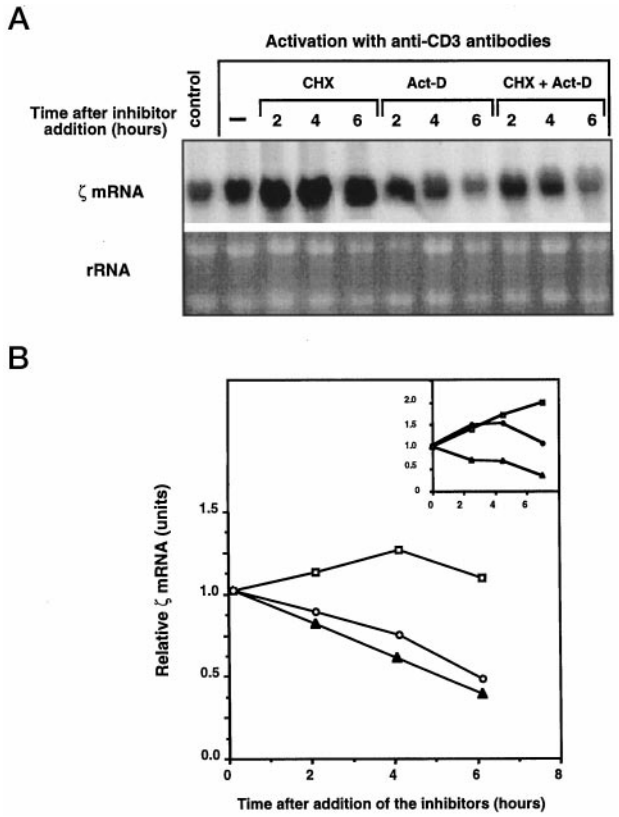

FIG. 2. **Effect of CHX and/or Act-D on activation-induced ζ mRNA up-regulation.** *A,* splenocytes (2×10^6/ml) were activated with anti-CD3ε antibodies (ascites 1:2000) or nontreated (control) for 12 h. At this time point the cells were harvested (−) or treated with CHX (10 μg/ml) and/or Act-D (5 μg/ml). After being cultured in the presence of inhibitors for the indicated periods of time, the cells were harvested and ζ mRNA was analyzed as described in the legend to Fig. 1. *B,* ζ mRNA values in *A* were estimated by densitometry and corrected for rRNA ethidium staining. Arbitrary ζ mRNA units (relative units) were calculated as: ζ mRNA in activated cells after inhibitor addition/ζ mRNA in activated cells, and plotted as a function of time after addition of the inhibitors. *Inset,* splenocytes (2×10^6/ml) were untreated or treated with CHX and/or Act-D for the indicated periods of time. ζ mRNA values were estimated and corrected for rRNA ethidium staining as in *B*. Relative ζ mRNA units were calculated as: ζ mRNA in treated cells/ζ mRNA in non-treated cells, and plotted as in *B*. □, CHX; ▲, Act-D; ○, CHX + Act-D.

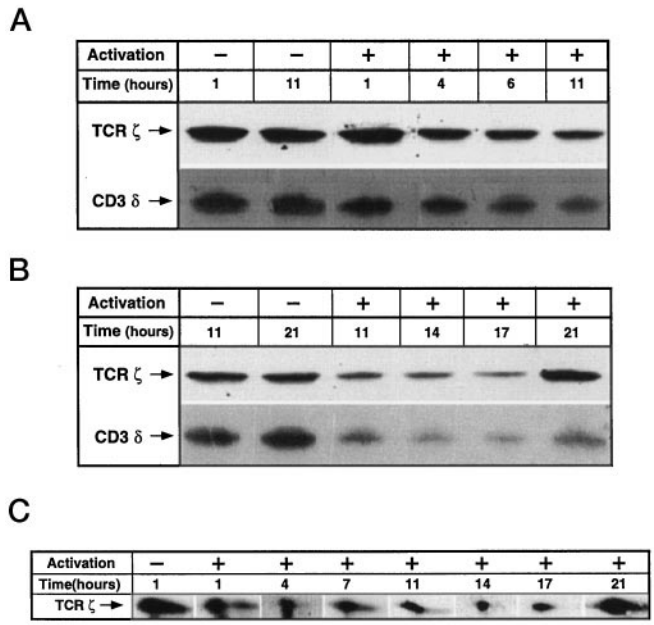

FIG. 3. **T-cell activation induces rapid ζ protein degradation followed by a return to normal levels following 21 h of activation.** Splenocytes (2×10^6/ml) were non-activated or activated with anti-CD3ε antibodies (ascites 1:2000) for short (*A*) or long (*B*) periods of time. The cells were harvested, washed, and lysed as described under "Experimental Procedures." The samples were reduced, and subjected to 13% SDS-PAGE. After being transferred to nitrocellulose filters, the filters were incubated with anti-ζ monoclonal antibodies (H146) or anti-CD3δ polyclonal antibodies, followed by washing and incubation with protein A-horseradish peroxidase. Specific proteins were detected using the enhanced chemiluminescence system (ECL). *C,* splenocytes were activated as in *A* and *B*. Cells were washed and lysed and the detergent-insoluble fraction was obtained as described under "Experimental Procedures." Samples were separated on a two-dimensional (non-reducing/reducing) SDS-PAGE and specific proteins were detected as described above.

characteristic of all T cell receptor genes.

Activation-dependent ζ mRNA Up-regulation Is Controlled Mainly at the Transcriptional Level—To determine at which level activation-dependent ζ mRNA up-regulation is controlled, we designed experiments using the transcriptional inhibitor Act-D and/or the translational inhibitor CHX. The inhibitors were added to the cells 12 h after their activation with anti-CD3 antibodies. Cells were harvested at different time intervals after the addition of the inhibitors and ζ mRNA was analyzed. In cells treated with CHX, ζ mRNA levels were superinduced, as compared with those in non-treated activated cells (Fig. 2, *A* and *B*), indicating that a short-lived protein controls ζ mRNA levels, either transcriptionally (repressor) or post-transcriptionally (RNase). When activated cells were treated with Act-D, ζ mRNA levels rapidly dropped to the level measured in non-activated cells (Fig. 2, *A* and *B*). The addition of CHX to activated cells together with Act-D neither prevented a reduction in ζ mRNA nor significantly stabilized ζ mRNA levels, as compared with the results obtained with Act-D alone (Fig. 2, *A* and *B*). These findings indicate that activation-induced ζ mRNA up-regulation is mainly controlled at the transcriptional level. Interestingly, treatment of resting cells with CHX and Act-D (Fig. 2*B, inset*) demonstrated that ζ mRNA steady-state levels are controlled at the transcriptional and post-transcriptional levels, suggesting that steady-state ζ expression and activation-dependent ζ mRNA up-regulation are controlled by distinct mechanisms. The results of the experiments presented here are in accord with our previous studies (21) demonstrating that the activity of the ζ gene 5′-flanking region is up-regulated following T cell stimulation. Our findings strongly indicate that activation-dependent ζ mRNA up-regulation is transcriptionally controlled.

T Cell Activation Induces Rapid ζ Chain Degradation followed by Recovery to Normal Levels—After finding that activation of normal T cells induces ζ mRNA up-regulation, we analyzed the effect of T cell activation on ζ protein expression. As shown in Fig. 3*A*, the ζ chain undergoes rapid degradation in activated T cells: between 1 and 4 h following stimulation 50% of the ζ protein was degraded. ζ Protein levels remain low during at least 17–19 h of continuous stimulation (Fig. 3*B*). Only after 21–24 h of activation did the ζ protein levels return to the basal level. Similar results were obtained for CD3 δ, albeit the recovery rate was slower. Immunoblotting with antibodies directed against the CD3γ and CD3ε chains revealed a pattern essentially similar to the one obtained for the δ chain (data not shown). These findings indicate that the rapid degradation observed following T cell stimulation is a property common to all the TCR subunits. However, the recovery rate of ζ and δ differs in that the latter does not attain the level of untreated cells even after 21–24 h of stimulation. These results

provide further evidence that in contrast to the CD3 γ, δ, and ϵ subunits, ζ protein levels are differentially regulated.

We have previously identified two TCR populations expressed on the T cell surface, one of which is linked to the actin-based cytoskeleton via the ζ chain and localized to the detergent-insoluble cytoskeleton-enriched fraction (Refs. 26 and 27 and reviewed in Ref. 28). The latter population includes 20–40% of the receptors. Furthermore, we (27) and others (29) have shown that upon T cell activation (within 2–15 min), 30–50% of the detergent-soluble ζ chains translocate to the cytoskeletal fraction and become detergent-insoluble. Thus, it was necessary to determine whether the reduced ζ protein levels observed following activation in the detergent-soluble fraction are due solely to degradation, or also involve massive translocation to the cytoskeleton. We found that the activation-dependent ζ protein degradation also occurs in the detergent-insoluble fraction (Fig. 3*C*) with kinetics closely resembling those of the soluble fraction (Fig. 3, *A* and *B*).

ζ mRNA Up-regulation Is a Prerequisite for ζ Protein Recovery to Normal Levels following TCR Stimulation—We demonstrated that upon T cell activation, ζ chain is rapidly degraded and its recovery to normal levels occurs 21–24 h after continuous stimulation (Fig. 3). In parallel, upon activation and prior to the recovery of the ζ protein, ζ mRNA was up-regulated (Fig. 1*B*). This sequence of events, graphically depicted in Fig. 4*A*, led to the supposition that ζ protein recovery depends on ζ mRNA up-regulation. To test this hypothesis, we used CsA to block activation-dependent ζ mRNA up-regulation. The rationale for using CsA was based on our computer analysis of the ζ gene 5′-flanking sequence, that allowed us to localize a putative NF-AT responsive element (21). It is well established that CsA exerts its inhibitory effect on the activation-dependent gene transcription of several cytokine and cell surface receptors, primarily by repressing the function of the transcription factor NF-AT (30, 31). This raised the possibility that CsA could inhibit activation-induced ζ mRNA up-regulation. To this end, we activated normal splenocytes in the presence or absence of CsA and analyzed ζ mRNA expression. As depicted in Fig. 4*B*, ζ mRNA was up-regulated in splenocytes following TCR-mediated activation. This response was largely impaired in the presence of CsA. The incomplete abrogation of activation-dependent ζ mRNA up-regulation by CsA was most likely due to the basal activation levels of the splenocytes used in the experiment: NF-AT has already been translocated to the nucleus. CD3δ expression was relatively unaffected under these experimental conditions. These results imply that the NF-AT transcription factor is directly or indirectly involved in T cell activation-induced ζ mRNA up-regulation. Furthermore, these observations are in accord with our results (Fig. 2) indicating that activation-induced ζ mRNA up-regulation is controlled primarily at the transcriptional level.

Based on the above findings, we next analyzed whether ζ mRNA up-regulation is required for the recovery of ζ protein expression to the normal level. To test this hypothesis, splenocytes were activated in the presence or absence of CsA and the levels of ζ protein were analyzed. Fig. 4*C* shows that CsA also impaired full recovery of ζ protein to normal levels after activation-induced degradation. Therefore, blockage of ζ mRNA up-regulation by CsA also prevents ζ protein recovery to normal levels. Hence, activation-induced ζ mRNA up-regulation appears to be required for assuring ζ protein recovery. However, the involvement of additional CsA-sensitive factors in ζ protein recovery, other than ζ mRNA, cannot be ruled out.

The Failure of Activated T Cells to Express Cell Surface TCR following 24 h of Activation Is Correlated with the Appearance of a Monomeric ζ Form—Following 24 h of activation, ζ protein

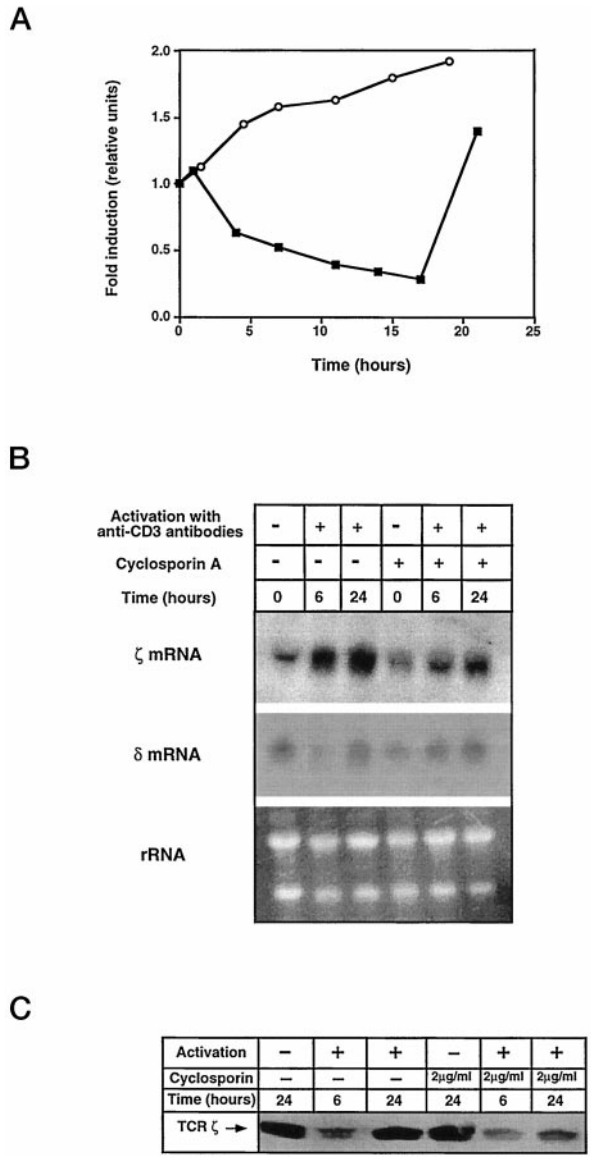

FIG. 4. **CsA blocks activation-induced ζ mRNA up-regulation and ζ protein recovery to normal levels.** *A,* fold induction of ζ (mRNA or protein from Figs. 1 and 3, respectively), was calculated as the ratio between the ζ values measured in activated cells and the ζ values measured in non-activated cells. The ζ mRNA (○) and protein (■) values (relative units) were estimated by densitometry and the ζ mRNA values were corrected for rRNA ethidium staining. ζ Fold induction was plotted as a function of activation time. *B,* splenocytes (2×10^6/ml) were non-activated or activated with anti-CD3ϵ antibodies (ascites 1:2000), in the presence or absence of 2 μg/ml CsA. At the indicated time points cells were harvested and specific mRNA was analyzed as described in the legend to Fig. 1. *C,* splenocytes (2×10^6/ml) were non-activated or activated with anti-CD3ϵ antibodies (ascites 1:2000), in the presence or absence of 2 μg/ml CsA. Cells were harvested and ζ protein expression was analyzed as described in the legend to Fig. 3.

levels return to normal and significant amounts of δ protein (Fig. 3*B*) and of the other TCR components (data not shown), are present as assessed by Western blot analysis. We next examined whether the newly synthesized TCR subunits are assembled and re-expressed on the cell surface. For this purpose, we activated the cells for 24 h and then labeled cell surface expressed proteins by biotinylation. Cells were lysed, immunoprecipitated with anti-CD3 antibodies, and the co-immunoprecipitated proteins were separated by two-dimensional (non-reducing/reducing) SDS-PAGE, as described under "Experimental Procedures." Fig. 5*A* (*left panels*) shows a typical experiment where the CD3 and α/β TCR subunits are readily

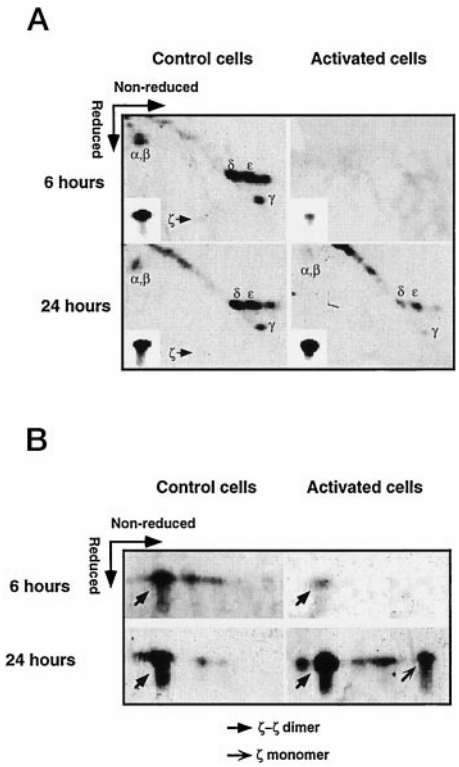

FIG. 5. **Failure of TCR surface re-expression following 24 h activation is correlative with the appearance of a monomeric ζ form.** *A,* splenocytes (2×10^6/ml) were activated with anti-CD3ε antibodies (ascites 1:2000) for 6 or 24 h. They were then harvested, washed with ice-cold phosphate-buffered saline, subjected to surface biotinylation as described under "Experimental Procedures," and lysed. The TCR components were immunoprecipitated with anti-CD3ε (2C11) monoclonal antibodies. Samples were separated by two-dimensional (non-reduced/reduced) SDS-PAGE and transferred onto nitrocellulose filters. Biotinylated proteins were visualized by incubating the filters with streptavidin-horseradish peroxidase conjugate, followed by the ECL reaction. The loading of comparable amounts of protein and the presence of co-immunoprecipitated ζ protein was confirmed by immunoblotting the filters with anti-ζ antibodies (*inset*). *B,* splenocytes (2×10^6/ml) were activated with anti-CD3ε antibodies (ascites 1:2000) for 6 or 24 h. The cells were then harvested, lysed, and ζ protein was immunoprecipitated using anti-ζ antibodies. Samples were separated by two-dimensional (non-reduced/reduced) SDS-PAGE and transferred to nitrocellulose filters. Specific proteins were detected by immunoblotting with anti-ζ antibodies. The position of the ζ-ζ dimer and the ζ monomer is indicated by *arrows*.

biotin-labeled whereas the ζ chain is poorly detected. Unlike the CD3 and α/β subunits, which comprise large extracellular domains containing 4–9 lysine residues (the major target sites for biotinylation), the ζ chain has an extracellular domain of nine amino acids with only one lysine residue. Therefore, ζ is less efficiently biotinylated than the other TCR subunits. The presence of the ζ protein in the co-immunoprecipitated complex was verified by immunoblotting with anti-ζ antibodies (Fig. 5*A*, *inset*). Cells activated for 6 h, expressed virtually no cell surface TCR, correlating with very low levels of ζ protein at this time (Fig. 5*A, inset,* and Fig. 3*A*). Following 24 h of activation, cell surface levels of TCR remained very low, even though the levels of ζ protein and the remaining TCR subunits were comparable to those of the control (Fig. 5*A, inset,* and Fig. 3*B*). Similar results were obtained by fluorescence-activated cell sorter analysis using anti-TCR α/β antibodies (data not shown).

Correlative with the failure of cell surface TCR re-expression following activation, we detected a previously undescribed monomeric form of the ζ chain (Fig. 5*B*), which migrated as a 16-kDa band. This was localized on the diagonal following separation on two-dimensional non-reducing/reducing SDS-PAGE. The identity of the 16-kDa monomer as the ζ chain was demonstrated by immunoprecipitation and immunoblotting, using both monoclonal and polyclonal anti-ζ antibodies directed against different ζ peptides. The ζ monomer was apparent about 7 h following activation (data not shown) and peaked at around 24 h. Interestingly, the ζ monomeric form could not be immunoprecipitated by anti-CD3 antibodies, as opposed to the ζ-ζ homodimer (Fig. 5*A*), indicating that only the latter can assemble with the other TCR subunits to form an intact complex.

DISCUSSION

In the present study, we focused on the characterization of the regulatory mechanisms controlling TCR ζ chain expression during activation of normal T cells, and assessed how the latter affects the expression of the entire TCR complex. We found that following T cell activation ζ mRNA is significantly up-regulated, while the CD3 δ, γ, and ε mRNA levels are relatively unchanged, indicating that ζ expression is differentially regulated. Interestingly, the TCRα and TCRβ mRNA were reported to be down-regulated under similar conditions (32), again emphasizing the unique regulation of the ζ chain. Our results indicate that activation-induced ζ mRNA up-regulation is transcriptionally controlled. This conclusion is supported by three findings: 1) up-regulation of the activity of the ζ gene 5′-flanking region following T cell stimulation, as demonstrated in our previous study (21). 2) Activation-dependent ζ mRNA up-regulation is transcriptionally controlled, as indicated in our present experiments with Act-D and CHX, and 3) blockage of ζ mRNA up-regulation by CsA treatment. Run-on analysis did not lead to conclusive results (data not shown), due to the limited sensitivity of this assay to detect a 2–3-fold increase in activation-induced ζ gene transcription. Cumulatively, our results strongly indicate that activation-induced ζ mRNA up-regulation is controlled mainly at the transcriptional level.

The finding that activation-induced ζ mRNA up-regulation is blocked by CsA enables us to add the ζ gene to the expanding list of genes that serve as targets for this immunosuppressive drug. It is well established that CsA impairs the function of the phosphatase calcineurin, which is required for inducing the translocation of NF-AT from the cytoplasm to the nucleus, thereby inhibiting NF-AT transcriptional activity (30, 31). Thus, CsA, through its effect on NF-AT, exerts its inhibitory activity on various genes critical to the immune response. These include genes encoding for a number of cytokines and the high affinity interleukin-2 receptor (31). Our data provide the first evidence that CsA also impairs the up-regulation of a member of the TCR complex. Although an indirect effect of CsA on ζ mRNA up-regulation cannot be ruled out, its specific inhibitory effect on activation-dependent ζ mRNA up-regulation, together with our previous observation that the ζ gene 5′-flanking region contains a putative NF-AT-binding site (21), strongly suggest that NF-AT is involved in controlling activation-dependent ζ mRNA up-regulation at the transcriptional level. The finding that CD3δ expression was not modified by CsA treatment is in accord with previously published studies, showing that δ expression is mainly controlled at the post-transcriptional level and that no NF-AT-binding sites were identified in the CD3δ enhancer (33–35). Our present results highlight the complexity of the mechanisms regulating ζ expression. While CsA blocked activation-induced ζ mRNA up-regulation, treatment of activated cells with CHX induced ζ mRNA superinduction. This effect may be due to the elimination of a short-lived repressor(s) present during ζ up-regulation. We have previously identified three functional regions localized upstream of the murine ζ gene: a basic promoter, an activator region, and a region displaying negative regulatory properties (21). The lat-

ter is a plausible candidate for binding the putative repressor, whose exact function remains to be elucidated.

We next assessed the effect of ζ mRNA up-regulation on ζ protein levels. To this end, we followed the fate of ζ protein expression at different time points after T cell activation. Surprisingly, a biphasic pattern of ζ expression was observed, beginning with rapid degradation and followed by recovery to basal levels. These results were obtained upon analysis of both the detergent-soluble and -insoluble receptors. Whether the ζ chain in each of the fractions is independently degraded or whether the detergent-soluble ζ chain is targeted to the cytoskeletal compartment for subsequent degradation, remains to be further investigated. The rapid degradation of the TCR components measured following short-term activation tallies with previously published studies (7–9), showing that T cell activation (up to 3 h) leads to the down-regulation and lysosomal degradation of the TCR subunits. We have extended their findings and followed the fate of ζ expression up to 24 h after activation. To the best of our knowledge, this is the first evidence demonstrating that ζ protein levels return to normal only after 21–24 h of continuous stimulation. Earlier in this "Discussion," we indicated that ζ and CD3δ are differentially controlled. This is also supported by the distinct recovery rates observed for CD3δ and ζ. Although CD3δ is also degraded following activation, it fails to return to normal levels within 21–24 h of continuous stimulation. Thus, ζ appears to be the first TCR chain to fully recover following activation. The consensus that ζ chain is the limiting factor for receptor formation, and the finding that the remainder of the TCR chains show partial recovery following 24 h activation, led us to question whether at this stage, the newly synthesized TCR chains could be assembled to form an intact complex and be transported to the cell surface. Our analysis revealed that levels of cell surface-expressed TCR were very low following 24 h of activation and remain low for up to 3 days provided the activating antibody is continuously present (Fig. 5 and data not shown). This is in agreement with previous studies (4) showing that activation-induced TCR down-regulation is a long-lasting phenomenon (at least 48 h). Thus, we found that although the levels of ζ and the other TCR subunits measured after 24 h of activation were relatively high, the receptor was not expressed on the cell surface. This indicates that re-expression of the TCR following activation is restricted by a yet unidentified post-translational mechanism. A putative factor that might play a role in this checkpoint is the oxidizing redox conditions in the cell which are known to be modified upon activation (36). In our analyses, we detected a previously undescribed monomeric ζ form, whose appearance correlated with the failure of the TCR complex to reach the cell membrane. Although the mechanisms controlling TCR assembly are poorly understood, it is well established that oxidizing redox conditions in the ER are essential to correct folding and disulfide bond formation (14, 15). Conceivably, the monomeric ζ form may be considered a "marker" indicating that the ER redox conditions following 24 h of activation do not permit correct TCR complex formation. It is also possible that the monomeric ζ form physically interferes with TCR complex assembly. It is generally assumed that surface expression of the TCR proteins reflects their assembly status, since unassembled TCR subunits or partial TCR complexes either fail to exit the ER or are degraded in the lysosome (16, 17). Cumulative evidence indicates that the ζ chain is the last component to join the partial TCR complex. Therefore, any obstacle impeding the correct assembly of the ζ dimer would lead to the formation of partial TCR complexes that cannot exit the ER (16, 17). The significance of the appearance of the monomeric ζ form in normal activated T cells and its possible role in preventing cell surface TCR re-expression, merits investigation.

Our findings indicate that the normal outcome of T cell activation is transient ζ degradation followed by a recovery to normal levels. We have also shown that this recovery can be blocked by CsA. These results might be relevant in explaining the specific down-regulation of ζ protein observed in T cells isolated from tumor-bearing hosts (37–42), HIV carriers (43), and patients with autoimmune disorders such as rheumatoid arthritis (44) and systemic lupus erythematosus (45). It is tempting to speculate that one or more unknown factors common to these pathological conditions could mimic the CsA effect, thus preventing ζ protein recovery after normal TCR engagement.

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Expression of the T Cell Antigen Receptor ζ Chain following Activation Is Controlled at Distinct Checkpoints: IMPLICATIONS FOR CELL SURFACE RECEPTOR DOWN-MODULATION AND RE-EXPRESSION

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