A Role for the Region Encompassing the c″ Strand of a TCR Vα Domain in T Cell Activation Events[1]

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The distinct strand topology of TCR Vα domains results in a flatter surface in the region encompassing the c″ strand than the corresponding region in Ig V domains. In the current study a possible role for this region in T cell activation has been investigated by inserting a potential glycosylation site at Vα residue 82. This residue is in proximity to the c″ strand and distal to the putative interaction site for cognate peptide:MHC ligand. An additional *N*-linked carbohydrate at this position would create a protrusion on the Vα domain surface, and this may interfere with TCR aggregation and/or recruitment of signaling molecules. The modified TCR has been expressed in transfected T cells, and the phenotype following stimulation has been compared with that of cells expressing the wild-type TCR. The mutation has significant effects on activation-induced cell death and TCR internalization, but, unexpectedly, does not affect IL-2 secretion. Furthermore, analyses with tetrameric, peptide:MHC class II complexes suggest that the mutation decreases the ability of the TCR to aggregate into a configuration compatible with avid binding by these multivalent ligands. ***The Journal of Immunology,*** **2000, 165: 820–829.**

For $\alpha\beta$ T cells, the Ag receptor comprises a clonotypic $\alpha\beta$ heterodimer noncovalently associated with invariant CD3-γ, -δ, and -ϵ subunits and a disulfide-linked ζ-ζ homodimer. The $\alpha\beta$ heterodimer forms the recognition unit for cognate antigenic peptides bound to restricting MHC class I or class II molecules, whereas the CD3 proteins are required for signal transduction (1, 2). T cell signaling via the TCR-CD3 complex involves a cascade of events that is initiated by phosphorylation of immunoreceptor tyrosine-based activation motifs on the ϵ- and ζ-chains by protein tyrosine kinases such as the Src family kinase p56^{lck} (reviewed in Ref. 3).

The affinity of a TCR for a given peptide:MHC complex plays a role in the outcome of the corresponding TCR:peptide:MHC interaction, and much data support affinity/avidity models of activation (4–9). In particular, the off-rate of the interaction appears to play a central role (5, 9, 10), and this is consistent with kinetic proofreading models of T cell activation (11, 12). In addition to the effect of affinity/avidity in determining the outcome of TCR:peptide:MHC contact, for optimal (i.e., agonist) signaling there appears to be a need for ordered oligomerization or aggregation of TCRs and associated components following ligand recognition (13–16). Thus, conformational and affinity models are not mutually exclusive, and both are relevant to sequential engagement models (13, 15, 17) for which the interaction needs to be sufficiently long-lived to allow organization of TCR, coreceptors, and other relevant proteins into higher order assemblies that are signaling competent. Much data support the concept that clustering of TCRs at the T cell:APC interface occurs during T cell activation (18–22). The segregation of TCR/CD3 and associated kinases into low density, detergent-insoluble rafts (or lipid rafts) following stimulation has also been reported (23–27). Furthermore, agents that cross-link TCRs are generally more effective in inducing T cell activation than monomeric ligands (28, 29), and data supporting a model for TCR dimerization following ligand interaction have recently been presented (14). Studies using monomeric, dimeric, and multimeric peptide-MHC class II complexes to activate T cells provide additional support for the need for TCR oligomerization (30, 31). Consistent with the concept that the TCR migrates into aggregates following ligand binding, quasi-elastic light-scattering studies with soluble molecules (TCR and peptide:MHC class II) indicate that the TCR forms hexameric complexes with cognate peptide:MHC (32). Thus, TCR aggregation may be an important step in T cell activation, but to date tools to investigate this in cell systems have not been available.

The x-ray crystallographic analysis of a murine Vα domain (Vα4.2-Jα40) derived from the 1934.4 T cell hybridoma (33) demonstrated a novel strand topology that allows the Vα domain to pack in the crystal form as dimers of dimers (34). This observation led to the proposal of a model for TCR dimerization following ligand binding (35, 36), although more recent x-ray crystallographic structures have argued against the validity of this "dimer of dimers" model (37–42). However, the novel strand topology results in a flatter surface than that observed in variable domains that have an Ig V domain-like topology (34). The strand switch

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also results in a reorientation of the Vα CDR2 by about 90° to produce a more compact Ag interaction site (reviewed in Ref. 43). This unusual strand topology is invariably observed for Vαs in other more recently solved TCR $\alpha\beta$ structures (37, 42; reviewed in Ref. 43). In Vβ domains the position of the c″ strand shows greater variation (44), but in one Vβ domain analyzed to date a switch similar to that present in the Vα domain was observed (40). Furthermore, analysis of Vα domain sequences indicate that there is an exclusion of potential glycosylation sites in the vicinity of the Vα c″ strand (34), in contrast to the corresponding region of Vβ domains.

The distinctive structural features of Vα domains prompted us to analyze a possible role for the region encompassing the Vα c″ strand in events subsequent to ligand recognition by the TCR. To do this, we have mutated a serine to asparagine to insert a potential glycosylation site on this surface of the Vα domain. Our rationale was that *N*-linked glycosylation at this asparagine would insert a protrusion in this region of the TCR that might affect receptor aggregation and/or association with other signaling molecules. Mutated α-chains have been expressed in association with the wild-type (WT)[8] β-chain in a TCR$\alpha^-\beta^-$ thymoma cell line, and the characteristics of the resulting T cell transfectants have been compared with those of similarly generated WT TCR transfectants. The results indicate that the additional *N*-linked carbohydrate differentially affects IL-2 secretion, apoptosis, and TCR internalization. In addition, analysis of transfectants with fluorescent tetrameric, cognate peptide:MHC class II complexes indicates that there are differences in the ability of the mutant and WT TCR to form a configuration that allows tetrameric ligand binding.

Materials and Methods

Cell lines, Abs, and reagents

The I-A^u-expressing B cell line, PL-8 (45) was provided by Dr. David Wraith (University of Bristol, Bristol, U.K.). The I-A^u-transfected derivative of BW4157, Utm6.15 (46, 47), was made available by Dr. Harden McConnell (Stanford University, Stanford, CA). The TCR-negative cell line 58$\alpha^-\beta^-$ (48), which is also CD4 negative, was provided by Dr. Stephen Hedrick (San Diego, CA) with permission from Dr. Bernard Malissen (Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique, Marseille-Luminy, France). The 1934.4 α- and β-chain expression vectors used in the transfections were constructed with α and β shuttle vectors (49) provided by Dr. Mark Davis (Stanford, CA). The anti-Vβ mAb F23.1 (50) was a gift from Drs. John Kappler and Philippa Marrack (University of Colorado Health Science Center, Boulder, CO). The anti-TCR α-chain mAb H28-710 was provided by Dr. Kelly Kearse (East Carolina University, Greenville, NC). FITC-labeled H57-597 (anti-mouse TCRβ), 145-2C11 (anti-mouse CD3ϵ), and annexin V-FITC were purchased from PharMingen (San Diego, CA). FITC-labeled anti-mouse, anti-rat, and anti-hamster IgG were obtained from Cappel (Warrington, PA). The N-terminal peptide (Ac1–11; acetylated at position 1) of rat myelin basic protein (MBP) and an analogue in which WT lysine at position 4 is substituted by tyrosine (Ac1–11[4Y]) were synthesized at the peptide synthesis unit of Howard Hughes Medical Institute, University of Texas Southwestern Medical Center (Dallas, TX). Streptavidin-PE was purchased from BioSource International (Camarillo, CA). The anti-phosphotyrosine Ab, 4G10, was purchased from Upstate Biotechnology (Lake Placid, NY). *N*-Glycosidase F was purchased from Roche (Indianapolis, IN). Enhanced chemiluminescence reagent (ECL) was purchased from Amersham (Poole, U.K.).

Plasmid construction

The 1934.4 Vα and Vβ sequences are derived from the 1934.4 TCR and have been described previously (51). The isolated genes (52) were used as templates in the PCR with primers designed to tailor the genes with splice sites and 5′ *Xho*I (α-chain), 5′ *Cla*I (β-chain), and 3′ *Not*I (α- and β-chain) sites, using an approach analogous to that described previously (49). The tailored genes were then cloned into a modified pUC119 vector with unique *Xho*I, *Cla*I, and *Not*I sites as *Xho*I-*Not*I (α-chain) and *Cla*I-*Not*I fragments (β-chain) and sequenced. In addition, Ser^{82} of the Vα domain gene was converted to Asn to generate the potential glycosylation site Asn-X-Ser using the mutagenic oligonucleotide S82N (5′-CAGCCGAGT CATTCTCTTCGACT-3′) and site-directed mutagenesis as previously described (53). All constructs were sequenced and recloned into the α and β shuttle vectors and resequenced before use in transfections.

Transfections

58$\alpha^-\beta^-$ cells were used as recipients. Plasmid DNA was linearized using *Sal*I and transfected into the cells by electroporation, and mycophenolic acid-resistant clones were selected as previously described (49). Transfectants were analyzed for surface expression of TCR by indirect immunofluorescence using F23.1 (anti-Vβ8) followed by FITC-labeled anti-mouse IgG. Analysis of staining was conducted using a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA) and the program WinMDI 2.7.

Analysis of the glycosylation state of the TCRs

T cell transfectants were washed with PBS and lysed at 4°C with 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF containing 1% Nonidet P-40 (1 ml/3 $\times$ 10^7 cells). The lysate was spun at 15,000 $\times$ *g*, and the TCRs from the supernatants were immunoprecipitated overnight at 4°C using the anti-Vβ mAb F23.1 cross-linked to Sepharose-4B, or protein A-Sepharose (Pharmacia Biotech, Piscataway, NJ). The beads were thoroughly washed with the lysis buffer followed by PBS, resuspended in PBS, and incubated with *N*-glycosidase F for 1 h at 37°C. Control beads were incubated with PBS alone. The beads were boiled in Laemmli sample buffer containing 2% 2-ME, and the supernatants were electrophoresed using 10% SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) or Immobilon-P (Millipore, Bedford, MA) membranes. The membranes were blocked with 1% nonfat dry milk in PBS and incubated with an anti-Cα hamster mAb, H28-710, for 1 h at room temperature. The strip was washed with PBS containing 0.05% Tween-20, incubated with HRP-labeled anti-hamster IgG for 1 h, and then developed using the ECL reagent.

T cell activation

T cells were washed with mycophenolic acid-free medium, resuspended in RPMI 1640 containing 10% FCS, and incubated with varying concentrations of the peptide Ac1–11[4Y] in the presence of an I-A^u-transfected thymoma line, Utm6.15, or B cell line, PL-8, at 37°C in a humidified CO_2 incubator. Control wells contained Utm6.15 and T cells but no peptide. The transfectants were also activated using PMA and calcium ionophore A23187 (Sigma, St. Louis, MO) or with plate-bound mAbs F23.1 (anti-Vβ8), 145-2C11 (anti-CD3ϵ), and recombinant MBP1–11[4Y]:I-A^u complexes (54). The production and characterization of the recombinant MBP1–11[4Y]:I-A^u complexes have been described previously (54). Abs and the recombinant molecules were coated onto 96-well plates for 2 h at 37°C (54). Twenty to 24 h later the supernatants were collected, and IL-2 levels were determined using an IL-2-dependent cell line, CTLL-2, as previously described (55) or by cytokine ELISA using JES6-1A12 (rat anti-mouse IL-2, PharMingen) as a capture Ab and biotinylated JES6-5H4 (rat anti-mouse IL-2, PharMingen) followed by Extravidin-HRP (Sigma) for detection.

Analysis of apoptosis

Following activation with the appropriate stimuli (as above), cells were washed once with 1% BSA/PBS. Propidium iodide (PI) was added to a final concentration of 10 μg/ml, and cells were analyzed by flow cytometry (56). To confirm that the death was apoptotic, in some experiments cells were stimulated for 6 h at 37°C with 1 μg/ml of recombinant MBP1–11[4Y]:I-A^u, stained with both annexin V-FITC (2.5 μg/ml) and PI (10 μg/ml), and analyzed by flow cytometry. For annexin V staining the buffer comprised 10 mM HEPES/NaOH (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, and 1.8 mM $CaCl_2$.

TCR down-regulation

For TCR down-regulation experiments the T cell transfectants were stimulated for 1–3 h at 37°C with recombinant MBP1–11[4Y]:I-A^u complexes or PMA and calcium ionophore A23187. After activation, TCR levels were determined by flow cytometric staining using an FITC-labeled anti-Cβ Ab (H57-597) or an anti-Vβ mAb, F23.1 followed by FITC-labeled anti-mouse IgG.

[8] Abbreviations used in this paper: WT, wild type; MBP, myelin basic protein; ECL, enhanced chemiluminescence; PI, propidium iodide; AICD, activation-induced cell death.

Anti-phosphotyrosine immunoblotting

T cells (2×10^7) were stimulated at 37°C using 100 μg/ml of Ac1–11[4Y] and 10^7 Utm16.5 cells. After 5 min cells were resuspended in 1 ml of lysis buffer (250 mM NaCl, 50 mM Tris-Cl, 0.5% Triton X-100, 1 μg/ml pepstatin, 1 mM Pefabloc, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM activated sodium orthovanadate, and 5 mM NaF) for 2 h at 4°C. The TCR/CD3 complex was immunoprecipitated using F23.1 cross-linked to protein A-Sepharose beads. Following washes with lysis buffer and PBS, the beads were boiled for 5 min in SDS-PAGE loading buffer. 2-ME was added to the supernatants at a final concentration of 2%; the samples were electrophoresed using 10% SDS-PAGE and transferred to an Immobilon-P membrane. The membrane was dried for 12 h at room temperature and then incubated with the anti-phosphotyrosine Ab, 4G10, followed by HRP-conjugated anti-mouse IgG. The blot was developed using the ECL reagent.

Flow cytometric analyses using MBP1–11[4Y]:I-A^u tetramers

MBP1–11[4Y]:I-A^u tetramers were prepared as described elsewhere.[9] T cells were incubated with the MHC class II tetramers labeled with PE or, as a control, with streptavidin-PE, in the presence of the anti-CD3ε Ab, 145-2C11. After 3 h, the cells were washed twice with PBS and analyzed by flow cytometry.

Analysis of TCR capping

Cells ($2–3 \times 10^6$) were incubated with 10 μg/ml 145-2C11 in 100 μl of 1% BSA/PBS at 37°C for 40 min. Cells were washed once in warm (37°C) PBS and incubated with 10 μg/ml Texas Red-conjugated anti-hamster Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) in 200 μl of 1% BSA/PBS at 37°C for 20 min. Cells were then washed three times with ice-cold PBS, fixed with 1% paraformaldehyde, and adhered to poly-lysine-treated coverslips. Coverslips were mounted using Polymount (Polysciences, Warrington, PA), and cells were visualized using a Zeiss Axiovert fluorescence microscope (New York, NY) and a Plan-Apochromat objective (×63). Images were captured with a Hamamatsu CCD camera (Hamamatsu Photonics K.K., Hamamatsu-City, Japan).

Results

TCR expression by the transfectants

Mycophenolic acid-resistant TCR-positive transfectants were generated by cotransfection of either the 1934.4 WT or αS82N mutant α-chain construct together with the β-chain shuttle plasmid into $58\alpha^-\beta^-$/CD4$^-$ cells. The location of the mutation site on the Vα4.2 structure (34) is shown in Fig. 1. The transfectants expressed different levels of TCR as determined by reactivity with the anti-Vβ8 mAb, F23.1 (Fig. 2*A*). Mutation of serine 82 to asparagine in the TCR α-chain does not have a significant effect on the surface expression of the TCR in transfectants (Fig. 2*A*, MUT-1 and MUT-2). However, despite analyzing about 20–30 mutant transfectants, expression levels as high as those seen for WT transfectants such as WT-1 were not observed. This suggests that the mutation may have a minor effect on the stability of the α-chain and/or the efficiency of assembly of the corresponding TCR. Immunoprecipitation of the expressed TCRs using F23.1 indicated that the α-chain containing the αS82N mutation had a higher m.w. than the WT α-chain (size difference of ~2–3 kDa using SDS-PAGE), and this size difference was eliminated following digestion with *N*-glycosidase F (Fig. 2*B*). The additional glycosylation site is therefore used in the αS82N mutant.

IL-2 secretion by the T cell transfectants

The 1934.4 TCR recognizes the N-terminal 11 residues (or nonamer) of MBP in association with the MHC class II molecule, I-A^u. For recognition, the antigenic peptide requires acetylation at residue 1 to block the N-terminal charge (57), and position 4 analogues of this peptide that bind with higher affinity to I-A^u than the WT peptide stimulate T cells more efficiently (46, 58). For example, replacement of position 4 lysine with tyrosine results in a peptide that gives >100-fold shifts in dose-response curves (58), and this is also observed for the transfectants (59). For this reason, the higher affinity analogue (Ac1–11[4Y]) has been used in the current study.

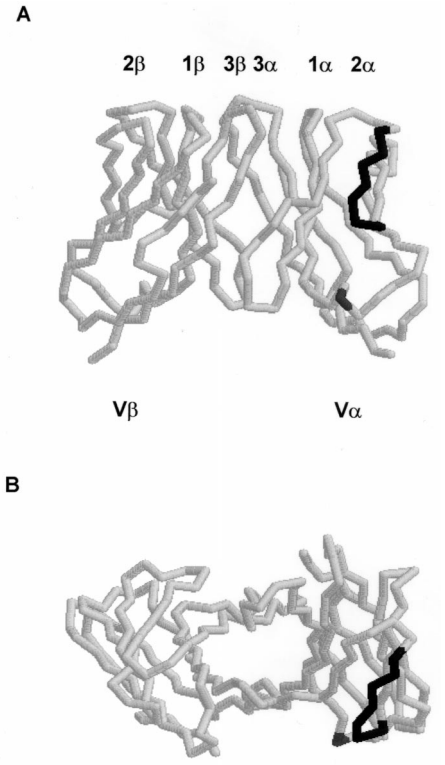

FIGURE 1. Structure of the 1934.4 VαVβ heterodimer with the location of the αS82N mutation indicated. *A*, α-Carbon trace viewed with the locations of the c″ strand (residues 54–60; shown in black) and Ser^{82} (dark gray) shown. CDRs are indicated by 1α, 2α, and 3α (Vα domain) and 1β, 2β, and 3β (Vβ domain). *B*, Same as in *A*, but with a 90° rotation, so that the presumed peptide-MHC docking site on the VαVβ heterodimer is seen. The figures were drawn using RASMOL (90).

Initially, transfectants were stimulated with plate-bound anti-Vβ8 mAb, F23.1, and anti-CD3ε Ab, 145-2C11. The responsiveness of the transfectants, assessed by quantitating IL-2 levels, was dependent on the levels of surface TCR (data not shown). This dependence on expression levels is consistent with the findings of other studies using TCR transfectants (49, 59). For this reason two transfectants (WT-3 and MUT-2), which showed only minor differences (~2-fold; Fig. 2*A*) in surface TCR levels, were used for all subsequent studies. Similar levels of responsiveness were seen when the T cells were stimulated by cross-linking the TCR with F23.1 or the anti-CD3-ε Ab 145-2C11 (Fig. 3, *A* and *B*). Thus, the αS82N mutation does not have a significant effect on IL-2 production in response to Ab-mediated cross-linking.

The transfectants were also activated with PMA plus the calcium ionophore A23187 and, to investigate IL-2 secretion in response to antigenic stimulation, with peptide-pulsed, I-A^u-expressing transfectants. Again, no significant differences were seen with the WT-3 and MUT-2 transfectants (Fig. 3, *C* and *D*).

Apoptosis induction in the TCR transfectants

To evaluate activation-induced cell death (AICD), recombinant MBP1–11[4Y]:I-A^u complexes adsorbed onto the wells of microtiter plates were used to stimulate the cells (Fig. 4). The MBP1–11[4Y]:I-A^u complexes were functionally expressed by insertion

[9] C. G. Radu, S. M. Anderton, M. Firan, D. C. Wraith, and E. S. Ward. Quantitative analysis of T helper cells specific for an immunodominant epitope of myelin basic protein. *Submitted for publication.*

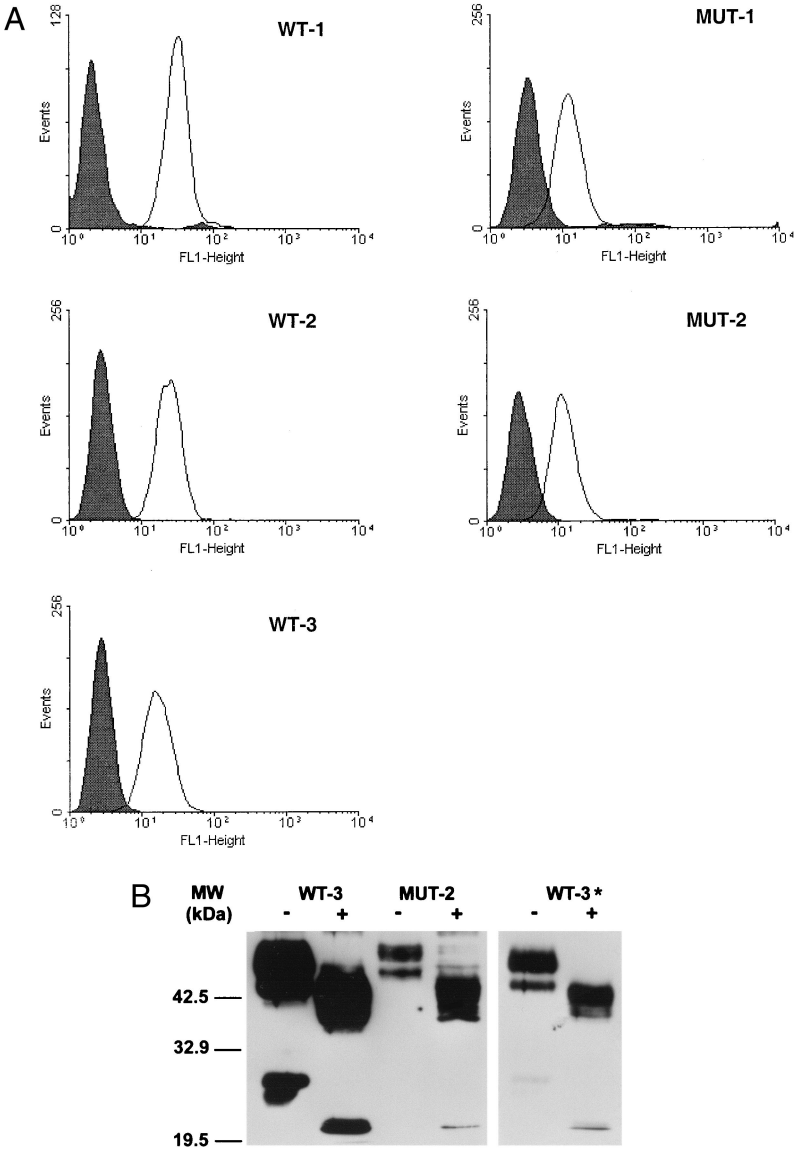

FIGURE 2. *A*, Flow cytometric analysis of TCR expression levels on transfectants expressing WT (WT-1, WT-2, and WT-3) and αS82N mutant (MUT-1 and MUT-2) TCRs. Cells were stained with anti-Vβ Ab F23.1 followed by FITC-labeled anti-mouse IgG (lines). Shaded curves show staining with FITC-labeled anti-mouse IgG only. *B*, Analysis of the glycosylation of the TCR in WT-3 and MUT-2 cells. TCRs expressed by the T cell transfectants were immunoprecipitated using the anti-Vβ mAb F23.1 and were incubated for 1 h at 37°C with (+) or without (−) *N*-glycosidase F. The samples were electrophoresed using 10% SDS-PAGE and transferred to Immobilon-P membranes. The TCR α-chain was detected using an anti-Cα mAb (H28-710). The *right panel* (WT-3*) shows a shorter exposure of the same membrane. Data are representative of three independent experiments.

of the N-terminal epitope of MBP between codons 2 and 3 of the gene encoding the mature β^u polypeptide to result in antigenic peptide covalently tethered to the I-A^u molecule (54). These molecules are potent and specific stimulators of T cell transfectants/hybridomas expressing the WT 1934.4 TCR (54). They are also efficient inducers of AICD, and these were used in apoptosis studies in preference to Ag-pulsed APCs because this simplified the flow cytometric analyses.

When stimulated with recombinant, plate-bound MBP1–11[4Y]:I-A^u complexes, the WT-3 cells consistently produced slightly higher levels of IL-2 than MUT-2 cells at low Ag concentrations (Fig. 4*A*). In contrast to the minor difference in IL-2 secretion levels following treatment with plate-bound MBP1–11[4Y]:I-A^u complexes, there was a marked difference between the WT-3 and MUT-2 transfectants in AICD, as assessed by PI staining. The MUT-2 cells were markedly more resistant to AICD (Fig. 4*A*). This could not be explained by differences in IL-2 levels, since Ag doses that induced similar levels of IL-2 production by the two transfectants resulted in significant differences in sensitivity to apoptotic death (Fig. 4*A*). Importantly, for these experiments IL-2 levels were determined by ELISA, and under the conditions of the assay, OD values corresponded in direct proportion to IL-2 concentrations. Annexin V staining was used to confirm that the death was apoptotic and showed the same difference in induction of programmed cell death for the WT and mutant transfectants as PI staining (Fig. 4*B*). Significantly, stimulation by PMA and calcium ionophore A23187 resulted in similar levels of IL-2 secretion and apoptotic death for both transfectants (Fig. 3*C* and 4*C*). Thus, the resistance to cell death by MUT-2 cells was only observed following antigenic stimulation and can be bypassed by activation of pathways distal to TCR triggering. Importantly, qualitatively similar results were observed for other WT and αS82N transfectants (data not shown), indicating that the resistance to AICD was not a peculiarity of MUT-2 cells.

TCR down-regulation

To analyze another important marker of T cell activation, TCR down-regulation, the surface levels of TCR on the transfectants following stimulation were analyzed by flow cytometry using the anti-Vβ8 Ab F23.1 or the anti-Cβ Ab H57-597. Following activation by the recombinant MBP1–11[4Y]:I-A^u complexes, mutant transfectants showed markedly reduced internalization of TCR compared with WT transfectants (Fig. 5*A*). The relatively low levels of down-regulation observed with the WT transfectants are

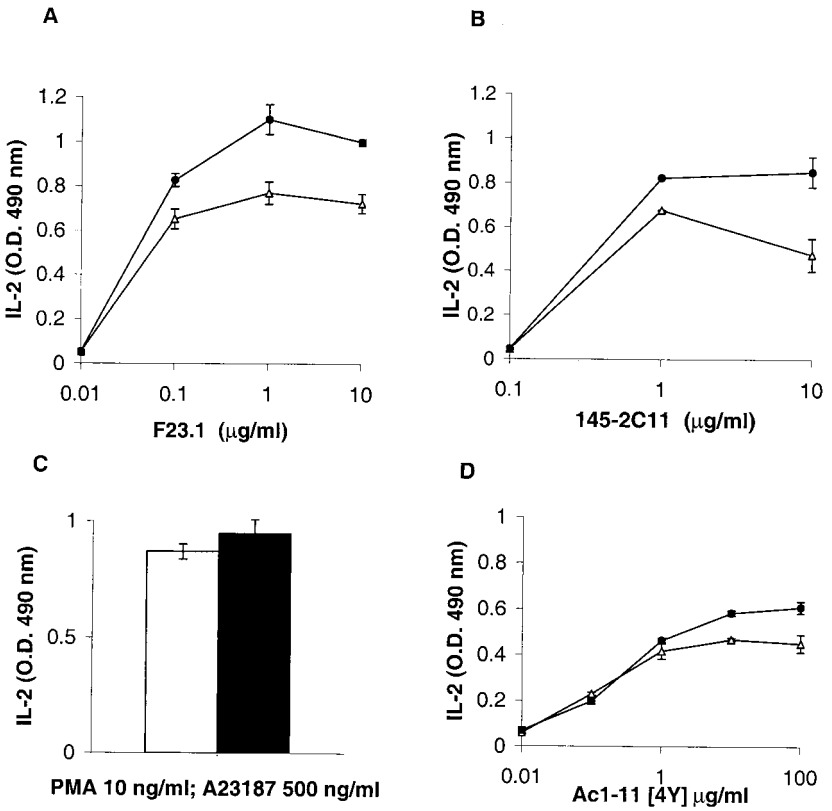

FIGURE 3. IL-2 secretion by the T cell transfectants WT-3 (△ or □) and MUT-2 (● or ■) in response to different stimuli. Transfectants (5×10^4/well) were stimulated with: *A*, plate-bound F23.1 (anti-Vβ8); *B*, plate-bound 145-2C11 (anti-CD3ϵ); *C*, 10 ng/ml PMA and 500 ng/ml calcium ionophore A23187; *D*, Ac1–11[4Y]-pulsed Utm6.15 (Utm6.15 were more effective APCs than PL-8; data not shown). For both *A* and *B*, the concentrations of Ab shown are those used to coat the plates. IL-2 levels were quantitated by ELISA, and the values represent the means of triplicates. Data are representative of three independent experiments.

consistent with the observations of others in studies in which immortalized T cells were compared with T cell clones (60).

To exclude the possibility that the lower level of TCR down-regulation for mutant cells relative to WT cells is due to the difference in expression levels, TCR internalization in response to phorbol ester (PMA)-mediated stimulation was also assessed. TCR down-regulation in response to PMA was almost abrogated in transfectants expressing the mutated TCR α-chain (Fig. 5*B*), whereas down-regulation was observed for WT transfectants. Similar results were observed for the other transfectants shown in Fig. 2 (data not shown).

Anti-phosphotyrosine immunoblotting

The observed differences between the WT and mutant transfectants prompted us to investigate the phosphorylation of TCR-associated proteins following Ag-specific stimulation, i.e., events proximal to TCR-mediated activation. The transfectants were activated for 5 min at 37°C with Ac1–11[4Y]-pulsed APCs, and the TCR/CD3 complexes were immunoprecipitated using F23.1 cross-linked to protein A-Sepharose beads. Immunoblotting of the F23.1-captured immunoprecipitates with the anti-phosphotyrosine Ab 4G10 demonstrated that the same TCR-associated phosphoproteins were present in the WT-3 and MUT-2 cells following stimulation with Ac1–11[4Y]-pulsed I-A^u-expressing cells (Fig. 6). Consistent with the F23.1 staining data (Fig. 2), the amount of immunoprecipitated phosphoproteins was lower for mutant cells than for WT cells. Importantly, for both transfectants the ratio of pp23 to pp21 forms of TCR-ζ was similar (Fig. 6). Taken together, the data indicate that the αS82N mutation and the resultant additional *N*-linked carbohydrate do not qualitatively affect the TCR-proximal phosphorylation events following antigenic stimulation.

Analyses of the transfectants using MBP1–11[4Y]:I-A^u tetramers

To further investigate the characteristics of the mutated TCR, flow cytometric analyses were conducted with fluorescently labeled MBP1–11[4Y]:I-A^u tetramers. These tetramers specifically stain MBP1–11[4Y]:I-A^u-responsive T cells and activate cognate T cells to secrete IL-2 when used in T cell stimulation assays.[9] Levels of staining were increased when the cells were coincubated with the anti-CD3ϵ Ab, 145-2C11 (see Footnote 9). This enhanced staining is most likely due to the ability of this Ab to cross-link TCRs and is consistent with the observations for tetramer staining of T cell hybridomas (61). This Ab was therefore used in all flow cytometry experiments with tetramers. The tetramers stained MUT-2 poorly relative to WT-3 cells, whereas staining of the transfectants with the anti-Cβ Ab H57-597 showed only minor differences that are consistent with the F23.1 staining data (Fig. 2). There are two possible explanations for the poor tetramer staining of MUT-2 cells. First, from studies in other peptide:MHC class II systems (62, 63) tetramer staining has been shown to correlate with TCR:ligand affinity, and the mutation may therefore result in a reduced affinity of the TCR for cognate ligand. However, the similarity of MUT-2 and WT-3 in dose responses to recombinant MBP1–11[4Y]:I-A^u complexes (Figs. 3 and 4) would make this seem improbable. Second, the mutation may prevent the aggregation of the αS82N TCR into a configuration that allows multivalent binding of the tetrameric complexes. The former possibility was investigated further by analyzing transfectants bearing a mutated derivative of the 1934.4 TCR in which mutation of glutamic acid 69 to alanine (E69A) results in a significant reduction in Ag responsiveness (59). Fig. 7, *A* and *B*, shows the mean fluorescence intensity for WT-3, MUT-2, and E69A-2 cells stained with either

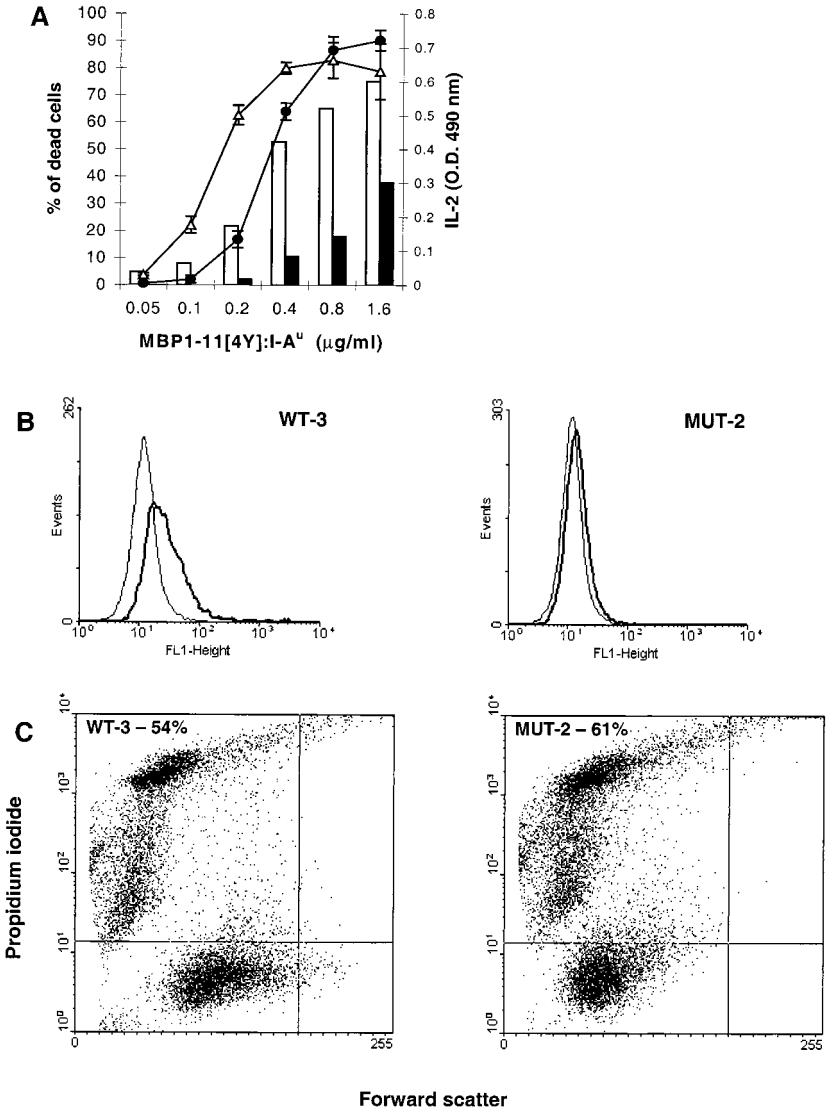

FIGURE 4. Analysis of IL-2 secretion and AICD in the T cell transfectants after stimulation with recombinant MBP1–11[4Y]:I-A^u molecules. *A*, Cells (5×10^4/well) were incubated for 24 h with different concentrations of the recombinant molecules coated onto 96-well plates. Supernatants were analyzed for IL-2 levels using an IL-2 ELISA for WT-3 (△) and MUT-2 cells (●). AICD was analyzed by staining the cells with PI and was expressed as the percentage of dead cells (□, WT-3 cells; ■, MUT-2 cells). Values for IL-2 levels are the means of triplicates. *B*, Annexin V-FITC staining of WT-3 and MUT-2 cells after 6 h of incubation with 1 μg/ml of plate-bound MBP1–11[4Y]:I-A^u complexes. Cells double positive for PI and annexin V-FITC (15% for WT-3 and 5% for MUT-2) were gated out. Annexin V staining of stimulated (thick lines) and unstimulated (thin lines) cells is shown. *C*, Analysis of AICD following 24-h incubation with 10 ng/ml PMA and 500 ng/ml of the calcium ionophore A21387. The percentage of PI-positive cells in the absence of stimulation was 13% for WT-3 cells and 22% for MUT-2 cells. Data shown in each panel are representative of at least two independent experiments.

anti-Cβ (H57-597) or tetramers. IL-2 levels produced by the transfectants in response to plate-bound MBP1–11[4Y]:I-A^u complexes were also analyzed (Fig. 7*C*) and for the WT-3 and MUT-2 cells are reminiscent of the data shown in Fig. 4. Similar results were obtained using biotinylated MBP1–11[4Y]:I-A^u immobilized on streptavidin-coated plates (not shown). As expected from our

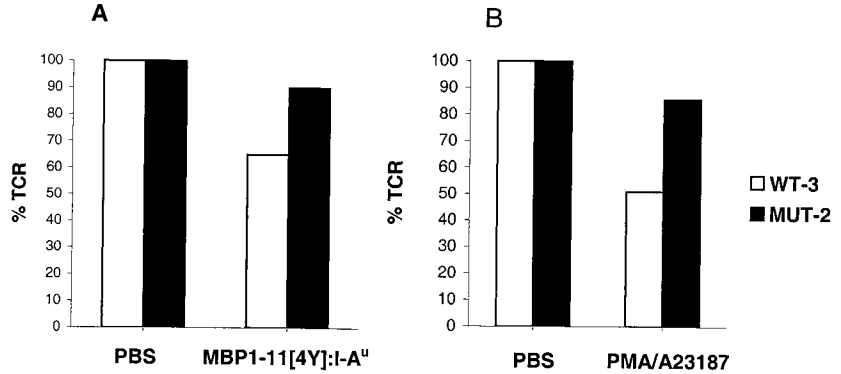

FIGURE 5. TCR down-regulation following stimulation of WT-3 and MUT-2 cells. Cells were treated with 1 μg/ml plate-bound MBP1–11[4Y]:I-A^u complexes (*A*) or 10 ng/ml PMA plus 500 ng/ml calcium ionophore (A23187; *B*). After a 2-h incubation, surface TCR levels were assessed by staining with F23.1 (anti-Vβ8) followed by FITC-labeled anti-mouse IgG or with FITC-labeled H57-597 (anti-Cβ). Data are expressed as the percent TCR, with 100% representing the expression levels for cells treated with PBS only. Data are representative of three independent experiments.

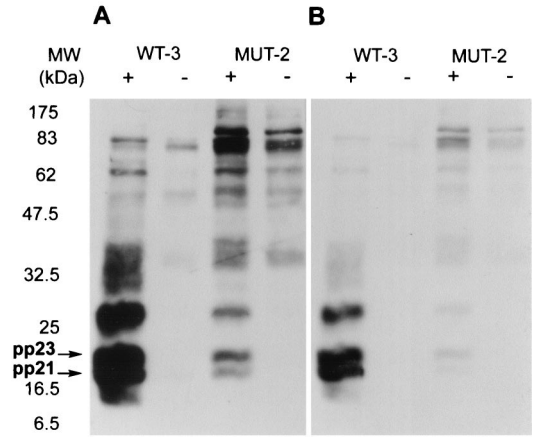

FIGURE 6. Anti-phosphotyrosine immunoblot of Ag-stimulated cells. The transfectants were stimulated for 5 min at 37°C using Utm16.5 cells pulsed with 100 μg/ml of Ac1–11[4Y] (+) or incubated with Utm16.5 cells alone (−). The anti-phosphotyrosine mAb, 4G10, was used for detection (see *Materials and Methods* for details). *A* and *B,* Different exposures of the same membrane. Data are representative of two independent experiments.

earlier analyses of the E69A transfectant (59), WT-3 cells are both more responsive to cognate ligand and stain at higher levels with tetramer than E69A cells. In fact, the fluorescence shift for WT-3 cells is about 2-fold greater with tetramer analysis than that for H57-597 staining, whereas for E69A cells these shifts are essentially the same (Fig. 7*B*). Most significantly, despite higher levels of tetramer binding by E69A transfectants compared with MUT-2 cells, IL-2 secretion by E69A cells in response to cognate ligand was very low (Fig. 7*C*). Thus, MUT-2 cells are significantly more responsive to MBP1–11[4Y]:I-A^u than E69A transfectants, and yet they bind tetramers of this ligand relatively poorly. Since in other systems Ag responsiveness has been shown to almost invariably correlate with the affinity (and particularly the off-rate) of the TCR for cognate ligand (4, 6–10, 64), this makes it improbable that the poor tetramer staining is due to reduced affinity of the αS82N TCR. Thus, the alternative explanation, that the TCRs with an additional *N*-linked carbohydrate in the proximity of the c″ strand cannot aggregate into a configuration compatible with multivalent tetramer binding, appears to be more likely. Finally, capping of the TCR using the anti-CD3ε Ab 145-2C11 indicated that at this level of resolution there were no significant differences among WT-3, MUT-2, and E69A cells (Fig. 7*D*). The MUT-2 TCR is therefore able to redistribute into the focal aggregates that are observed following T cell activation (19).

Discussion

The present study involves analysis of the role of the region encompassing the c″ strand (residues 54–60) of the TCR Vα domain in T cell recognition and signaling. Although earlier crystallographic studies suggested a role for this region of the TCR in dimerization, more recent structural data have questioned the validity of this model (37–42, 44). However, the unusual nature of this surface, which is distinct from the corresponding region in the majority of Vβ domains analyzed to date (34; reviewed in Ref. 43), prompted us to investigate a possible role in T cell function. A potential glycosylation site was therefore introduced at residue 82, which is in close proximity to the c″ strand and is distal to the putative Ag binding site (Fig. 1). Glycosylation at this site would be predicted to introduce a protrusion in this region of the α-chain, and biochemical data indicate the presence of additional carbohydrate in the mutated TCR α-chain.

The activity of T cell transfectants carrying the mutated α-chain together with a WT β-chain was compared with the activity of those expressing the WT TCR. The effects on IL-2 secretion, apoptosis, and TCR down-regulation following stimulation were analyzed. The data show that the αS82N mutation has a minor, if any, effect on IL-2 secretion by the T cells in response to either Ab-mediated cross-linking or cognate ligand. Significantly, this demonstrates that modification of the TCR α-chain did not affect the ability of the TCR to recognize cognate peptide-MHC complexes. Thus, insertion of the *N*-linked carbohydrate in this region of the TCR did not affect the signaling cascade that resulted in IL-2 secretion. However, the mutation significantly affected AICD and TCR down-regulation, and this provides a functional separation between TCR internalization or AICD and IL-2 production. Earlier studies involving analysis of T cells expressing TCR β-chains mutated in the transmembrane region (65, 66) or CD3γ,δ/TCR α-chains with truncations in their cytoplasmic tail (67, 68) resulted in T cells that, although not affected in cytokine secretion, were defective in either apoptosis or down-regulation, respectively. Therefore, the current study is distinct in that it shows functional defects in both AICD and internalization due to an alteration in a membrane-distal region, i.e., the Vα domain, of the TCR. Furthermore, in contrast to the studies involving the mutated TCR β-chain (65), ζ-chain phosphorylation following activation appears to be normal in MUT-2 cells.

TCR down-regulation following ligand recognition is believed to be a consequence of serial engagement of multiple TCRs that results in sustained T cell activation (69) and has been shown to play a role in the regulation of T cell responses (70–72). However, analyses of TCRs bearing an α-chain, CD3δ, or CD3γ with cytoplasmic tail truncations indicate that down-regulation is not essential for cytokine production (67, 68), and this is consistent with our observations. The molecular mechanism(s) of internalization is not fully understood, and both tyrosine and serine protein kinases have been implicated in the process of down-regulation (73). Furthermore, because ligation of a TCR with a peptide:MHC complex or with anti-TCR Abs also induces activation of protein kinase C, it was assumed previously that TCR internalization in response to PMA stimulation and TCR triggering might follow similar mechanisms. However, more recent data suggest that this might not be the case (67, 74, 75). For example, phosphorylation of serine 126 in the CD3γ cytoplasmic tail, which is required for PMA-induced down-regulation, does not appear to be necessary for ligand-induced TCR internalization (75). Furthermore, TCRs internalized following PMA down-regulation are recycled, whereas agonist-induced down-regulation results in lysosomal degradation (74, 76). In the current study insertion of an *N*-linked carbohydrate in the proximity of the c″ strand of the α-chain affected both PMA-induced and peptide:MHC-mediated down-regulation, suggesting that this structural modification affected the recruitment or activation of a common machinery that is used in response to the mechanistically distinct stimuli. For example, the modification may affect the recruitment/activation of Rab5 GTPase and/or of clathrin-coated vesicle adaptor proteins that are known to be involved in TCR internalization (72, 75, 77, 78). In this context, clathrin coassembles with membrane-associated adaptor proteins into a polyhedral lattice to bring about receptor internalization (79, 80). It is therefore possible that the additional glycosylation might not allow the mutated TCR-CD3 complexes to configure into aggregates that are permissive for lattice formation.

A possible explanation for the observed phenotype of cells expressing the αS82N mutant TCR is that the protrusion induced by

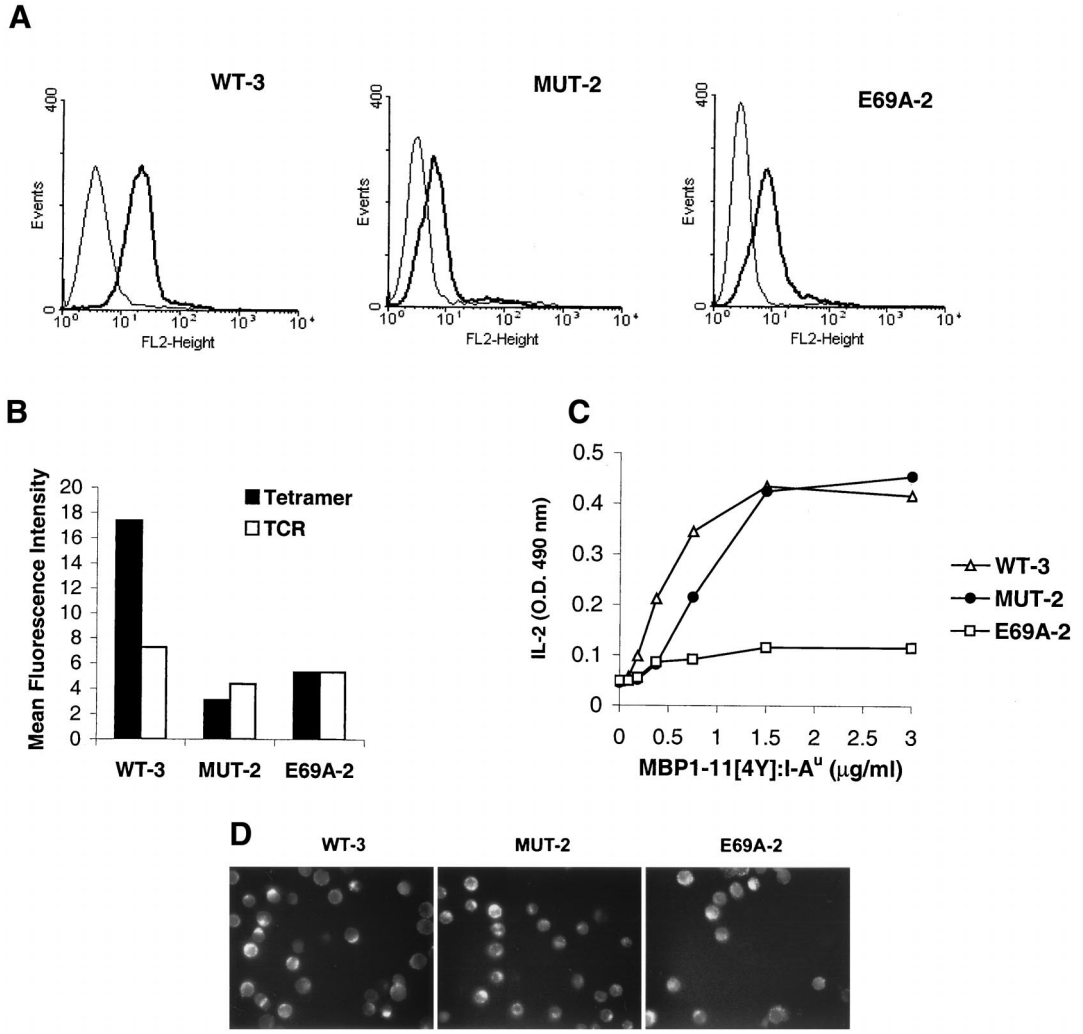

FIGURE 7. Analysis of TCR aggregation on WT-3, MUT-2, and E69A-2 cells. *A*, Staining of the transfectants with tetrameric (streptavidin-PE-labeled) MBP1–11[4Y]:I-A^u complexes (thick lines) and streptavidin-PE (thin lines). *B*, Staining of transfectants with anti-Cβ (H57-597) or tetrameric MBP1–11[4Y]:I-A^u complexes, expressed as the mean fluorescence intensity after background subtraction. *C*, IL-2 secretion by transfectants following incubation with plate-bound MBP1–11[4Y]:I-A^u complexes. IL-2 levels were determined by ELISA, and values are the averages of duplicates. Data are representative of three independent experiments. *D*, Capping analysis of WT-3, MUT-2, and E69A-2 transfectants. Cells were treated with 145-2C11 (anti-CD3ϵ) followed by Texas Red-labeled anti-hamster Ig. Cells were visualized by fluorescence microscopy. The fields shown are representative of data obtained from two independent experiments. Cells treated with Texas Red-labeled anti-hamster IgG Ab resulted in diffuse, almost undetectable staining (not shown).

glycosylation affects the association and/or activity of two or more distinct cellular components that are exclusively involved in two bifurcating processes (AICD and down-regulation) that do not involve any common intermediates. This is consistent with the effects of PMA and ionomycin on the transfectants; although the difference in down-regulation is still observed following treatment with these stimuli, apoptosis is induced to similar extents in both WT-3 and MUT-2 cells. The marked reduction in tetramer (MBP1–11[4Y]:I-A^u) staining by the mutant transfectants indicates that the additional carbohydrate severely limits aggregation of the TCRs into a configuration that allows multivalent binding by the tetramer. Whether this configuration is the same as that required for association of the proteins involved in AICD and/or down-regulation is not clear from the current experiments. However, it is probable that the loss of tetramer binding is a manifestation of the steric effects of the additional *N*-linked carbohydrate, and these effects may also impact on alternative configurations of the TCR and associated proteins necessary for either down-regulation or programmed cell death. Consistent with a role for the architecture of the TCR and associated proteins in programmed cell death, a recent study has demonstrated that altered peptide ligands that induce focal aggregation/capping of the TCR also induce apoptosis (81). Significantly, capping analyses indicate that the mutated TCR is able to aggregate following Ab-mediated cross-linking, demonstrating that this modified TCR retains the ability to undergo surface redistribution.

Recent studies indicate that there might be a redundancy in the TCR-proximal signaling mechanisms operative during down-regulation of the TCR and AICD. For example, constitutive p56^{lck} activation targets TCRs to lysosomal compartments (82), and this kinase has been suggested to play a critical role during cell death in T cells by up-regulating FasL expression (83, 84). Evidence supports involvement of the transcription factors NF-κB and NF-AT in regulating Fas ligand expression (85–87), but the unaffected IL-2 secretion by MUT-2 cells suggests that these factors are functionally intact. However, it has recently been shown that cytokine secretion and AICD are differentially sensitive to NF-κB deprivation (88). Thus, it is possible that while a signaling competent configuration with respect to IL-2 secretion is induced by cognate ligand in the mutant transfectants, the configuration for

$p56^{lck}$ to optimally phosphorylate substrates to levels necessary for apoptosis and TCR internalization does not form. These substrates may be as yet unidentified and may be distinct for the two processes. This (partial) defect in $p56^{lck}$ activity could be due to either sequestration of the putative substrates and/or inappropriate orientation of $p56^{lck}$. By analogy, proximity and spatial orientation of the tyrosine kinase ZAP-70 have been shown to be key parameters in T cell signaling (89).

In conclusion, the results indicate that following stimulation, a specific configuration of the TCR complex is needed for AICD and receptor internalization that is distinct from that needed for IL-2 production. The generation of this configuration appears to correlate with tetrameric ligand binding and involves a region of the TCR Vα domain that, due to a specific strand rearrangement, has a flatter surface than the corresponding surface in Ig V domains and the majority of TCR Vβ domains analyzed to date. The resulting complex may form an optimal module necessary for recruitment of/interaction with the molecular machinery critical for TCR down-regulation and AICD. The transfectants described in this study could provide a useful system to dissect mechanisms of T cell apoptosis and TCR internalization, and this may lead to an improved understanding of the requirements for a particular higher order configuration of the TCR and associated polypeptides in the two processes. Finally, the resistance of the transfectants bearing the mutated TCR to programmed cell death suggests that our studies are of relevance to diseases involving dysregulated T cell homeostasis, such as neoplasia and autoimmunity.

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