CHARACTERIZATION OF A THIRD FORM OF THE HUMAN T CELL RECEPTOR γ/δ

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A subpopulation of peripheral blood T lymphocytes express a novel non-α, non-β T cell receptor (TCR) heterodimer that is associated with the CD3 complex. This heterodimer is composed of TCR-γ and TCR-δ polypeptides which are distinct from each other on the basis of immunochemical criteria (1-3) and by peptide mapping (4), and are encoded by different genes.

The TCR-γ (5, 6) and TCR-δ (7-9) genes are composed of variable (V), joining (J), and constant (C) region segments that undergo somatic rearrangement in T cells (7, 10, 11), as is the case for TCR-α, TCR-β, and Ig genes. In the human germline, six or seven functional TCR-γ variable gene segments have been identified (12-14) that are located 5' of two constant region gene segments, designated Cγ1 and Cγ2 (15). The TCR-γ constant gene segments consist of three exons, namely the CI exon (encoding an extracellular, Ig-like constant domain), a short CII exon (encoding a connecting peptide), and the CIII exon (encoding the membrane spanning region and a short intracellular tail).

In man, two protein forms of the TCR-γ/δ have been well described. The first form is composed of 40 kD TCR-γ polypeptides, associated by a disulfide bridge with a TCR-δ protein (16-18). In contrast, the other form consists of a 55 kD TCR-γ protein that is noncovalently associated with a TCR-δ polypeptide (1, 17, 19).

cDNA clones representing the transcripts for the disulfide-linked TCR-γ polypeptide on T cell clone PBL-C1 demonstrated that its constant region is encoded by the Cγ1 gene segment (4). The single CII exon in Cγ1 encodes an extracellular, unpaired cysteine that is implicated in disulfide linkage with the TCR-δ subunit. cDNA clones encoding the 55-kD non-disulfide-linked TCR-γ have been analyzed from the IDP2 and PEER cell lines (4, 20). They use the Cγ2 gene segment and reveal
the presence of three CII exons (CII exon copy a, b, and c). Interestingly, although the three CII exons of the Cy2 gene show a high degree of sequence identity with the Cy1 counterpart, each of the three copies of the Cy2 CII exons is unique. The cysteine present in the single Cy1 CII exon is replaced with another amino acid in all three Cy2 CII exon copies. Moreover, CII exon copies b and c each bear potential N-linked glycan attachment sites, accounting for the larger size of the non-disulfide-linked Cy2 (55 kD) versus the disulfide-linked Cy1 (40 kD) polypeptide subunits.

Besides these TCR-y/6 types, an additional form characterized by a 40-kD, non-disulfide-linked TCR-y chain exists (9, 17). It contains structural features that could not be accounted for by the known Cy1 (40-kD, disulfide-linked polypeptide) or the known Cy2 (55-kD, non-disulfide-linked polypeptide) gene segments. This third form is not disulfide linked to its partner (similar to Cy2) but reveals a short (40-kD) polypeptide (similar to Cy1). In the present study, we characterize this third TCR-y/6 protein structure and compare it with the two other forms. We present the primary structure of this TCR-y form determined through cDNA sequence analysis and propose an explanation for the observed cell surface protein structure.

Materials and Methods

**Antibodies.** mAbs used were anti-Leu-4 (anti-CD3) (21), βF1 (anti-TCR-β) (22), anti-TCR-δ1 (anti-TCR-δ) (3), P3 (control) (secreted by P3X63.Ag8) (23), I87.1 (rat anti-mouse κ light chain) (24), and WT31 (stains TCR-a/β lymphocytes brightly) (25). Anti-Cyβ peptide serum (anti-TCR-γ) generated against a 22-amino acid synthetic peptide (Gln-Leu-Asp-Ala-Asp-Val-Ser-Pro-Lys-Pro-Thr-Ile-Phe-Leu-Pro-Ser-Ile-Ala-Glu-Thr-Lys-Cys) was generously provided by T Cell Sciences Inc., Cambridge, MA.

**Cell Lines.** PEER (19) and MOLT-13 (isolated by J. Minowada, Fujisaki Cell Center, Okayama, Japan) (9) are T leukemic cell lines. Umbilical cord blood-derived clone WM-14 (26), peripheral blood-derived cell line IDP2 (1) and thymus-derived Clone II (27) were cultured as described earlier. Peripheral blood-derived cell line 2 (PBL-L2) was isolated by sorting peripheral blood-isolated lymphocytes that did not stain with mAb WT31. The isolated cells were then expanded in vitro in RPMI 1640 medium supplemented with 10% (vol/vol) conditioned medium containing IL-2 and 10% (vol/vol) human serum, and stimulated every 3 wk with irradiated autologous feeder cells.

**Iodination and Immunoprecipitation.** 2 x 10^7 cells were isolated by Ficoll-diatrizoate (Organon Teknika Corp., Durham, NC) centrifugation and iodinated on ice as described before (1). Cells were solubilized overnight in detergent supplemented TBS1 (50 mM Tris-Base, pH 7.6, 140 mM NaCl) containing 1 mM PMSF and 8 mM iodoacetamide (IAA). As indicated, different detergents used in this study were 0.3% (wt/vol) 3-[3-cholamidopropyl] dimethylammonio] 1-propane-sulfonate (CHAPS), 1% (wt/vol) digitonin, and Triton X-100 (TX-100). After 20 min of centrifugation at 10,000 g to remove insoluble material, detergent lysates were precleared by a 30-min incubation with 4 μl of normal rabbit serum (NRS) and 400 μl of 187.1 hybridoma culture medium, followed by addition of 200 μl of a 10% (wt/vol) cell suspension of fixed Staphylococcus aureus Cowan I (PANSORBIN; Calbiochem-Behring Corp., San Diego, CA). After a 1-h incubation Pansorbin was removed by centrifugation. Specific precipitations were carried out by adding 0.25 μl βF1 ascites, 1 μl anti-Leu-4 (1 mg/ml) or 0.25 μl P3 ascites, together with 150 μl of I87.1 culture supernatant to each sample, followed by a 1-h incubation. The efficiency of βF1-immunoprecipitation was improved by the addition of TX-100 detergent to a final concentration of 1% (vol/vol). 100 μl of 10% (vol/vol) protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was added and the mixture was rocked for 1 h at 4°C. Immunoprecipitates were washed five times with 0.1% (vol/vol)

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1 Abbreviation used in this paper: TBS, Tris-buffered saline.
TX-100 containing TBS and analyzed by SDS-PAGE (28). Immunoprecipitations with the anti-Cyb peptide serum were performed as described before (3).

Biosynthetic Labeling. 4 x 10^7 exponentially growing cells were resuspended in 4 ml of methionine- and cysteine-free RPMI 1640 (Select-Amine kit; Gibco Laboratories, Grand Island, NY) supplemented with 10% dialyzed FCS and 20 mM Heps, pH 7.4. After a 30-min starvation period at 37°C, 1 mCi of [35S]methionine and 1 mCi of [35S]cysteine were added, allowing a 15-min labeling period. Cells were harvested and solubilized in 2% (vol/vol) TX-100, TBS. Preclearing and immunoprecipitations were performed as described above. The immunoprecipitates were washed four times in 0.5% (vol/vol) TX-100, 0.5% (wt/vol) deoxycholic acid, 0.05% (wt/vol) SDS, TBS followed by three washes in 0.5% (vol/vol) TX-100, 0.5 M NaCl, 5 mM EDTA, 50 mM Tris, pH 7.6. The samples separated in SDS-PAGE and visualized by standard fluorography procedures (29).

Gel Purification of TCR-8 Proteins. Surface-iodinated cells were solubilized in 0.3% (wt/vol) CHAPS/TBS and immunoprecipitated using 50 µl of anti-Leu-4-coupled Sepharose beads. The immunoprecipitated species were resolved by SDS-PAGE under nonreducing conditions and the wet gel was exposed for 24 h at 4°C on XAR-5 film (Eastman Kodak Co., Rochester, NY) to visualize radiolabeled TCR-8 proteins. The gel regions corresponding to TCR-6 were excised, incubated in 5% (vol/vol) 2-ME containing sample buffer, and resolved a second time by SDS-PAGE. Because of the characteristic SDS-PAGE mobility shift upon reduction, TCR-8 protein could be separated from contaminants. TCR proteins were eluted from gel slices by overnight incubation in 0.05% (wt/vol) SDS, 50 mM ammonium bicarbonate buffer at 37°C, and were lyophilized.

Endoglycosidase Digestion. For endoglycosidase H digestions, immunoprecipitated material or gel purified protein was boiled for 3 min in a 40 µl 1% (wt/vol) SDS solution containing 0.14 M 2-ME. After cooling, the mixture was diluted with 360 µl of 0.15 M acetate buffer, pH 5.5, containing 1 mM PMSF. 5 µl Endo H (1 U/ml; Endo-β-N-acetylglucosaminidase H; Genzyme, Boston, MA) was incubated for 16 h at 37°C, while the other half was mock incubated.

For N-glycanase digestion, gel-purified material was boiled for 3 min in 35 µl of 0.5% (wt/vol) SDS, 0.10 M 2-ME. Then, 100 µl of 0.2 M sodium phosphate (pH 8.6), 1.25% (vol/vol) TX-100 was added. Half of the mixture was incubated with 1 µl N-glycanase (250 U/ml, peptide- N-[N-acetyl-β-glucosaminyl]asparagine amidase; Genzyme) and incubated for 16 h at 37°C, while the other half was mock treated.

After digestion, 10 µg BSA was added as carrier and samples were recovered by TCA precipitation. Protein pellets were then taken up in sample buffer containing 5% (vol/vol) 2-ME.

Production of mAb Anti-CyM1. Part of the Cy C1 and C1I exons of HPB-MLT pTy-1 was isolated using the Bam H1 and Pst I sites at nucleotide positions 571 and 848 (30) and was cloned into expression vector pRIT2T (Pharmacia Fine Chemicals). The resulting protein A-fusion protein was expressed in Escherichia coli N4830. Bacteria were lysed with lysozyme and the fusion protein was isolated by purification over an IgG Sepharose column. Mice were injected intraperitoneally with 100 µg of fusion protein in Freund's adjuvant at days 0, 7, and 28. 28 d later 100 µg of fusion protein in PBS was injected intravenously. After 3 d splenocytes were isolated and fused with the myeloma P3X63Ag8.653 as described (22). Hybridomas were screened by ELISA. 96-well flat-bottomed plates (Linbro; Flow Laboratories Inc., McLean, VA) were incubated overnight with 0.4 µg of fusion protein or nonfused protein in PBS. Nonspecific binding sites were blocked at 23°C with 0.25 mg/ml normal rabbit IgG (Sigma Chemical Co., St. Louis, MO) in PBS containing 50% (vol/vol) FCS. 50 µl of hybridoma supernatant was added for 1 h at 4°C, followed by a similar incubation in 50 µl of a 5 µg/ml solution of peroxidase-conjugated anti-mouse IgG (Cappel Laboratories, Cochranville, PA). All described incubations were interspersed with washing steps, using 10% (vol/vol) FCS, 0.1% (wt/vol) BSA, PBS. The ELISA was developed with 0.08% (wt/vol) O-phenylene diamine in 0.012% (wt/vol) hydrogen peroxide containing phosphate-citrate buffer, pH 5.0. Hybridoma number three, secreting anti-CyM1 mAb, was selected for further study.

Although anti-CyM1 does not recognize the native TCR-γ/δ/CD3 complex in cytological analysis nor the TCR-γ/δ heterodimer from TX-100 solubilized cells in immunoprecipitation (data not shown), it does recognize biosynthetically labeled TCR-γ precursor, as
well as mature TCR-γ proteins after separation of CD3/TCR-γ/δ proteins into individual chains. After separation CD3/TCR-γ/δ complexes into individual chains by boiling in 1% (wt/vol) SDS, anti-CγM1 was shown to specifically recognize the TCR-γ protein (Fig. 2 B, lane 3). Since this mAb was generated against a constant region-encoded peptide, and reacts with both Cγ1- and Cγ2-encoded TCR-γ chains, it is likely to possess framework reactivity against all TCR-γ polypeptides (unpublished observations).

**Isolation and Sequencing of a MOLT13 TCR-γ cDNA Clone.** Poly(A)⁺ RNA was prepared from MOLT-13 cells by urea/lithium chloride precipitation followed by oligo(dT)-cellulose affinity chromatography. A λgt10 cDNA library was prepared from poly(A)⁺ RNA by the method of Huynh et al. (31) using Mung Bean Nuclease (Promega Corp., Madison, WI) for the hairpin loop cleavage. The cDNA library was amplified on the E. coli strain C600 Hfl and screened by plaque filter hybridization with 32P-labeled pTy1 (30). Positive clones were analyzed for size and restriction enzyme map, and cDNA clone M13k was selected for sequencing. The cDNA of M13k was excised from λgt10 phage with the endonuclease Eco RI and further digested with appropriate restricting enzymes. The fragments were subcloned into M13 vectors and sequenced by the dideoxy chain termination method (32) using the modified T7 polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH).

Clone M13k corresponds to a full length, in-frame, TCR-γ transcript, including 36 nucleotides of 5' untranslated region and 72 nucleotides of 3' noncoding region (Fig. 3). The nucleotide sequence of the V region is identical to the genomic Vγ1.3 sequence (12, 14), except for a C to T (Ile to Val) change of nucleotide 53 in the putative signal sequence. The J region is identical to the Jγ2.3 sequence (33, 34). Interestingly, eight nucleotides occur at the V-J junction that do not appear to be encoded by the genomic V or J sequences and presumably comprise an N region. The C region sequences match the corresponding genomic sequence (15), with the exception of nucleotide 559 (G to C; Val to Ile) and nucleotide 908 (T to C; Met to Thr).

**Results**

**Third Form of the TCR-γ/δ.** In an attempt to delineate the non-disulfide-linked 40-kD TCR-γ form, we produced and characterized a number of cell lines derived from normal human donors. Peripheral blood lymphocytes were stained with mAb WT31, which brightly stains resting TCR-α/β lymphocytes. Cells that did not stain were isolated by cell sorting and then expanded in vitro in IL-2-containing medium.

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**Figure 1.** Three forms of the TCR-γ/δ. The antibodies used for immunoprecipitation are anti-Leu-4 (anti-CD3), 8F1 (anti-TCR-γ), anti-TCR-δ1 (anti-TCR-δ), anti-Cy2 serum (anti-TCR-γ), and P3 (unlabeled lanes, control) as shown at the top of each lane, while the 125I-labeled cell line used is shown at the bottom of each 10% SDS-PAGE autoradiograph. R indicates that the immunoprecipitate was resolved under reducing conditions and N indicates that nonreducing conditions were used. M, (10⁻³) markers are shown to the left. An open arrow indicates the position of TCR-δ under reducing conditions, whereas the closed arrow denotes the position of TCR-δ under nonreducing conditions. (A) Non-disulfide-linked TCR-γ (40 kD) on PBL-L2. In lanes 1-6 the radiolabeled cells were solubilized in 0.3% CHAPS detergent which preserves the TCR-CD3 association, whereas in lanes 7 and 8, immunoprecipitates were performed after chain separation (see Materials and Methods). Note that the arrows point to TCR-δ positions under reducing and nonreducing conditions. (B) Non-disulfide-linked TCR-γ (55 kD) on IDP2 cells. In lanes 1-4 radiolabeled cells were solubilized in 0.3% CHAPS detergent, whereas in lanes 5 and 6 immunoprecipitates were carried out after chain separation. (C) Disulfide-linked TCR-γ (40 kD) on WM-14 cells. All lanes correspond to immunoprecipitations from 1% digitonin solubilized radiolabeled cells. (D) Non-disulfide-linked TCR-γ (40 kD) on thymic Clone II cells. Radiolabeled cells were solubilized in 1% digitonin (lanes 1-4) or in 0.1% TX-100 (lanes 5 and 6), whereas in lanes 7 and 8 immunoprecipitations were carried out after chain separation. (E) Non-disulfide-linked TCR-γ (40 kD) on MOLT13 T leukemia cells. In lanes 1-4 immunoprecipitations were carried out after solubilization of cells in 0.3% CHAPS detergent, whereas in lanes 5 and 6 immunoprecipitations were carried out after chain separation.
Peripheral blood lymphocyte line 2 (PBL-L2) obtained in this way proved to be homogeneously CD3⁺ CD4⁻ CD8⁻ (data not shown), a cell surface phenotype typical of TCR-γ/δ lymphocytes.

To visualize TCR-γ/δ complexes on PBL-L2 cells, immunoprecipitations with an anti-CD3 mAb were carried out from cell surface ¹²⁵I-labeled cells solubilized in CHAPS or digitonin. In these detergents, the physical association between the CD3 complex and TCR-γ/δ subunits is preserved. SDS-PAGE of anti-CD3 immunoprecipitates from PBL-L2 cells resolved 40-kD and 44-kD proteins (referred to as 40 kD) that were identified as TCR-γ subunits by anti-Cyb serum, an antiserum directed against a TCR-γ constant region peptide (Fig. 1 A; see Materials and Methods section).

These TCR-γ proteins on PBL-L2 are noncovalently associated with a TCR-δ subunit, which is visible as a weakly iodinated protein in the anti-CD3 immunoprecipitation analyzed under nonreducing conditions (Fig. 1 A, lane 6, closed arrow). This weakly iodinated protein represents the TCR-δ subunit on PBL-L2 cells, since it is not recognized by anti-Cyb serum (Fig. 1 A, lane 8). In addition, it displays the same SDS-PAGE mobility shift comparing analysis under nonreducing and reducing conditions as was noted for the TCR-δ proteins on IDP2 and PEER cells (see below) (17). The TCR-δ protein could not be visualized after reduction (Fig. 1 A, lane 3), because it migrated with a mobility of 40 kD (see below) and then was obscured by the similar sized TCR-γ protein (open arrow).

This TCR-γ/δ form is not only present on normal peripheral blood T lymphocytes, but is also observed on thymus-derived Clone II cells (Fig. 1 D) (27), and on the T-leukemic cell line MOLT-13 (Fig. 1 E) (9). These three cell lines possess TCR-γ species that display differential glycosylation resulting in a TCR-γ protein doublet observed on PBL-L2 (40 kD and 44 kD; Fig. 1 A, lane 8) and Clone II cells (40 kD and 44 kD; Fig. 1 D, lane 8) or a diffusely labeled TCR-γ protein band observed on MOLT-13 cells (40-46 kD; Fig. 1 E, lane 6). Two-dimensional gel analysis (nonequilibrium pH gradient electrophoresis [NEPHGE] followed by SDS-PAGE) of the MOLT-13 TCR-γ protein band resolved two TCR-γ species (40 kD and 44 kD), of which the 44-kD TCR-γ species contained an additional high mannose (or hybrid) N-linked glycan compared with the 40-kD TCR-γ species (data not shown). We conclude that the TCR-γ subunits of this receptor complex isolated from three different cell sources (peripheral blood, thymus, and a leukemia) reveal cell surface species of 40 kD that are noncovalently associated with TCR-δ partner chains.

For comparison to the TCR-γ/δ form on PBL-L2, Clone II, and MOLT-13 cells, we examined the previously characterized forms on the IDP2 and WM-14 cell lines. The IDP2 cell line (1) contains a larger, 55-kD TCR-γ protein, that is recognized by anti-Cyb serum (Fig. 1 B). When the anti-CD3 immunoprecipitate is examined under nonreducing conditions, it is evident that the IDP2 TCR-γ protein is associated noncovalently with its TCR-δ partner chain (Fig. 1 B, lane 4, closed arrow). Upon reduction, the TCR-δ protein displays a decrease in SDS-PAGE mobility to a relative molecular mass of 40 kD (compare Fig. 1 B, lane 4, closed arrow, with Fig. 1 B, lane 2, open arrow).

In contrast to the noncovalently associated TCR-γ/δ forms, the umbilical cord blood-derived T cell clone, WM-14, bears a disulfide-linked TCR dimer of 70 kD (Fig. 1 C, lane 7, closed arrow), which was recognized by anti-Cγ serum (26). This
FIGURE 2. Peptide backbone sizes of TCR-γ from PEER and MOLT-13 cells. mAbs used for immunoprecipitation are anti-Leu-4 (anti-CD3), anti-CyM1 (anti-TCR-γ), anti-TCR-δ1 (anti-TCR-δ), and P3 (unlabeled lane, control) as shown at the top of each lane. The labeled cell lines used are shown at the bottom of each 10% SDS-PAGE autoradiograph or fluorograph. All samples were resolved under reducing conditions. Mr (10^5) markers are indicated to the left. (A) Peptide backbone sizes of TCR-γ from PEER and MOLT-13 cells. Cells were biosynthetically labeled with [35S]cysteine and [35S]methionine for 15 min. Samples were either treated with Endo H (+) or mock treated (−). Immunoprecipitation with anti-CyM1 shows the positions of immature TCR-γ of PEER cells (lane 3) and of MOLT-13 cells (lane 7), while the corresponding polypeptide backbone sizes are visualized after treatment with endo H (lanes 4 and 8). (B) Specificity of anti-CyM1 mAb. Cell surface-radiolabeled cells were solubilized in 0.3% CHAPS detergent and the TCR-γ/δ-CD3 complex was isolated with anti-CD3 mAb (lane 1). Anti-CyM1 specifically immunoprecipitates the TCR-γ subunit (lane 3) after separating the chains of the isolated TCR-γ/δ-CD3 complexes, compared with anti-TCRδ1 which specifically immunoprecipitates the TCR-δ chain (lane 4). (C) Glycosylation of TCR-δ from MOLT-13 cells. 125I-labeled cells were immunoprecipitated with anti-CD3 mAb and the TCR-δ polypeptides were gel purified (see Materials and Methods). Samples were treated with Endo H (lane 2) or with N-glycanase (lane 4), or mock treated (lanes 1 and 3). The decrease of 2.5 x 10^3 in Mr after endo H digestion (lane 2) indicates the presence of one high mannose or hybrid N-linked glycan on the TCR-δ chain. A further 2.5 x 10^3 decrease in Mr, followed digestion with N-glycanase (lane 4).

dimer is also recognized by anti-TCRδ1 (3), a mAb directed against the TCR-δ subunit (Fig. 1 C, lane 5), and therefore represents a TCR-γ/δ heterodimer. Analysis under reducing conditions reveals TCR-γ proteins of 40 kD and 36 kD (referred to as 40 kD) and a 43-kD TCR-δ protein (Fig. 1 C, lane 6, open arrow). These subunits were identified by recognition with anti-Cyb serum and by two-dimensional gel analysis (data not shown).

Thus, the CD3-associated complex on PBL-L2, Clone II, and MOLT-13 cells constitute a TCR-γ/δ heterodimer that is distinct from, but related to, the other known forms, since its TCR-γ subunit is 40 kD (similar in size to the disulfide-linked, Cyγ-
encoded TCR-γ protein on WM-14 cells), yet it is not disulfide linked to its partner chain (similar to the 55-kD, Cy2-encoded TCR-γ protein on IDP2 cells). To understand the molecular basis of this complex a more detailed structural analysis of its TCR-γ and TCR-δ subunits was carried out, using the MOLT-13 cell line as an example.

**Core Polypeptide Size of MOLT-13 TCR-γ Subunit.** To determine the size of the TCR-γ core polypeptide of MOLT-13 cells (40-kD TCR-γ glycoprotein), and compare it with that of PEER cells (55-kD TCR-γ glycoprotein), both cell lines were biosynthetically labeled for 15 min in the presence of [35S]methionine and [35S]cysteine, solubilized in Triton X-100 and then immunoprecipitated with anti-CyM1, a new mAb that specifically recognizes the TCR-γ chain (Fig. 2B, see Materials and Methods). Immunoprecipitated material was digested with endoglycosidase H (Endo H) to remove the immature N-linked glycans. The MOLT-13 TCR-γ polypeptide backbone has a relative molecular mass of 35 kD (Fig. 2A, lane 8), which is 5 kD smaller than the PEER TCR-γ core polypeptide (40 kD; Fig. 2A, lane 4) or the IDP2 TCR-γ core polypeptide (40 kD) (17). This 5-kD difference in polypeptide core size cannot solely account for the 15-kD difference in size between the MOLT-13 (40 kD) and PEER (55 kD) cell surface proteins. The remaining 10-kD difference in size must result from differences in posttranslational processing between two TCR-γ chains. While 15–20 kD of relative molecular mass can be accounted for by posttranslational processes on the PEER and IDP2 TCR-γ glycoproteins (55–60 kD surface size minus 40-kD core size), only 5–11 kD of size on the mature MOLT-13 TCR-γ glycoprotein is accounted for by posttranslational processes (40–46-kD surface size minus 35-kD core size). Experiments using tunicamycin to inhibit the addition of N-linked carbohydrates to the polypeptide chain confirmed that the posttranslational processing is largely, if not totally accounted for, by the addition of N-linked glycans (data not shown). Assuming that each glycan accounts for ~3 kD of relative molecular mass, we predict that two or three N-linked glycans are attached to the MOLT-13 TCR-γ protein, while five N-linked glycans are added to the TCR-γ polypeptides on PEER and IDP2 cells.

**Primary Sequence of MOLT-13 TCR-γ.** To understand the structure of the constant region gene segment encoding the MOLT-13 TCR-γ subunit, the sequence of a cDNA clone representing the MOLT-13 TCR-γ transcript was determined. A λgt10 library from MOLT-13-derived poly (A)+ RNA was constructed and probed with a human TCR-γ cDNA clone, pIγ1 (30). Based on size and limited restriction enzyme mapping one clone, M13k, was selected and its nucleotide sequence was determined (Fig. 3). Clone M13k represents a full length, in-frame TCR-γ transcript, using a Vγ1.3 gene segment joined to a Jγ2.3 gene segment (12, 33; nomenclature based on references 14, 34). The constant region sequence was found to be encoded by two CII exons, copies b and c, similar to a recently reported nonfunctional TCR-γ (36) and to the Cy2 genomic sequence containing two CII exons (15).

The deduced amino acid sequence of this cDNA clone predicts a polypeptide backbone size of 34.8 kD, which is in good agreement with biochemical data described above. Surprisingly, six potential N-linked carbohydrate attachment sites are encoded by this transcript. Since the biochemical data indicate that only two or three N-linked glycans are attached to the polypeptide chain, we predict that most of the potential glycan acceptor sites are not used.
FIGURE 3. Nucleotide sequence of MOLT-13 TCR-γ. (A) Sequencing strategy of clone M13k. A partial restriction map of the 1.1-kb cDNA clone M13k is shown. (B) Nucleotide and deduced amino acid sequence of clone M13k. Signal sequence (S), variable (V), N region (N), joining (J), and constant (C, Clb, ClIc, and ClII) region gene segments are indicated by arrows and were identified by comparison to genomic sequences (for S and V: 12, 14; for J: 33, 34; for C: 15, 36). The deduced amino acid sequence beginning at the initiator methionine is presented below the nucleotide sequence, using single-letter codes. Extracellular cysteines are highlighted by boxes, and potential N-linked carbohydrate attachment sites (N-X-S or N-X-T) (35) are indicated by brackets. These sequence data have been submitted to the EMBL/GenBank Data Libraries under accession number Y00790.

To reflect Cy gene segment usage for purpose of nomenclature, we have denoted the disulfide-linked TCR-γ/δ form expressed by PBL-C1 and WM-14 as "form 1," since such disulfide-linked TCR-γ chains use the Cy1 gene segment. The large (55 kD), non-disulfide-linked TCR-γ subunit of the TCR-γ/δ form expressed on IDP2 and PEER cells is encoded by Cy2 gene segments containing three CII exons (copies a, b, and c) and therefore this TCR-γ/δ form will be called "form 2abc." Correspond-
Unequal use of TCR-γ/δ forms. Freshly isolated peripheral blood mononuclear cells from healthy donors were \(^{125}\text{I}-\)labeled and solubilized in 1% Triton X-100. Immunoprecipitates with P3 control antibody (lanes 1 and 3) and anti-TCRδ1 (lanes 2 and 4) were analyzed under nonreducing (N; lanes 1 and 2) and reducing (R; lanes 3 and 4) conditions. \(M_r\) (× 10\(^{-3}\)) markers are shown on the left.

| Subject 1 | Subject 2 | Subject 3 |
|-----------|-----------|-----------|
| 1 | 2 | 3 | 4 |
| N | R | N | R | N | R |

Unequal \(\gamma\) Gene Segment Usage. To determine the presence of these three TCR-γ/δ forms in freshly isolated peripheral blood we analyzed the mononuclear cells from 10 healthy subjects using biochemical analysis with mAb anti-TCRδ1. This reagent appears to react with all TCR-γ/δ lymphocytes (3, our unpublished observations). Representative results from this panel are shown in Fig. 4. In subject 1, anti-TCRδ1 immunoprecipitates (analyzed under nonreducing conditions) demonstrate the presence of both disulfide-linked TCR-γ/δ complexes as a 70-kD protein band (form 1) and non-disulfide-linked TCR-γ/δ complexes as a broad 40-kD protein band (form 2bc) (Fig. 4, lane 2). This indicates that the \(\gamma\)1 and \(\gamma\)2 constant regions are both used by the expressed γ/δ T cell receptors of this individual. However, the amount of form 2bc varied among individuals. Note the smaller fraction of form 2bc in subject 2 compared with subject 1 by comparing the intensity of the 40-kD protein bands in both individuals (compare lane 2 of subject 2 with lane 2 of subject 1). Even more strikingly, only disulfide-linked TCR-γ/δ complexes could be detected on the mononuclear cells of 3 of the 10 individuals examined, even after long exposure of the autoradiographs (see subject 3). None of the analyzed individuals revealed the 55-kD, non-disulfide-linked TCR-γ/δ complex (form 2abc) in peripheral blood.

Glycosylation of the TCR-δ Subunit. In contrast to the striking structural differences in size and glycosylation of the TCR-γ proteins, the TCR-δ subunits from different
cell sources were remarkably similar. The relative molecular mass of the TCR-δ glycoprotein on MOLT-13 cells was directly determined to be 40 kD using the anti-TCR-δ mAb (Fig. 2 B, lane 4), confirming that it is similar in size to the TCR-δ glycoprotein on IDP2 cells (Fig. 1 B, lane 2, open arrow).

To also determine its polypeptide backbone size, cell surface $^{125}$I-labeled TCR-δ protein of MOLT-13 was digested with N-glycanase to remove asparagine-linked glycans (of the high mannose, hybrid, and complex type) (37, 38). The TCR-δ core polypeptide of MOLT-13 cells has a relative molecular mass of 35 kD (Fig. 2 C, lane 4), which is similar to that of the TCR-δ backbone of IDP2 cells (35 kD) (3). Digestion with endoglycosidase H (Endo H, removing only high mannose and certain hybrid N-glycans) (39, 40) caused a decrease in relative molecular mass of 2.5 kD (Fig. 2 C, lane 2), consistent with the presence of one carbohydrate moiety, leaving a relative mass of 2.5 kD of Endo H-resistant carbohydrates attached to the polypeptide.

Since there are two potential N-glycan attachment sites present in the TCR-δ constant domain (8, 9), these data directly show that both are used, but that their N-glycans are differently processed, namely one as a high mannose N-glycan (Endo H-sensitive) and the other as a complex N-glycan (Endo H-resistant, but N-glycanase-sensitive) and the other as a complex N-glycan (Endo H-resistant, but N-glycanase-sensitive). In contrast to the different amounts of attached N-linked carbohydrate on TCR-γ polypeptide chains, the TCR-δ subunits expressed on PEER, IDP2, and MOLT-13 cells all revealed the same peptide core sizes and the presence of two N-linked glycans (Fig. 2 C, and data not shown).
Discussion

In this study, three protein forms of the human TCR-γ glycoprotein are compared, namely the disulfide-linked 40-kD TCR-γ (form 1), the non-disulfide-linked 55-kD TCR-γ (form 2abc), and the non-disulfide-linked 40-kD TCR-γ protein (form 2bc). All three forms are shown to be associated with a TCR-δ subunit. CDNA sequences representing the first two TCR-γ forms have been reported previously (4, 20). The constant region of TCR-γ form 1 (on PBL-CI) is encoded by the Cγ1 gene segment containing a single CII exon, while TCR-γ form 2abc (on IDP2 and PEER cells) uses the Cγ2 gene segment containing CII exon copies a, b, and c. The cDNA sequence corresponding to a TCR-γ chain of form 2bc (on MOLT-13 cells, this study) was shown to contain a Cγ2 gene segment using only two CII exon copies, namely copy b and copy c. Similarly, it seems likely that the gene structure of the TCR-γ connector region of clone II and PBL-L2 (non-disulfide-linked, 40-kD TCR-γ protein) will also be like the MOLT-13 structure, namely of form 2bc. Since the TCR-δ constant region used is the same in all three forms (8, 9) (Brenner, M. B., and M. Krangel, unpublished observations) a complete comparison of the structures of the three TCR-γ/δ forms in man can be made (Fig. 5).

The fact that two Cγ2 polymorphic genomic forms exist (15, 36) suggests that the two transcript forms (form 2abc and form 2bc) are the product of these different allelic types. Interestingly, the dramatic difference in TCR-γ cell surface protein size between form 2abc (55 kD) and form 2bc (40 kD) is largely determined by the amount of attached N-linked carbohydrates, most likely reflecting the number of N-linked glycans. Backbone sizes of IDP2 TCR-γ (form 2abc) and MOLT-13 TCR-γ (form 2bc) proteins have been measured to be 40 kD and 35 kD, respectively, on the basis of SDS-PAGE, which correlates well with their predicted molecular masses of 36.6 and 34.8 kD, respectively, calculated on the basis of cDNA sequences. It is clear that this small difference in backbone size (5 kD in SDS-PAGE), accounted for mainly by one CII exon encoded peptide of 16 amino acids could not solely explain the observed difference in molecular mass between the 55-kD and 40-kD non-disulfide-linked TCR-γ surface forms. Form 2abc TCR-γ polypeptides possess five potential N-linked glycan attachment sites that are probably all used, in contrast to the MOLT-13 TCR-γ polypeptide which bears one additional potential attachment site, while carrying only two or three N-linked glycans. The reason for this limited use of potential attachment sites is unknown, but may result from the influence of the CII exon encoded peptides on the confirmation of the TCR-γ protein. The CII exon-encoded peptides and their neighbouring amino acids make up a connector region between the plasma membrane and the Ig-like constant domain. This connector region contains most of the N-linked glycan attachment sites (Fig. 5). We conclude that the CII exon copies determine the protein form not only by determining polypeptide backbone size, and by creating the ability to disulfide-link chains, but also by influencing the amount of attached carbohydrates.

TCR-δ cDNAs of IDP2 (8), PEER (9), and MOLT-13 (40a) cells have been sequenced and were found to be identical, except for the diversity/N region interspacing the variable and constant region gene segments. The TCR-δ protein on WM-13 cells has a relative molecular mass of 43 kD, which is similar to the TCR-δ proteins described by Borst et al. (16) and Lanier et al. (18), but is 3 kD larger than the other
TCR-δ chains. These 43 kD TCR-δ proteins might indicate the presence of different TCR-δ variable domains.

Since structural differences comparable to those described for TCR-γ constant region segments have not been observed for TCR-α and TCR-β genes (41-44), the significance of the dramatic structural diversity of these three TCR-γ/δ forms in man remains a mystery. However, there is possible similarity in the number of human CII exon repeats with the length in murine Cy regions, of which the Cy1, Cy2, and Cy4 constant regions encode 15, 10, and 33 amino acid connector regions, respectively (45, 46).

The ability to correlate protein size and presence or absence of disulfide linkage with TCR-γ constant region gene structure allowed us to examine constant region gene usage of expressed TCR-γ/δ complexes in peripheral blood of healthy subjects. Importantly, the human TCR-γ/δ forms are not used equally. The mechanism(s) responsible for the unequal usage of the TCR-γ/δ forms remains to be determined. If selection does occur, it may be at the level of the constant region per se, or possibly through the usage of J regions linked to these constant region gene segments. It is also intriguing to consider the possibility that the structural diversity may prove relevant to functional differences in these TCR-γ/δ complexes.

**Summary**

A subpopulation of the CD3+ peripheral T lymphocytes express the TCR-γ/δ complex. Three distinct TCR-γ forms that differ in size and in the ability to form a disulfide bridge with the TCR-δ subunit have been described. In this study we analyze the structural difference between the non-disulfide-linked 55-kD and 40-kD TCR-γ chains. The 40-kD TCR-γ form contains a smaller polypeptide backbone and carries less carbohydrate compared with the 55-kD TCR-γ form. A cDNA clone corresponding to the 40-kD TCR-γ subunit lacks one copy of the second exon of the constant region that is present in the other TCR-γ subunit. This exon copy encodes part of the connector region that is located between the constant domain and the membrane spanning region. We show that the number of potential N-linked glycan attachment sites are the same for the two TCR-γ forms. Since these attachment sites are located in the connector region we conclude that the connector region influences the amount of N-linked carbohydrates added to the core TCR-γ polypeptide, probably by affecting the conformation of the protein. In contrast to the TCR-β constant region usage, the TCR-γ constant regions are unequally expressed. Virtually exclusive usage of disulfide-linked complexes were found in some individuals, while both the disulfide-linked and the 40-kD, non-disulfide-linked TCR-γ forms were detected in other subjects. The ability to distinguish these TCR-γ/δ forms now makes it possible to study the mechanisms that govern their selection and to determine if they correspond to functionally distinct isotypes.

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