Characterization of Thymus-derived Lymphocytes Expressing Tiα-βCD3γδεζ-ζ, Tiα-βCD3γδεη-η or Tiα-βCD3γδεζ-ζ/ζ-η Antigen Receptor Isoforms: Analysis by Gene Transfection

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Summary

To characterize the function of the CD3η subunit of the T cell receptor (TCR), we have used cDNAs encoding CD3ζ, CD3η, or both to reconstitute a variant of a cytochrome c–specific, I-E^k-restricted murine T cell hybridoma, termed MA5.8, which lacks CD3ζ and CD3η proteins. We provide direct evidence that assembly and surface expression of TCRs can be mediated by either of these subunits separately or together. However, the level of TCR expression on ζ transfectants is up to one order of magnitude greater than that on η transfectants, implying that CD3η is weakly associated with the pentameric Tiα-βCD3γδε complex and/or inefficient at salvaging the incomplete TCR from lysosomal degradation. As a component of the TCR, the CD3η subunit preferentially forms a heterodimer with CD3ζ, but is also able to form a CD3η-η homodimer. Crosslinking of Tiα-βCD3γδεζ-ζ, Tiα-βCD3γδεη-η, or Tiα-βCD3γδεζ-ζ/ζ-η TCR isotypes with anti-CD3ε monoclonal antibody or a cytochrome c peptide epitope on I-E^k antigen-presenting cells mediates signal transduction resulting in reversible cell-cycle arrest of transfected clones. Given the potential for diversity of signals generated by these functional TCR isotypes and the expression of the CD3 η gene product in the thymus, CD3η is likely to play a role in selection and/or activation of thymocytes during development.

The TCR, a molecular complex consisting of at least seven subunits, is responsible for recognition of foreign antigen in the context of a particular MHC product (reviewed in references 1–4). The clonally unique antigen-specific binding component of this receptor has been termed Ti and exists as a disulfide-linked α-β heterodimer on the majority of peripheral T cells. The subunits of Ti are Ig-like and are composed of variable and constant regions. In turn, the Ti structure is noncovalently associated with a set of invariant molecules referred to as the CD3 subunits. CD3γ, CD3δ, and CD3ε are structurally related polypeptides encoded by closely linked genes located on human chromosome 11 and mouse chromosome 9 (5–8). In contrast, the structurally distinct CD3ζ molecule is encoded on chromosome 1 in both man and mouse (9, 10). CD3γ, CD3δ, and CD3ε exist primarily as monomeric components in the TCR, although a minor component of CD3ε disulfide-linked homodimers has been found in human and murine TCRs (10a). CD3ζ is found primarily as a homodimer within the CD3-Ti complex (11). Recent work has suggested an additional level of complexity of TCR structure in that ~10% of CD3ζ subunits exist as disulfide-linked heterodimers, bonded to another subunit,

termed CD3η (11). It is particularly interesting that studies have suggested that although the copy number of the TCR-associated CD3ζ-η heterodimer is one order of magnitude less than that of CD3ζ-ζ homodimers, CD3ζ-η may be important in coupling the TCR to phosphoinositide hydrolysis (12). Furthermore, other studies using murine T-T hybridomas have suggested that CD3ζ-η may be important in TCR-mediated programmed cell death (13).

Given the potential importance of the CD3η subunit of the TCR, we recently characterized the primary structure of murine CD3η as deduced from protein microsequencing and cDNA cloning (14). The mature CD3η protein was predicted to consist of a 9 amino acid extracellular segment, a 21 amino acid transmembrane segment harboring a negatively charged residue characteristic of CD3 subunits, and a 155 amino acid cytoplasmic tail. The NH$_2$-terminal sequences of CD3η and CD3ζ were found to be identical through the first 122 amino acids of each mature protein and to diverge in their COOH-terminal regions. These findings were consistent with the possibility that CD3η and CD3ζ were alternatively spliced products of a common gene. This supposition has been verified by direct genomic cloning and

sequencing (Clayton, L.K., L. D'Adamio, M. Sieh, R. E. Hussey, S. Kuyasu, and E. L. Reinherz, manuscript submitted for publication). This differential splicing results in a cytoplasmic domain of CD3η which is 42 amino acids longer than that of CD3ζ. In addition, CD3η lacks one of six potential tyrosine phosphorylation sites and, perhaps more importantly, a putative nucleotide binding site previously identified in CD3ζ (15).

To directly analyze the consequences of these structural differences in CD3η and CD3ζ, we have used transfection techniques to express CD3η and CD3ζ cDNAs in eukaryotic expression vectors separately or together in murine T cells lacking CD3η and CD3ζ proteins. The results to be reported below demonstrate for the first time that the CD3η subunit in the form of a CD3η-η homodimer is able to associate with and transport Tiα-βCD3γδε pentamers to the cell surface. Thus, TCR assembly, surface expression and function are not restricted to the CD3ζ-ζ homodimer or the CD3ζ-η heterodimer. Substantial differences in TCR copy number resulting from CD3η vs. CD3ζ cDNA transfection using the same expression vectors suggest that CD3η and CD3ζ isoforms may differentially regulate surface TCR expression. In addition, functional studies with transfectants expressing Tiα-βCD3γδεζ-ζ, Tiα-βCD3γδεη-η, and Tiα-βCD3γδεζ-ζ/ζ-η indicate that each TCR isoform is competent to mediate signal transduction. The implications of these findings for differential TCR triggering during thymic ontogeny are discussed.

Materials and Methods

BALB/c mice and B10.BR mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (German Town, NY). New Zealand white rabbits were from Pine Acres Rabbitry (Brattleboro, VT). RPMI 1640 medium, L-glutamine, penicillin-streptomycin, and G418 (geneticin) were purchased from Gibco Laboratories (Grand Island, NY). FCS and propidium iodide were from Sigma Chemical Co. (St. Louis, MO). FITC-conjugated IgG fraction of goat antiserum against mouse IgG was from Whittaker M.A. Bioproducts (Walkersville, MD). FITC-conjugated IgG fraction of goat antiserum against hamster IgG that does not cross-react with mouse IgG was from Caltag Laboratories (So. San Francisco, CA). Alkaline phosphatase-conjugated IgG fraction of goat antiserum against rabbit IgG and prestained molecular weight markers were from Biorad Laboratories (Richmond, CA). RNase A was from Worthington Biochemical Corp. (Freehold, NJ). Succinimydyl-4-6 maleimidophenyl butyrate (S-SMPB) was from Pierce Chemical Co. (Rockford, IL). Immulon plates (96-well, round-bottomed ELISA plates) were from Dynatech Laboratories (Chantilly, VA). Polyvinyldifluoride (PVDF)[1] membrane was from Millipore Corp. (Bedford, MA). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH). RPMI 1640 medium was generally supplemented with 50 U/ml penicillin G, 50 μg/ml streptomycin, and 4 mM L-glutamine.

A hamster-mouse somatic B cell hybridoma 145.2C11 producing a mAb (2C11) against murine CD3ε (16), was generously provided by J.A. Bluestone (National Institutes of Health, Bethesda, MD). 2C11 mAb was purified from culture supernatant of the hybridoma.

Anti-human Ti mAb 11C5 (IgG1) specific for an irrelevant TCR clonotype used as a control antibody was made in our lab. mAb A2B4.2 specific for the α chain of the 2B4.11 TCR (17) was the generous gift of J. Ashwell (National Institutes of Health). A synthetic cytochrome c epitope corresponding to the residues 86–103 of moth cytochrome c and lacking the residues 90–92 (18) was synthesized on an automated peptide synthesizer (model 431A; Applied Biosystems Inc., Foster City, CA) and purified by reverse-phase HPLC using a C18 column. The sequence of the peptide is as follows: KKANDLIAYLKQATK.

Cells and Growth Conditions. The cytochrome c–specific, I-E^k-restricted murine T cell hybridoma 2B4.11, its variant MA5.8 (19), and the antigen-presenting B cell hybridoma LK35.2 were generous gifts of J. Ashwell (National Institutes of Health). An ovalbumin-specific, I-A^d-restricted murine T cell hybridoma 3DO54.8 was a kind gift of P. Marrack (National Jewish Hospital, Denver, CO). Cells were maintained in RPMI 1640 supplemented with 10% FCS. Transfectants were maintained in RPMI 1640 containing 10% FCS and 500 μg/ml G418. For determination of cell growth after TCR crosslinking, T cell hybridomas and transfectants were plated into 96-well, round-bottomed Immulon plates with or without 2C11 mAb (4 μg/well) and cultured in RPMI 1640 containing 10% (vol/vol) FCS at 2 × 10^4 cells/200 μl/well. Cell numbers were counted in duplicate samples on days 1, 2, and 3. To examine whether growth arrest after CD3 crosslinking is reversible, cells were collected from the wells coated with 2C11 on day 1, replated into nontreated wells, and cell numbers were counted on days 2 and 3.

Construction of Expression Plasmids. The inserts of pBS17 (murine CD3η) and pBS23 (murine CD3ζ) (14) were excised with EcoRI and blunted with DNA PolI (Boehringer Mannheim Biochemicals, Indianapolis, IN). The inserts were ligated to pPink-2 (20) which had been cut with XbaI and blunted as described above. Ligations were transformed into HB101 and positive colonies were identified by colony hybridization with CD3ζ- or CD3η-specific probes prepared by digesting pBS23 with EcoNI and EcoRI and pBS17 with AvaII and EcoRI, respectively. The 3' fragments of the cDNAs (14), were isolated in low melting agarose gels and specific probes were labeled by the random-priming method (21). Plasmid DNA was isolated from correct orientation constructs of pPink-2/ζ and pPink-2/η by double banding in CsCl or by Qiagen plasmid kits (Studio City, CA) following the manufacturer's recommendations. Plasmid DNA was prepared for transfection by linearization with XmnI. All restriction enzymes were from New England Biolabs (Beverly, MA).

Generation and Flow Cytofluorometric Analysis of Transfectants. Transfections into MA5.8 cells were performed on a Bethesda Research Laboratories (Gaithersburg, MD) Cell-Porator using 200 V and a capacitance of 800 μF. 15 μg pPink-2/ζ, pPink-2/η, or both were transfected. After transfection, cells were plated in 6-well plates in RPMI 1640 containing 10% FCS at a concentration of 2 × 10^5 cells/2 ml/well. After 24 h, G418 was added at 1 mg/ml and medium was changed every 2–3 d. G418-resistant clones were selected over 2–3 wk and subcloning was carried out by limiting dilution method at 0.3 cells/well. Clones of transfectants are designated by the original number of the transfectant followed by a decimal point and an additional number, i.e., MAη61.9 is a clone of MAη61. Cell surface TCR expression of transfectants was quantified by indirect immunofluorescence using 2C11 (anti-CD3ε) and 11C5 (control) antibodies and FITC-conjugated goat anti-hamster antibody.

RNA Analysis. Total RNA was isolated from 2B4.11, MA5.8, and transfectants using guanidine isothiocyanate (22). RNA concentrations were measured spectrophotometrically and 10 μg of

total RNA was run per lane on a 1% agarose gel containing 2.2 M formaldehyde and 1× MOPS buffer solution (22). The RNA was transferred to nitrocellulose in 20× SSC and the RNA blot was hybridized to the CD3η-specific probe described above. Hybridization was in 50% formamide, 5× SSC, 5× Denhardt's, 0.1% SDS, 250 μg/ml denatured salmon sperm DNA, 50 mM NaPO₄, pH 6.5, at 42°C for 16–20 h with 1 × 10⁶ cpm/ml probe. After washing in 2× SSC, 0.1% SDS for 15–30 min at room temperature and 0.1× SSC, 0.1% SDS at 50°C for 30 min, the blot was exposed to Kodak X-Omat AR x-ray film at −70°C for the indicated times. The blot was then stripped by boiling in distilled water for 10 min and exposed for 12–14 h to insure all signals had been removed. The filter was next hybridized to the CD3ζ-specific probe using conditions as described for the CD3η specific probe, washed, and autoradiographed.

¹²⁵I Surface Labeling, Immunoprecipitation, and Two-Dimensional (2-D) Reducing/Nonreducing SDS-PAGE Analysis. 5 × 10⁷ cells were labeled with 1 mCi of ¹²⁵I by lactoperoxidase-catalyzed surface iodination. They were then lysed in 1 ml of digitonin lysis buffer solution (1% digitonin, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 10 mM iodoacetamide, 20 mM Tris/HCl, pH 7.4). After 2 h at 4°C, the lysates were centrifuged at 13,000 g for 15 min at 4°C. The postnuclear supernatant was immunoprecipitated overnight at 4°C with 25 μl of Sepharose beads coupled with 2C11 (3–5 mg/ml). Thereafter, the pelleted beads were washed once with 0.05% digitonin, 150 mM NaCl, 20 mM Tris/HCl, pH 7.4, twice with 150 mM NaCl, 20 mM Tris/HCl, pH 7.4 (TBS), and once with 20 mM Tris/HCl, pH 7.4. The protein was eluted from the beads by boiling for 5 min in 25 μl of Laemmli's nonreducing SDS sample buffer solution (23). Samples were subject to electrophoresis in 12.5% polyacrylamide using the nonreducing buffer system of Laemmli. The gel was then equilibrated with reducing Laemmli's SDS sample buffer solution for 20 min before the second dimension electrophoresis in a 12.5% polyacrylamide gel. The gels were dried and autoradiographed with Kodak X-Omat AR x-ray film at −70°C for 1–3 d.

Heteroantiserum Production. A rabbit polyclonal antiserum, NS-2, was produced against a CD3η-related peptide, CLWSPWPPS-SSSQL, comprising the COOH-terminal 13 amino acids of CD3η with an NH₂-terminal cysteine residue added for coupling purposes. The peptide was synthesized with a peptide synthesizer (model 431A; Applied Biosystems), cleaved from resin in trifluoroacetic acid, and purified on a Waters Binary Gradient HPLC using an RCM μ Bondapak C18 25 × 100 mm column. The peptide was coupled to BSA pretreated with S-SMPB and 200 μg of the BSA-peptide conjugate in CFA (diluted 1:1 with PBS) injected intradermally into ∼20 sites in the backs of two New Zealand white rabbits. At 2 wk, the rabbits were boosted with 100 μg BSA-peptide conjugate in IFA. Beginning at 4 wk, the rabbits were bled weekly from ear veins and the serum used in Western blot analysis.

Western Blotting. 2-D SDS-PAGE of 2C11 immunoprecipitates was performed as described above. 1 × 10⁸ cells were used in each Western blot. Immediately after the 2nd dimension SDS-PAGE, electrophoretic transfer to a PVDF membrane was carried out essentially according to Legendre and Matsudaira (24) with a buffer solution consisting of 25 mM Tris, 192 mM glycine, 20% methanol in a trans-blot apparatus (Bio-Rad Laboratories) following the manufacturer's directions. The PVDF membrane was removed from the transblot, preincubated in TBS containing 1% BSA for 2 h at room temperature and then incubated with anti-η antiserum NS-2 (1:200 dilution) in TBS containing 1% BSA with gentle shaking overnight. After three 15-min washes in TBS containing 0.1% Triton X-100, the PVDF membrane was incubated with alka-

line phosphatase–conjugated goat anti–rabbit antibody (1:2,000 dilution) in TBS for 2 h. After three 15-min washes in TBS containing 0.1% Triton X-100 and two 15-min washes in TBS, the color was developed using Biorad alkaline phosphatase color developing kit following the manufacturer's instructions. After electrophoretic transfer, all experiments were performed at room temperature.

Activation of T Cells with Anti-CD3ε mAb or Antigen-pulsed Presenting Cells. For the cell stimulation by crosslinking with antibody-coated plates, we cultured 2 × 10⁴ cells/200 μl in triplicate in 96-well Immulon plates. The wells were precoated for at least 2 h at room temperature with 2C11 (4 μg/well) or media. The plates were washed twice with PBS. After 24 h of incubation the cells were plated with 1 μCi/well [³H]TdR for 4 h and incorporation was measured by scintillation counting. For stimulation of the cells with peptide antigen, 5 × 10⁴ LK35.2 cells irradiated with 10,000 rad were pulsed in 100 μl of media containing 1–100 μM moth cytochrome c peptide for 2 h in triplicate in 96-well, round-bottomed microtiter plates. Subsequently 5 × 10⁴ transfectants in 100 μl of media were added to each well. After 24 h of cultivation, DNA synthesis was determined by [³H]TdR incorporation as above.

Cell Cycle Analysis. Cells (2 × 10⁵) were cultured for 24 h in 10 wells of Immulon plates with or without 2C11 mAb, collected, and washed once with RPMI 1640 containing 10% (vol/vol) FCS. After centrifugation, cell pellets were mixed with 0.5 ml of a solution consisting of 50 μg/ml propidium iodide, 0.05% NP40, 10 μg/ml RNase A, and 0.1% sodium citrate. After incubation for 1 hr on ice, flow cytometry was performed using a FACScan (Becton Dickinson, Mountain View, CA) and the results were analyzed by Dean's method (25).

Results

Production of CD3ζ, η and ζ + η Transfectants. To directly examine the role of CD3η in TCR assembly, surface expression, and signal transduction, CD3ζ and CD3η cDNAs were cloned into the eukaryotic expression vector pPink-2 (20) by standard methodology. In this vector (Fig. 1), expression of the cDNA insert is under the control of the spleen focus forming virus (SFFV) long terminal repeat (LTR) and the presence of the neomycin resistance gene provides the selection marker for the antibiotic G418. As shown in Fig. 1, CD3ζ and CD3η inserts are identical from nucleotide 50 to 568 being derived from the same gene locus. They differ in a short stretch of 5' untranslated region (bp 1 to 49) and within the coding and 3' untranslated region from bp 569 to the vector insertion site (numbering according to CD3η in reference 14). pPink-2/η and pPink-2/ζ plasmids were electroporated into MA5.8 separately or together and transfectants selected after 10–14 d in G418.

Individual transfectants were characterized by flow cytometry for expression of the TCR using either the anti-CD3ε mAb 145.2C11 (2C11) (Fig. 2) or the anti-clonotypic anti-Ti mAb A2B4.2. Analyses were carried out on the original transfectants or clones derived from the original transfectants. Clones are designated by the number of the transfectant from which they are derived followed by a decimal point and number; since all transfectants used were derived from MA5.8, they were given the prefix "MA." The original transfectants
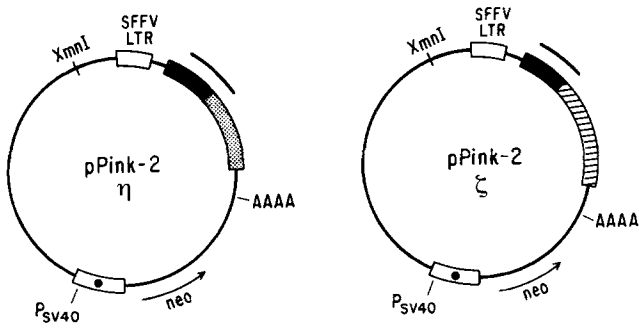
Figure 1. Expression constructs for transfection. cDNA encoding CD3η and CD3ζ were subcloned into the pPink-2 vector as described in Materials and Methods. This places the insert under control of the SFFV LTR with SV40 encoded poly(A) addition and splicing signals, the neomycin-resistance gene and SV40 origin of replication. The solid bars within the inserts indicate sequences common to CD3η and CD3ζ. The dotted or hatched bars represent sequence differences at the 5' and 3' ends of the inserts. The 5' end of the CD3ζ insert begins in the common sequences of CD3ζ and CD3η. There are 49 bp more on the 5' end of the CD3η insert, including 34 bp of sequence unique to CD3η and divergent from the 5' end of CD3ζ (14). The vector-encoded polyadenylation signal for the insert is indicated by AAAA. The line outside of the inserts indicates the coding region for CD3ζ or CD3η. Plasmids were linearized with XmnI before electroporation.
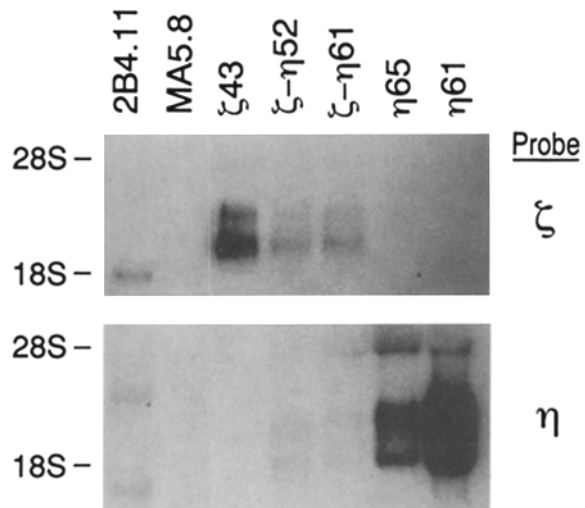
and clones both behaved identically in all assays. As shown in Fig. 2 and consistent with prior observations, the MA5.8 mutant expresses 10-fold less TCR than the parental cell line 2B4.11 as judged by 2C11 reactivity. Transfection of MA5.8
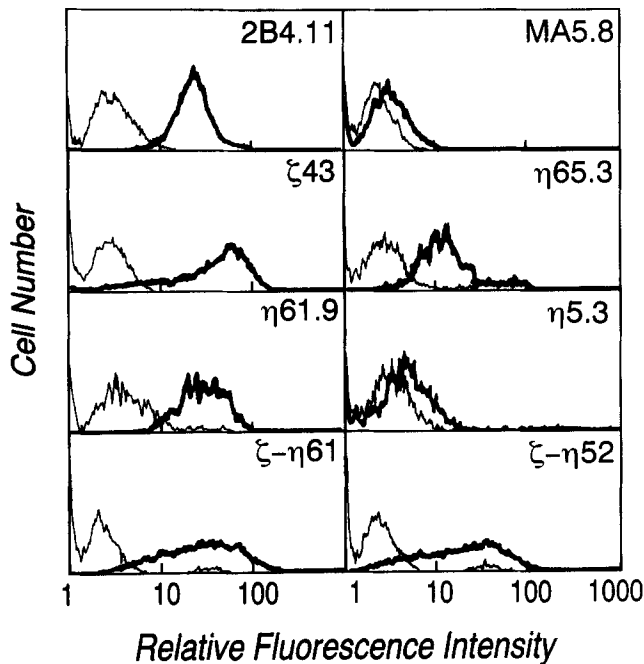
with CD3ζ resulted in surface TCR expression three to four-fold greater than 2B4.11 as judged by 2C11 reactivity. A similar increase in reactivity was observed when transfectants were analyzed with the anti-Ti mAb A2B4.2 (data not shown). The high level TCR expression observed for the CD3ζ transfectants was found in each of 15 transfectants examined. In contrast, 43 of 45 CD3η transfectants examined showed little anti-CD3ε mAb (see Fig. 2, MAη5.3) or anti-Ti MAb (data not shown) reactivity above that observed for MA5.8 alone. 2 of 45 CD3η transfectants, as represented by MAη61.9 and MAη65.3, express significantly more TCR than the MA5.8 cell line (Fig. 2); levels of expression are close to those of 2B4.11 and threefold less than that of ζ transfectants. The ζ-η double transfectants and clones showed, perhaps not surprisingly, a more heterogeneous level of TCR expression.

Northern Analysis of ζ and η Transfectants. RNA was prepared from representative transfectants, size fractionated by agarose gel electrophoresis, transferred to nitrocellulose membranes, and hybridized with a ^{32}P labeled cDNA probe specific for CD3η. Subsequently, the same blot was stripped and rehybridized with a CD3ζ specific probe. As shown in Fig. 3, the 1.7-kb CD3η mRNA is present in 2B4.11 but absent in MA5.8. In addition, a 3.4-kb band hybridizing with the CD3η probe is detected in 2B4.11 exclusively. The significance of this species is presently unknown. The lanes containing the RNA from CD3η transfectants MAη65 and MAη61 lack the 1.7-kb endogenous CD3η mRNA but rather demonstrate intense 2.9- and 2.2-kb hybridizing bands. The latter represent CD3η mRNA that are derived from the transfected plasmid DNA. The unique sizes of these transcripts

Figure 2. Flow cytometry analysis of CD3-Ti cell surface expression. 2B4.11, MA5.8, and MA5.8 transfectants expressing CD3ζ, CD3η or both were stained with anti-CD3ε mAb 2C11 (*thick line*) or control mAb 11C5 (*thin line*) followed by FITC-conjugated goat anti–hamster antibody.

Figure 3. Expression of CD3ζ and η mRNAs in 2B4.11, MA5.8, and representative ζ, η, or ζ-η MA5.8 transfectants. 10 μg of total RNA isolated from the indicated cells were size-fractionated on a 1% agarose gel containing formaldehyde and transferred to nitrocellulose. The filter was hybridized with a CD3η-specific probe, washed, and exposed at −70°C for 42 h with an intensifying screen. The filter was then stripped, reprobed with a CD3ζ-specific probe and exposed for 16 h. Positions of ribosomal 28S and 18S RNAs are indicated.

are probably due to differences in transcriptional initiation and/or termination sites of the DNA introduced into MAη65 and MAη61 via the pPink-2 vector; both the insert and the vector contain polyadenylation signals. MAη5.3 shown in Fig. 2 also expresses CD3η mRNA derived from transfected cDNA (data not shown). In contrast and as expected, RNA from the representative CD3ζ transfectant, MAζ43, lacks reactivity with the CD3η-specific probe. In ζ-η double transfectants, MAζ-η52 and MAζ-η61, a weak but definite set of hybridizing bands identical in size to that found in the η transfectants are detected. Reanalysis of the same blot with a CD3ζ probe identified the 2-kb CD3ζ mRNA in 2B4.11 which is absent in MA5.8. The CD3ζ probe hybridized to two mRNA species of 3.2- and 2.5-kb in ζ and ζ-η transfectants but not in η transfectants. Again, endogenous and exogenous CD3ζ mRNA bands could be readily detected in 2B4.11 and transfectants, respectively.

Given that the level of CD3η mRNA in η transfectants was as great or greater than the level of CD3ζ mRNA in ζ transfectants, the lower copy number of surface TCR complexes on the η transfectants relative to ζ transfectants could not be explained based simply on differences in steady-state mRNA expression. Furthermore, in view of the observation that synthetic RNA derived from CD3η and CD3ζ cDNA clones translates equivalently in a wheat germ in vitro system (data not shown), it is not obvious that translational differences account for the differential TCR surface expression in these transfectants. The observed differences are clearly post-transcriptionally regulated. It appears that CD3η is less able than CD3ζ to facilitate surface TCR expression when transfected into MA5.8. This is also observed in 2B4.11 where the levels of mRNA for CD3ζ and CD3η are very similar (Fig. 3), while the level of CD3η protein associated with surface TCR is an order of magnitude less than that of CD3ζ (see below and reference 11). We cannot exclude the possibility that the steady-state level of CD3η protein is less than CD3ζ protein in the T cell.

Biochemical Analysis of TCR Isoforms in Transfectants. To next characterize the TCR components in individual transfectants, cells were surface labeled with ^{125}I by the lactoperoxidase method, solubilized in digitonin, and immunoprecipitated with anti-CD3ε mAb. The resulting complex was analyzed in a 2-D nonreducing/reducing gel. As shown in the autoradiogram of 2B4.11 and as depicted schematically in Fig. 4 (*bottom right panel*), the off-diagonal Tiα-β heterodimer at 40–60 kD and CD3γ, CD3δ, and CD3ε subunits at 20, 25, and 26 kD, respectively, are readily apparent. The CD3ζ-ζ homodimer (16-kD subunits) and the CD3ζ-η heterodimer (16- and 22-kD subunits) are evident and the frequency of incorporation of CD3ζ into the homodimer is ~10-fold greater than its incorporation into the CD3ζ-η heterodimer. A 26-kD spot below the diagonal in 2B4.11 represents the CD3ε-ε homodimer (10a). The CD3ε-ε homodimer spot serves as an internal marker of the CD3ζ-η heterodimer, appearing just above and to the left of the latter. The set of off-diagonal CD3ζ spots to the right of the CD3ζ-ζ homodimer likely represent CD3ζ complexed to a smaller set of ~10-kD proteins. In addition, an 8-kD dimer run-

ning at 16 kD in the nonreduced dimension is noted. The significance of these spots is presently unknown but may represent other components of the TCR complex and/or include CD3ζ degradation products.

In contrast to 2B4.11, the anti-CD3ε immunoprecipitate of MA5.8 contains no CD3ζ or CD3η spots; only Tiα-β and CD3γ, CD3δ, and CD3ε monomers and CD3ε-ε homodimer spots are apparent. However, ζ transfectants of MA5.8 such as MAζ43 have a readily detectable CD3ζ-ζ homodimer spot as well as CD3ζ complexed to 10-kD structures. No CD3η is apparent in these cells, consistent with earlier studies showing that CD3η is not a post-translationally modified form of CD3ζ (26). On the other hand, the representative CD3η transfectant MAη61 contains a homodimer appearing as an off-diagonal spot at 22 kD in the reducing dimension, directly below the CD3ε-ε homodimer, presumably representing CD3η. No CD3ζ is detected, again demonstrating that CD3ζ and CD3η are distinct proteins. Finally, the ζ-η
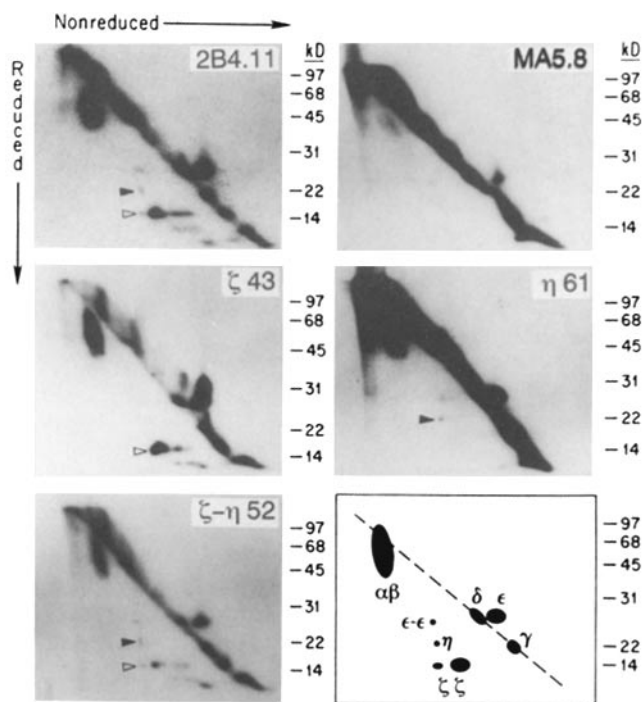

Figure 4. 2-D gel analysis of surface-labeled TCR components on 2B4.11, MA5.8, and representative transfectants. Cells were surface labeled with ^{125}I using lactoperoxidase, solubilized in digitonin and immunoprecipitated with anti-CD3ε mAb 2C11. Immunoprecipitates were run on 2-D nonreducing/reducing SDS-PAGE and autoradiographed. A schematic diagram (*lower right*) depicts the positions of Tiα, Tiβ, CD3γ, CD3δ, CD3ε, CD3ζ, and CD3η components. Note that while the 26-kD off-diagonal spot is designated as a CD3ε-ε homodimer, we cannot exclude the possibility that CD3ε forms a heterodimer with unknown protein of the same molecular weight (10a). Black arrowheads indicate the position of CD3η in the CD3η transfectant MAη61, the double transfectant MAζ-η52 and 2B4.11. The open arrowheads in the MAζ-η52 double transfectant, MAζ43, and 2B4.11 indicate the position of CD3ζ. Molecular mass markers are as follows: 97 kD, phosphorylase B; 68 kD, BSA; 45 kD ovalbumin; 31 kD, carbonic anhydrase; 22 kD, soybean trypsin inhibitor; and 14 kD, lysozyme.

double transfectant MAζ-η52 contains both the CD3ζ-η heterodimer and the CD3ζ-ζ homodimer in the anti-CD3ε immunoprecipitate. The absence of the putative η-η homodimer in the double transfectants and relative abundance of CD3ζ-ζ over CD3ζ-η dimers implies that the preference for subunit assembly in a stable TCR complex is CD3ζ-ζ > CD3ζ-η > CD3η-η. Note that while the surface TCR level of MAη61 is equivalent to that of 2B4.11, the amount of labeled CD3η protein in anti-CD3ε immunoprecipitates of MAη61 is much less than the total amount of CD3ζ plus CD3η in immunoprecipitates of 2B4.11, raising the possibility that CD3η is more loosely associated with the TCR than CD3ζ.

Direct Identification of the CD3η Gene Product in TCR Complexes by Means of Specific Antibody. To directly assess individual transfectants for the presence or absence of CD3η protein, we developed a rabbit antiserum, NS-2, against a CD3η-specific peptide for use in Western blot analysis of anti-CD3ε mAb immunoprecipitates from cells lysed in digitonin. The antiserum NS-2 was produced against a synthetic peptide corresponding to COOH-terminal amino acids 173–185 of CD3η, which are not shared by CD3ζ (see Materials and Methods). As shown in Fig. 5 A, a specific 22-kD spot below the diagonal is seen in lysates of the ovalbumin-specific, I-A^d-restricted T cell hybridoma 3DO54.8. This spot is not present when the lysates were reacted with preimmune sera from the same rabbit. Furthermore, while the CD3η spot is also detected in 2B4.11 lysates, it is absent in the MA5.8 blot consistent with the lack of CD3η mRNA in MA5.8. These data prove the specificity of the anti-η antiserum NS-2.

The results of examination of transfectant lysates for CD3η protein are shown in Fig. 5 B and demonstrate an expected set of patterns. CD3η is present in the representative ζ-η transfectant MAζ-η52 and η transfectants MAη61 and MAη65. In contrast, no NS-2 reactivity with the CD3η protein is observed in the ζ transfectant MAζ43. Collectively, Northern analysis, surface labeling and immunoprecipitation studies as well as western blot analysis precisely delineate the differences in T cell receptor components of these transfectants: η transfectants express a TCR composed of Tiα-βCD3γδεη-η, ζ transfectants express a TCR composed of Tiα-βCD3γδεζ-ζ and ζ-η transfectants express both Tiα-βCD3γδεζ-ζ and Tiα-βCD3γδεζ-η TCR complexes. In the latter case, we cannot exclude the possibility that CD3ζ-ζ and CD3ζ-η dimers are present within the same TCR complex.

Crosslinking of TCR Isoforms Results in Signal Transduction Leading to Cell Cycle Arrest. Having characterized individual transfectant populations, it was important to assess the signal transduction potential of their different TCR isoforms. To this end, transfectants were stimulated in Immulon microtiter plates with or without anti-CD3ε mAb. In additional experiments, the TCR of transfectants was crosslinked with I-E^k expressing LK35.2 cells pulsed with moth cytochrome c peptide (KKANDLIAYLKQATK) which together define the antigen/MHC specificity of the 2B4.11 TCR (18). Earlier analyses (27) demonstrated that TCR crosslinking of T-T hybridomas including 2B4.11 results in cell cycle arrest and hence, a reduction in proliferation as measured by [^{3}H]TdR
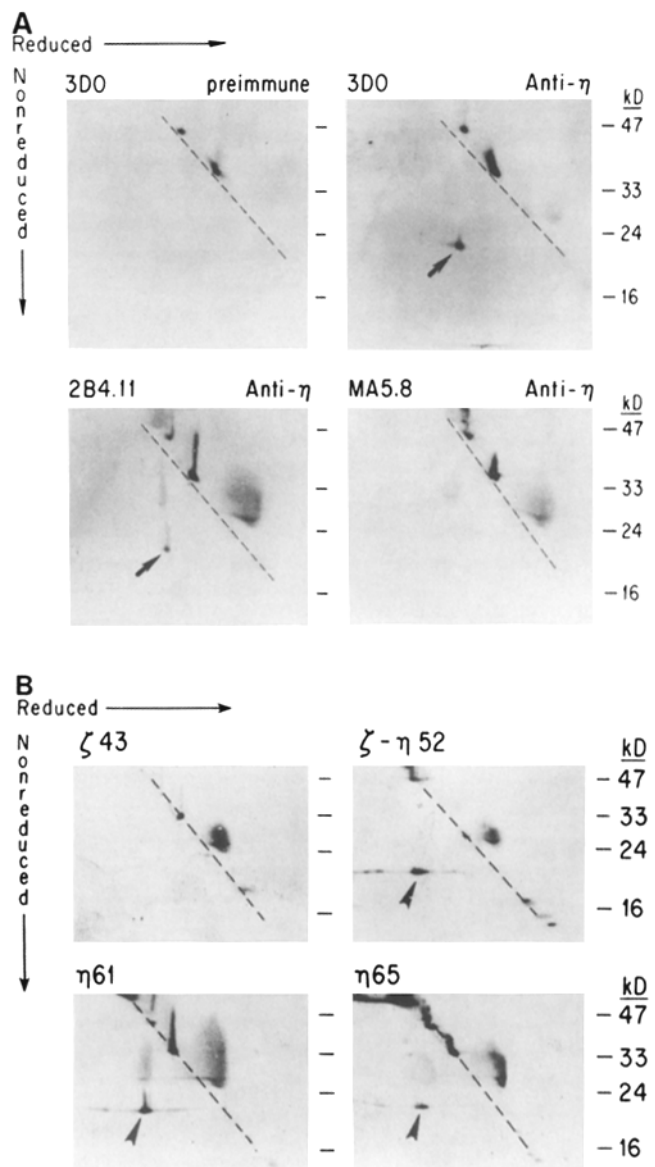
Figure 5. Western blot analysis of CD3η expression in 3DO54.8, 2B4.11, MA5.8 (A), and representative transfectants (B). Cells were lysed in digitonin, immunoprecipitated with anti-CD3ε mAb 2C11, and the resulting immunoprecipitates were run on 2-D nonreducing/reducing SDS-PAGE. Gels were blotted to PVDF membrane by electrophoretic transfer and the filters were incubated with preimmune serum or anti-η polyclonal antiserum NS-2 raised against a CD3η peptide (see Materials and Methods). The CD3η protein was detected using an alkaline phosphatase–conjugated goat anti–rabbit antibody and Bio-Rad Laboratories alkaline phosphatase developing reagents. Prestained molecular mass markers were; 47 kD, ovalbumin; 33 kD, carbonic anhydrase; 24 kD, soybean trypsin inhibitor; 16 kD, lysozyme. Arrowheads denote the position of the CD3η protein.

incorporation. As shown in Table 1, TCR crosslinking with anti-CD3ε mAb resulted in a significant decrease in [^{3}H] TdR incorporation of 2B4.11 and all transfected lines. For example, in comparison to media control, 2C11 reduced [^{3}H]TdR of MAζ43.2 by up to 91%. Similar effects were seen with all transfectants tested, regardless of their CD3ζ or CD3η isotype. Only MA5.8 which expresses a low copy

number of surface TCR complexes and lacks CD3ζ and CD3η showed no effect. Similar results were obtained when the cells were stimulated with antigen/MHC. The cytochrome c peptide at 10–100 μM also significantly reduced [^{3}H]TdR incorporation of 2B4.11 and all transfectants but was without significant effect on the antigen unresponsive MA5.8 line or the T-T hybridoma 3DO54.8 which has a different antigen specificity.

To determine whether the basis for the cell cycle arrest mediated by TCR crosslinking was the same or different in individual transfectants, representative cells were cultured in plates in the presence or absence of anti-CD3ϵ mAb for 24 h and then cell cycle analysis performed by flow cytometry using propidium iodide to stain the nuclear DNA. As shown in Fig. 6, before TCR crosslinking, 2B4.11, MA5.8, MAζ43, MAζ-η52, and MAη65 populations consisted of 51–57% of cells in G$_1$, 33-38% in S and 5–15%, in G$_2$/M. After TCR crosslinking, all populations with the exception of MA5.8 cells showed a reduction in cells in S phase (to 15–20%). In addition, the number of cells in G$_2$/M was reduced (see Fig. 6). These data indicate that TCRs comprising CD3ζ-ζ, CD3η-η, or CD3ζ-η transduce signals that arrest cell growth at the G$_1$/S boundary of the cell cycle. Whether the lack of cell cycle arrest in the MA5.8 cells is a result of the low number

of surface TCRs being insufficient to trigger signals or results from qualitative signaling differences due to the absence of CD3ζ and CD3η is not known at present.

Reversible Growth Inhibition Induced by Crosslinking of TCR Isoforms. To examine the effects of TCR signal transduction on cell growth of transfectants and the potential reversibility of cell cycle arrest, an additional set of experiments was performed. T cell hybridomas and transfectants were cultured in the presence or absence of anti-CD3ϵ mAb and cell numbers determined sequentially on each of 3 d in culture. In some cases, cells were removed from 2C11 coated wells after 24 h and replated into untreated wells. TCR crosslinking by 2C11 mAb resulted in growth arrest of 2B4.11 (Fig. 7) and 3DO54.8 (data not shown), and this inhibition was irreversible. In contrast, the growth of MA5.8 was unaffected by TCR crosslinking. Importantly, ζ, η or ζ-η transfectants also showed growth arrest, but in each case, the growth inhibition was reversible upon removal of the cells from the crosslinking stimulus as shown in Fig. 7 (*triangles*). Although not shown, no transfectants lysed upon TCR stimulation, as judged by ^{51}Cr release. Essentially the same results were obtained when 2B4.11, MA5.8, and transfectants were stimulated with I-E^k-expressing B10.BR splenocytes pulsed with the cytochrome c peptide (data not shown). These data show that neither

Table 1. *Growth Inhibition of Transfectants upon Stimulation with Antigen and MHC or Anti-CD3ϵ mAb Crosslinking*

Cells	Peptide concentration:	[^{3}H]TdR incorporation			
		0 μM	1 μM	10 μM	100 μM
		cpm			
2B4.11		19,505	24,336 (0)*	19,042 (2.4)	4,881 (74.4)
MA5.8		102,430	112,516 (0)	99,479 (2.9)	85,120 (16.8)
MAζ43.2		96,531	74,353 (22.9)	54,580 (43.5)	10,151 (89.5)
MAζ15.4		115,753	89,052 (23.1)	60,552 (47.7)	8,623 (92.6)
MAζ-η 52.9		121,896	119,543 (1.9)	106,256 (12.8)	59,530 (51.2)
MAζ-η 52.11		138,806	104,556 (24.7)	99,758 (28.1)	69,856 (49.7)
MAη61.9		119,300	99,294 (16.8)	82,280 (31)	18,709 (84.3)
MAη65.3		114,335	103,377 (9.6)	ND	71,275 (37.7)
3DO54.8		69,738	54,872 (21)	69,544 (0)	69,290 (0)
	Crosslinker:	None	Anti-CD3ϵ		
2B4.11		10,476	2,141 (79.6)		
MA5.8		57,331	56,853 (0.8)		
MAζ43.2		46,884	4,445 (90.5)		
MAζ15.4		51,432	8,369 (83.7)		
MAζ-η 52.9		23,217	14,154 (39)		
MAζ-η 52.11		37.391	26,591 (28.9)		
MAη61.9		55,561	29,604 (46.7)		
MAη65.3		29,473	14,305 (51.5)		
3DO54.8		32,021	1,441 (95.5)		

* Number in parentheses represents percent inhibition. Standard deviation was generally <10%.
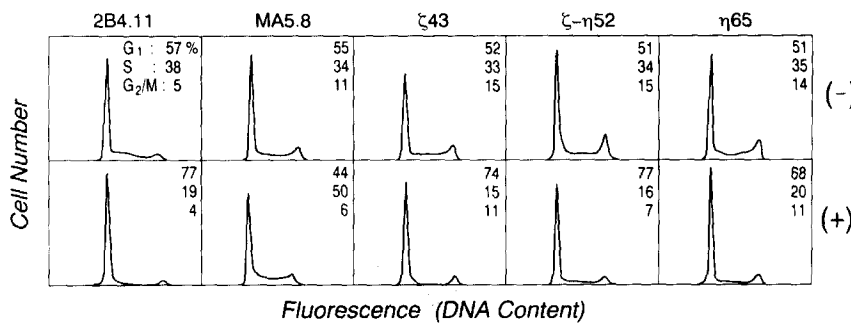
Figure 6. Cell-cycle analysis of the effect of TCR crosslinking on 2B4.11, MA5.8, and representative transfectants. 2×10^5 cells were cultured for 24 h in ten wells of plates coated with (+, *bottom panels*) or without (−, *top panels*) anti-CD3ε mAb 2C11. Nuclear DNA was stained with 50 μg/ml propidium iodide and analyzed for DNA content by flow cytometry on a FACScan. Numbers in the panels represent percentages of cells in G_1, S, or G_2/M as shown in the top left panel for 2B4.11.

CD3η nor CD3ζ, alone or in combination, is responsible for irreversible cell cycle arrest in this system. Likewise, crosslinking of TCRs expressing CD3η does not mediate cell death as assayed herein. The irreversible growth inhibition of 2B4.11 cells after TCR crosslinking is thus peculiar to T cell hybridomas such as 2B4.11 and 3DO54.8 and not simply a consequence of CD3η protein expression.

Discussion

In the present study, we have transfected cDNAs encoding CD3ζ or CD3η separately or together into the MA5.8 clonal variant of the murine T-T hybridoma 2B4.11. This clone is an ideal recipient with which to study the role of the CD3ζ and CD3η subunits since MA5.8 itself lacks both CD3ζ and

CD3η mRNA but expresses the other known TCR components, namely CD3γ, CD3δ, CD3ε, Tiα, and Tiβ subunits (19). The findings reported herein demonstrate that expression of CD3ζ or CD3η mRNA under the control of the SFFV LTR in stably transfected cell lines and clones results in the production of protein subunits which form disulfide-linked dimers that non-covalently associate with the other TCR components. This association is shown in anti-CD3ε precipitates of digitonin lysates from transfected cells and by the ability of the transfected subunits alone or together to augment surface TCR expression as judged by quantitative flow cytofluorometry. In η transfectants, the TCR-associated CD3η component in anti-CD3ε mAb immunoprecipitates is present as a homodimer. Likewise, ζ transfectants express a predominant TCR associated CD3ζ-ζ homodimer. In contrast, ζ-η double transfectants expressed both a CD3ζ-ζ homodimer and a CD3ζ-η heterodimer associated with the TCR. The absence of a detectable CD3ζ-η heterodimer in ζ transfectants is consistent with the finding of earlier studies involving selective transfer of CD3ζ into MA5.8 (28) and our recent finding that CD3η is an alternatively spliced product of the common gene which encodes both CD3ζ and CD3η proteins (14; Clayton, L. K., et al., manuscript submitted for publication). The lack of detectable TCR associated CD3η-η homodimers in the double transfectants implies that the formation of this homodimer is least favored relative to CD3ζ-ζ and CD3ζ-η dimers.

Northern blot analysis of ζ-η double transfectants indicates that the level of steady-state CD3ζ and CD3η mRNA is comparable in these cells. Nevertheless, the amount of TCR-associated CD3ζ protein in the CD3ζ-η heterodimers was at least one order of magnitude less than the amount of TCR-associated CD3ζ protein in the CD3ζ-ζ homodimer. Given that the extracellular segments of CD3ζ and CD3η are identical, this disparity could not be readily explained on the basis of differential ^{125}I labeling of the two subunits. Rather, in view of the fact that the cDNAs are driven by identical regulatory elements in the pPink-2 vector, the difference in the level of CD3ζ and CD3η associated with the TCR must be post-transcriptional.

Prior cytofluorometric analysis of MA5.8 revealed that the absence of CD3ζ correlated with a marked decrease in cell surface expression of the CD3-Ti complex in this cell line (19). Restoration of CD3ζ biosynthesis upon transfection reconstituted surface TCR expression. Furthermore, CD3ζ
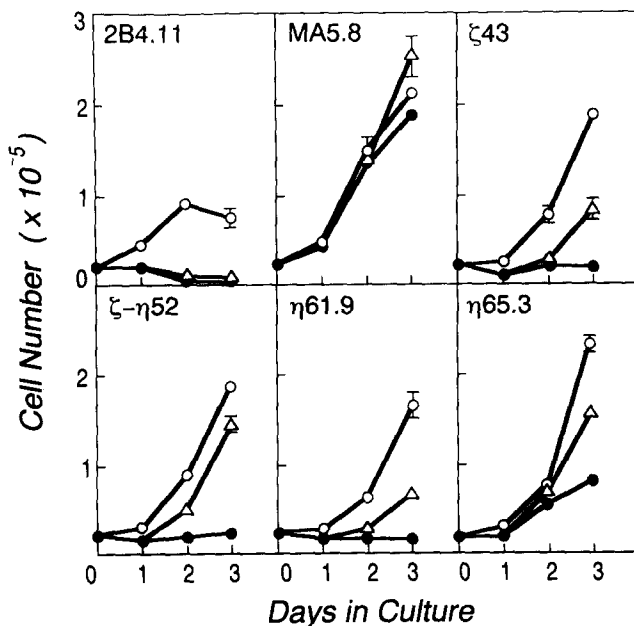
Figure 7. Effect of TCR crosslinking on cell growth in 2B4.11, MA5.8 and representative transfectants. Cells were plated at 2×10^4/well in plates with (O) or without (●) 2C11 mAb. Cell numbers were determined in duplicate on days 1, 2, and 3. To determine reversibility of inhibition, samples of cells incubated for 24 h in 2C11-coated wells were collected and replated into nontreated wells; cell numbers were then determined on days 2 and 3 (△).

protein level as quantitated by immunoblotting correlated with surface TCR expression (28). Biosynthesis studies have shown that although the pentameric $Ti\alpha$-βCD3$\gamma\delta\epsilon$ structure is assembled in the endoplasmic reticulum and transits into the Golgi apparatus in the absence of CD3ζ, the pentamers are transported to the lysozomes where they are degraded (29, 30). The increased surface TCR expression in T-T hybridomas resulting from η transfection is $\sim$5–10-fold less than the level resulting from ζ transfection. Even when the cells express high levels of CD3η mRNA as in MAη61.9, the level of surface TCR is still three to fivefold less than in ζ transfectants. It should be noted that 43 out of 45 η transfectants showed TCR expression as low as MA5.8 cells. For example, the level of TCR in MAη5.3 is indistinguishable from that of MA5.8 (Fig. 2), although this clone expresses CD3η mRNA derived from the transfected cDNA (data not shown). In contrast, all of the 15 ζ transfectants tested expressed high levels of TCR. These results imply that either CD3η is less efficient than CD3ζ in associating with the TCR pentamer or, once associated, cannot salvage the complex from lysozomal degradation as efficiently as CD3ζ. These possibilities are not mutually exclusive. A short half-life of CD3η protein and/or the unique structural feature of CD3η could account for such inefficiencies. Clearly, comparison of steady-state levels of CD3η and ζ proteins in transfectants will be informative. Furthermore, if the physical association of the CD3η-η homodimer with the TCR pentamer is weaker than that of the CD3ζ-ζ homodimer, then this may explain our inability to detect the CD3η-η receptor structure in immunoprecipitates with anti-CD3ϵ in cells expressing both CD3ζ and CD3η protein.

The present studies also have uncovered an additional complexity within TCR components as evidenced in the ζ transfectants. Anti-CD3ϵ immunoprecipitates of ^{125}I surface-labeled ζ transfectants identified all the known components of the TCR heptamer, $Ti\alpha$-βCD3$\gamma\delta\epsilon\zeta$-$\zeta$. In addition, a series of CD3ζ components linked to a heterogeneous set of $\sim$10-kD proteins were detected. These heterodimeric forms are also present in the parental 2B4.11 T-T hybridoma but absent from MA5.8, consistent with the notion that the 10-kD structures bond specifically to CD3ζ. Although we cannot exclude the possibility that some of the 10-kD spots represent degradation products of CD3ζ, in view of the CD3ζ subunit's ability to complex either with itself or the related CD3η subunit, the possibility that CD3ζ could disulfide bond to other molecules is likely. The recently identified structural homology between the FcϵRIγ subunit and the CD3ζ subunit (31, 32) raises the possibility that FcϵRIγ or a related protein might comprise one subunit of these heterodimers. Alternatively, the partner involved in heterodimeric formation might be another structure. The identity of the 8-kD homodimer in 2B4.11 also must be determined. Clearly, further biochemical analysis is required.

Crosslinking of surface TCRs on ζ, η, and ζ-η transfectants with either CD3ϵ mAb or a specific cytochrome c peptide antigen and I-E^k MHC restricting element expressed on LK35.2 cells or B10.BR splenocytes resulted in signaling in each cell type, as judged by cellular growth inhibition. Thus,

in all cases, when LK35.2 cells were pulsed with 10–100-μM concentrations of the cytochrome c peptide, [3H]TdR incorporation of those transfectants diminished substantially. Similarly, exposure of transfectants to anti-CD3ϵ mAb-coated plates resulted in reduced [3H]TdR incorporation. In contrast, MA5.8 was unaffected by both types of crosslinking stimuli. Cell-cycle analysis documented that this inhibition of [3H]TdR incorporation was secondary to cycle arrest at the G1/S boundary (Fig. 6). This block was, however, reversible since removal of transfectants from anti-CD3ϵ mAb–coated plates resulted in restoration of cell growth as shown in Fig. 7. Thus, in no case did ζ, η or ζ-η transfectants die after TCR triggering.

Mercep et al. have recently suggested the interesting possibility that activation-driven programmed cell death is linked to CD3ζ-η expression (13). This hypothesis was based on the observation that ζ transfectants of MA5.8, lacking CD3ζ-η heterodimers, underwent little, if any, cell lysis and death upon antigen/MHC or anti-CD3ϵ mAb crosslinking. In contrast, the CD3ζ-η expressing parental 2B4.11 hybridoma was readily killed. Unexpectedly, however, the present studies with η and ζ-η transfectants clearly document that expression of CD3η-η homodimers without CD3ζ-ζ homodimers or CD3ζ-η heterodimers with CD3ζ-ζ homodimers in MA5.8 cells does not lead to cell death. Consistent with results of Ashwell et al. (27), however, we did observe death of the CD3ζ-η expressing T-T hybridomas, 2B4.11 (Fig. 7) and 3DO54.8 (data not shown), upon anti-CD3ϵ mAb crosslinking. Collectively, the results imply that if the CD3η subunit is involved in providing a death signal after TCR crosslinking, it, itself, is not sufficient to mediate programmed cell death. Rather, since the MA5.8 clonal variant was isolated from 2B4.11 cells by chemical mutagenesis, this line may lack an important gene product required for the death process.

The structural differences between CD3ζ and CD3η are such that the CD3η cytoplasmic domain lacks one of six potential tyrosine phosphorylation sites and a putative nucleotide binding site (14); consequently, the signals emanating from TCRs comprised of $Ti\alpha$-βCD3$\gamma\delta\epsilon\zeta$-$\zeta$, $Ti\alpha$-βCD3$\gamma\delta\epsilon\eta$-η, and $Ti\alpha$-βCD3$\gamma\delta\epsilon\zeta$-$\eta$ components are likely to be different. It will be important to determine whether phosphoinositide turnover, calcium mobilization, protein phosphorylation, and induction of gene programs including that of IL-2 production are modulated in the same or different manner when such receptors are crosslinked. In this regard, the reduced phosphoinositide turnover triggered by TCR crosslinking of CD3ζ-η^{low} variants of 2B4.11 and MA5.8 ζ transfectants reported previously is consistent with this view (12, 13).

The potential for expression of surface receptors with identical ligand specificities and different functional capabilities is reminiscent of isotypy originally described for immunoglobulin molecules. Given that the ratio of CD3η to CD3ζ mRNAs is at least as great in thymocytes as in splenic T cells of neonatal and adult mice (Clayton, L. K. et al., manuscript submitted for publication), CD3η containing receptors are apparently important in thymic development. One can imagine that their expression as either TCR associated CD3η-η

homodimers or CD3ζ-η heterodimers could be involved in the selection process. For example, if CD3η containing TCR isotypes in conjunction with as yet unidentified other proteins transmit a signal for programmed cell death, then CD3η-η or CD3ζ-η isotypes might regulate the negative selection process. Restricted expression of such isotypes within the thymus would insure that TCR crosslinking in the peripheral lymphoid compartment does not result in cell death. Perhaps additional heterogeneity in composition of subunits yet to be defined also contributes to the signaling mechanism in thymic selection.

Aside from putative differential signaling by the TCR structures, levels of surface TCR expression could be affected by TCR isotypes and thereby influence the thymic selection process. For instance, if a thymocyte expresses a relatively small number of TCRs, it might be eliminated by negative selection only if its TCR has an extremely high affinity for self MHC molecules. While it has generally been construed that the basis for the low level of surface TCR expression in the CD4$^+$CD8$^+$ double-positive thymocytes is a consequence of low level of TCR subunit gene(s) expression, this issue needs to be reexamined in light of the reduced TCR surface expression shown herein to be associated with TCRs containing CD3η components, particularly receptors expressing the CD3η-η isoform. Clearly, analysis of TCR isoforms within the thymus and functional studies of thymocytes and transfectants expressing these isoforms is needed at present. Perhaps negative and positive selection of thymocytes will be explicable in structural terms dependent on signal transduction isotypes and their regulated surface expression.

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