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

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Summary
To characterize the function of the CD3ζ 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-Ek-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 Tia-βCD3γδεζ 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 CD3ζ-η homodimer. Crosslinking of Tia-βCD3γδεζ-ζ, Tia-βCD3γδεη-η, or Tia-βCD3γδεζ-ζ/ζ-η TCR isotypes with anti-CD3e monoclonal antibody or a cytochrome c peptide epitope on I-Ek 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.
sequencing (Clayton, L.K., L. D'Adamo, M. Sieh, R. E. Hussey, S. Kuyasu, and E. L. Reinherz, manuscript submitted for publication). This differential splicing results in a cytoplasmic domain of CD3\(\eta\) which is 42 amino acids longer than that of CD3\(\xi\). In addition, CD3\(\eta\) lacks one of six potential tyrosine phosphorylation sites and, perhaps more importantly, a putative nucleotide binding site previously identified in CD3\(\xi\) (15).

To directly analyze the consequences of these structural differences in CD3\(\eta\) and CD3\(\xi\), we have used transfection techniques to express CD3\(\eta\) and CD3\(\xi\) cDNAs in eukaryotic expression vectors separately or together in murine T cells lacking CD3\(\eta\) and CD3\(\xi\) proteins. The results to be reported below demonstrate for the first time that the CD3\(\xi\) subunit in the form of a CD3\(\xi\)/CD3\(\xi\) homodimer is able to associate with and transport Tia-1, CD3\(\gamma\)6Er, Tia-RCD3\(\gamma\)6Er, Tia-RCD3\(\gamma\)6Er, and Tia-RCD3\(\gamma\)6Er-q, and Tia-RCD3\(\gamma\)6Er-r1 heterodimer. Substantial differences in TCR copy number resulting from CD3\(\eta\) vs. CD3\(\xi\) cDNA transfection using the same expression vectors suggest that CD3\(\eta\) and CD3\(\xi\) isoforms may differentially regulate surface TCR expression. In addition, functional studies with transfectants expressing Tia-1, CD3\(\gamma\)6Er, Tia-RCD3\(\gamma\)6Er, and Tia-RCD3\(\gamma\)6Er-q, and Tia-RCD3\(\gamma\)6Er-r1 heterodimer.

trents are not restricted to the CD3\(\xi\)/CD3\(\xi\) homodimer or the CD3\(\xi\)/CD3\(\xi\) heterodimer. Substantial differences in TCR copy number resulting from CD3\(\eta\) vs. CD3\(\xi\) cDNA transfection using the same expression vectors suggest that CD3\(\eta\) and CD3\(\xi\) isoforms may differentially regulate surface TCR expression. In addition, functional studies with transfectants expressing Tia-1, CD3\(\gamma\)6Er, Tia-RCD3\(\gamma\)6Er, and Tia-RCD3\(\gamma\)6Er-q, and Tia-RCD3\(\gamma\)6Er-r1 heterodimer. Substantial differences in TCR copy number resulting from CD3\(\eta\) vs. CD3\(\xi\) cDNA transfection using the same expression vectors suggest that CD3\(\eta\) and CD3\(\xi\) isoforms may differentially regulate surface TCR expression.

Construction of Expression Plasmids. The inserts of pBS17 (murine CD3\(\eta\)) and pBS23 (murine CD3\(\xi\)) (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 CD3\(\xi\)- or CD3\(\eta\)-specific probes prepared by digesting pBS23 with EcoRI and EcoRI and pBS17 with AvaI 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/\(\xi\) and pPink-2/\(\eta\) by double banding in CoCl 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).

Results and Discussion. The inserts for different 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 (Germantown, 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 antiserum against rabbit IgG and prestained molecular weight markers were from Biorad Laboratories (Richmond, CA). RNase A was from Worthington Biochemical Corp. (Freehold, NJ). Succinimidyl-4-6 maleimidophenyl butyrate (S-SMPB) was from Pierce Chemical Co. (Rockford, IL). Immulon plates (96-well, round-bottomed) were from Dynatech Laboratories (Chantilly, VA). Polyvinylidifluoride (PVDF) 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 \(\mu\)g/ml streptomycin, and 4 mM L-glutamine.

A hamster-mouse somatic cell hybridoma 145.2C11 producing a mAb (2C11) against murine CD3e (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 \(\alpha\) 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: KKANDLIAYLQATK.

Cells and Growth Conditions. The cytochrome c-specific, 1-B'-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, 1-A'-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 \(\mu\)g/ml G418. For determination of cell growth after TCR crosslinking, T cell hybridomas and transfectants were plated at 96-well, round-bottomed Immulon plates with or without 2C11 mAb (4 \(\mu\)g/well) and cultured in RPMI 1640 containing 10% (vol/vol) FCS at 2 x 10^5 cells/200 \(\mu\)l/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.

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Concentration and Flow Cytométric 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 \(\mu\)F. 15 \(\mu\)g pPink-2/\(\xi\) and pPink-2/\(\eta\) or both were transfected. After transfection, cells were plated in 6-well plates in RPMI 1640 containing 10% FCS at a concentration of 2 x 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., MA\(\eta\)61.9 is a clone of MA\(\eta\)61. Cell surface TCR expression of transfectants was quantified by indirect immunofluorescence using 2C11 (anti-CD3\(\xi\)) 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 \(\mu\)g 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 CD3\textgamma-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 NaPO\textsubscript{4}, pH 6.5, at 42°C for 16–20 h with 1 × 10\textsuperscript{6} 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 ensure all signals had been removed. The filter was next hybridized to the CD3\textgamma-specific probe using conditions as described for the CD3\textgamma specific probe, washed, and autoradiographed.

**121 Surface Labeling, Immunoprecipitation, and Two-Dimensional (2-D) Reducing/Nonreducing SDS-PAGE Analysis.** 5 × 10\textsuperscript{5} cells were labeled with 1 mCi of \textsuperscript{35}S 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 Moclodocetamide, 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 subjected 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 CD3\textgamma-related peptide, CLWSPWPPS-SSQSL, comprising the COOH-terminal 13 amino acids of CD3\textgamma with an NH\textsubscript{2}-terminal cysteine residue added for coupling purposes. The peptide was synthesized with a peptide synthesizer according to Legendre and Matsudaira (24) with a buffer consisting of 25 mMTris, 192 mMglycine, 20% methanol, and purified on a Waters Binary Gradient HPLC using an RCM \textgamma 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 × 10\textsuperscript{6} 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 Matsudaïra (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-\textgamma antisemur 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 alkaline 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-CD3\textgamma mAb or Antigen-pulsed Presenting Cells.** For the cell stimulation by crosslinking with antibody-coated plates, we cultured 2 × 10\textsuperscript{6} 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 \textsuperscript{3}H]ThR for 4 h and incorporation was measured by scintillation counting. For stimulation of the cells with peptide antigens, 5 × 10\textsuperscript{4} 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 × 10\textsuperscript{4} transfectants in 100 µl of media were added to each well. After 24 h of cultivation, DNA synthesis was determined by \textsuperscript{3}H]ThR incorporation as above.

**Cell Cycle Analysis.** Cells (2 × 10\textsuperscript{5}) 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 h on ice, flow cytometry was performed using a FACSscan (Becton Dickinson, Mountain View, CA) and the results were analyzed by Dean’s method (25).

**Results**

**Production of CD3\textgamma, \texteta, and \textphi + \texteta Transfectants.** To directly examine the role of CD3\textgamma in TCR assembly, surface expression, and signal transduction, CD3\textgamma and CD3\texteta 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, CD3\textgamma and CD3\texteta 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 by 569 to the vector insertion site (numbering according to CD3\texteta in reference 14). pPink-2/\texteta and pPink-2/\textphi 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-CD3\textgamma mAb 145.2C11 (2C11) (Fig. 2) or the anti-clonotypic anti-\textphi 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 CD3\(\eta\) and CD3\(\zeta\) 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 CD3\(\eta\) and CD3\(\zeta\). The dotted or hatched bars represent sequence differences at the 5' and 3' ends of the inserts. The 5' end of the CD3\(\eta\) insert begins in the common sequences of CD3\(\eta\) and CD3\(\zeta\). There are 49 bp more on the 5' end of the CD3\(\eta\) insert, including 34 bp of sequence unique to CD3\(\eta\) and divergent from the 5' end of CD3\(\zeta\) (14). The vector-encoded polyadenylation signal for the insert is indicated by AAAA. The line outside of the inserts indicates the coding region for CD3\(\eta\) or CD3\(\zeta\). 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 CD3\(\zeta\) 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 CD3\(\zeta\) transfectants was found in each of 15 transfectants examined. In contrast, 43 of 45 CD3\(\eta\) transfectants examined showed little anti-CD3\(\eta\) mAb (see Fig. 2, MA\(\eta\)5.3) or anti-Ti MAb (data not shown) reactivity above that observed for MA5.8 alone. 2 of 45 CD3\(\eta\) transfectants, as represented by MA\(\eta\)61.9 and MA\(\eta\)65.3, 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 \(\zeta\) transfectants. The \(\zeta\)-\(\eta\) double transfectants and clones showed, perhaps not surprisingly, a more heterogeneous level of TCR expression.

Northern Analysis of \(\zeta\) and \(\eta\) 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 CD3\(\eta\). Subsequently, the same blot was stripped and rehybridized with a CD3\(\zeta\) specific probe. As shown in Fig. 3, the 1.7-\(\text{kb}\) CD3\(\eta\) mRNA is present in 2B4.11 but absent in MA5.8. In addition, a 3.4-\(\text{kb}\) band hybridizing with the CD3\(\eta\) probe is detected in 2B4.11 exclusively. The significance of this species is presently unknown. The lanes containing the RNA from CD3\(\eta\) transfectants MA\(\eta\)65 and MA\(\eta\)61 lack the 1.7-\(\text{kb}\) endogenous CD3\(\eta\) mRNA but rather demonstrate intense 2.9- and 2.2-\(\text{kb}\) hybridizing bands. The latter represent CD3\(\eta\) 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 CD3\(\zeta\), CD3\(\eta\) or both were stained with anti-CD3\(\eta\) mAb 2C11 (thick line) or control mAb 11C5 (thin line) followed by FITC-conjugated goat anti-hamster antibody.

Figure 3. Expression of CD3\(\zeta\) and \(\eta\) mRNAs in 2B4.11, MA5.8, and representative \(\zeta\), \(\eta\), or \(\zeta\)-\(\eta\) MA5.8 transfectants. 10 \(\mu\)g 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 CD3\(\eta\)-specific probe, washed, and exposed at -70°C for 42 h with an intensifying screen. The filter was then stripped, reprobed with a CD3\(\zeta\)-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 MAg5.8 and MAg61 via the pPink-2 vector; both the insert and the vector contain polyadenylation signals. MAg5.3 shown in Fig. 2 also expresses CD3γ mRNA derived from transfected cDNA (data not shown). In contrast and as expected, RNA from the representative CD3ε transfectant, MA43, lacks reactivity with the CD3γ-specific probe. In γ-γ double transfectants, MAgγ52 and MAgγ61, a weak but definite set of hybridizing bands identical in size to that found in the γ transfectants are detected. Reanalysis of the same blot with a CD3γ probe identified the 2-kb CD3γ mRNA in 2B4.11 which is absent in MA5.8. The CD3γ probe hybridized to two mRNA species of 3.2- and 2.5-kb in γ and γ-γ transfectants but not in γ transfectants. Again, endogenous and exogenous CD3γ mRNA bands could be readily detected in 2B4.11 and transfectants, respectively.

Given that the level of CD3γ mRNA in γ transfectants was as great or greater than the level of CD3ε mRNA in γ transfectants, the lower copy number of surface TCR complexes on the γ transfectants relative to γ 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 CD3γ and CD3ε 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 posttranscriptionally regulated. It appears that CD3ε is less able than CD3γ to facilitate surface TCR expression when transfected into MA5.8. This is also observed in 2B4.11 where the levels of mRNA for CD3ε and CD3γ are very similar (Fig. 3), while the level of CD3γ protein associated with surface TCR is an order of magnitude less than that of CD3ε (see below and reference 11). We cannot exclude the possibility that the steady-state level of CD3γ protein is less than CD3ε 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 125I 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 indicated schematically in Fig. 4 (bottom right panel), the off-diagonal Tia-β heterodimer at 40–60 kD and CD3γ, CD3δ, and CD3ε subunits at 20, 25, and 26 kD, respectively, are readily apparent. The CD3ε-ε homodimer (16-kD subunits) and the CD3ε-γ heterodimer (16- and 22-kD subunits) are evident and the frequency of incorporation of CD3ε into the heterodimer is ~10-fold greater than its incorporation into the CD3ε-γ 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 CD3ε-γ heterodimer, appearing just above and to the left of the latter. The set of off-diagonal CD3ε spots to the right of the CD3ε-γ homodimer likely represent CD3ε complexed to a smaller set of ~10-kD proteins. In addition, an 8-kD dimer running 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 CD3ε degradation products.

In contrast to 2B4.11, the anti-CD3ε immunoprecipitate of MA5.8 contains no CD3ε or CD3γ spots; only Tia-β and CD3γ, CD3δ, and CD3ε monomers and CD3ε-ε homodimer spots are apparent. However, γ transfectants of MA5.8 such as MA43 have a readily detectable CD3ε-ε homodimer spot as well as CD3ε complexed to 10-kD structures. No CD3γ is apparent in these cells, consistent with earlier studies showing that CD3γ is not a post-translationally modified form of CD3ε (26). On the other hand, the representative CD3γ transfectant MAγ61 contains a homodimer appearing as an off-diagonal spot at 22 kD in the reducing dimension, directly below the CD3ε-ε homodimer, presumably representing CD3γ. No CD3ε is detected, again demonstrating that CD3ε and CD3γ are distinct proteins. Finally, the γ-γ...
double transfectant MAβη52 contains both the CD3ζ-η heterodimer and the CD3ζ-ζ homodimer in the anti-CD3e 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-CD3e 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-CD3e 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-Ad-restricted T cell hybridoma 3D054.8. This spot is not present when the lysates were reacted with preimmune sera from the same rabbit. Furthermore, while the CD3ζ 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 η transfectant MAζη52 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 ΤηαβCD3γδεζη, ζ transfectants express a TCR composed of ΤηαβCD3γδεζζ and ζ-η transfectants express both ΤηαβCD3γδεζζ and ΤηαβCD3γδεζη 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-CD3e mAb. In additional experiments, the TCR of transfectants was crosslinked with I-Ek 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 T1 hybridomas including 2B4.11 results in cell cycle arrest and hence, a reduction in proliferation as measured by [3H]Tdr incorporation. As shown in Table 1, TCR crosslinking with anti-CD3e mAb resulted in a significant decrease in [3H]Tdr incorporation of 2B4.11 and all transfected lines. For example, in comparison to media control, 2C11 reduced [3H]Tdr of MAζη43.2 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 [³H]TdR incorporation of 2B4.11 and all transfectants but was without significant effect on the antigen unresponsive MA5.8 line or the 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, MAγ43, MAζη52, and MAη65 populations consisted of 51–57% of cells in G1, 33–38% in S and 5–15%, in G2/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 G2/M was reduced (see Fig. 6). These data indicate that TCRs comprising CD3ζ, CD3ζη, or CD3ζη transduce signals that arrest cell growth at the G1/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 ζη 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 ⁵¹Cr release. Essentially the same results were obtained when 2B4.11, MA5.8, and transfectants were stimulated with I-Ek-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: | 0 μM   | 1 μM   | 10 μM  | 100 μM  |
|-----------|------------------------|--------|--------|--------|--------|
| 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) |
| MAγ43.2   |                        | 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) |
| MAζη52.9  |                        | 121,896 | 119,543 (1.9) | 106,256 (12.8) | 59,530 (51.2) |
| MAζη52.11 |                        | 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          | | | | |
| 2B4.11    | Anti-CD3ε         | 10,476 | 2,141 (79.6) | | |
| MA5.8     |                        | 57,331 | 56,853 (0.8) | | |
| MAγ43.2   |                        | 46,884 | 4,445 (90.5) | | |
| MAζ15.4   |                        | 51,432 | 8,369 (83.7) | | |
| MAζη52.9  |                        | 23,217 | 14,154 (39) | | |
| MAζη52.11 |                        | 37,391 | 26,591 (28.9) | | |
| MAη61.9   |                        | 55,561 | 29,604 (46.7) | | |
| MAη65.3   |                        | 29,873 | 14,305 (51.5) | | |
| 3DO54.8   |                        | 32,021 | 1,441 (95.5) | | |

* Number in parentheses represents percent inhibition. Standard deviation was generally <10%.
CD3η nor CD3ζ, alone or in combination, is responsible for irreversible cell cycle arrest in this system. Likewise, cross-linking of TCRs expressing CD3ζ 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 CD3ζ 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 CD3ζ 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 CD3ζ-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 125I 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...
protein level as quantitated by immunoblotting correlated with surface TCR expression (28). Biosynthesis studies have shown that although the pentameric Tia-β CD3γδε structure is assembled in the endoplasmic reticulum and transits into the Golgi apparatus in the absence of CD3ζ, the pentamers are transported to the lysosomes where they are degraded (29, 30). The increased surface TCR expression in T-T hybridomas resulting from η transfection is ~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 lysosomal degradation as efficiently as CD3ζ. These possibilities are not mutually exclusive. A short half-life of CD3ζ protein and/or the unique structural feature of CD3ζ 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 CD3ζ-η homodimer with the TCR pentamer is weaker than that of the CD3ζ-ζ homodimer, then this may explain our inability to detect the CD3ζ-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 125I surface-labeled ζ transfectants identified all the known components of the TCR heptamer, Tia-β CD3γδεζ-ζ. In addition, a series of CD3ζ components linked to a heterogeneous set of ~10-kD proteins were detected. These heterodimeric forms are also present in the parental 2B4.11 TF 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 CD3ζ subunit, the possibility that CD3ζ could disulfide bond to other molecules is likely. The recently identified structural homology between the FceRIγ subunit and the CD3ζ subunit (31, 32) raises the possibility that FceRIγ 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-Ε 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 CD3ζ-η homodimers without CD3ζ-ζ homodimers or CD3ζ-ζ 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 TT hybridomas, 2B4.11 (Fig. 7) and 3D054.8 (data not shown), upon anti-CD3ε mAb crosslinking. Collectively, the results imply that if the CD3ζ 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 Tia-β CD3γδεζ-ζ, Tia-β CD3yδεζ-η, and Tia-β CD3γδεζ-ζ-η 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 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 CD3ζ-η
homodimers or CD3\(\gamma\)\(\delta\) heterodimers could be involved in the selection process. For example, if CD3\(\gamma\) containing TCR isotypes in conjunction with as yet unidentified other proteins transmit a signal for programmed cell death, then CD3\(\gamma\)-\(\delta\) isotypes might regulate the negative selection process. Restricted expression of such isotypes within the thymus would ensure 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 CD3\(\gamma\) components, particularly receptors expressing the CD3\(\gamma\)-\(\delta\) isoform. Clearly, analysis of TCR isotypes within the thymus and functional studies of thymocytes and transfectants expressing these isotypes 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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Note added in proof: In very recent studies, we find that TCR crosslinking by anti-CD3\(\epsilon\) mAb results in phosphoinositide turnover of IL-2 production in each of the above transfectants (Bauer, A. et al., manuscript in preparation).

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