T3 glycoprotein is functional although structurally distinct on human T-cell receptor γ T lymphocytes

(activation/antigen receptor/glycosylation/intracellular calcium/phosphorylation)

MICHAEL S. KRANGEL*, BARBARA E. BIERER†‡, PETER DEVLIN*, MARTHA CLABBY*, JACK L. STROMINGER*, JOANNE MCLEAN*, AND MICHAEL B. BRENNER*§

Divisions of *Tumor Virology and †Pediatric Oncology, Dana-Farber Cancer Institute, and Departments of ‡Medicine and §Rheumatology and Immunology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

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ABSTRACT The T-cell receptor (TCR) γ gene product occurs in association with T3 (CD3) polypeptides on the surface of human T lymphocytes. TCR γ lymphocytes express arrays of T3 polypeptides distinct from those typically observed on TCR $\alpha\beta$ lymphocytes. This report demonstrates that identical T3 γ , δ , and ϵ polypeptides are synthesized by TCR γ lymphocytes and TCR $\alpha\beta$ lymphocytes. However, the processing of T3 δ oligosaccharides is distinct in the two cell types. This observation may suggest distinct quaternary structures of these receptor complexes. Despite these structural differences, the T3 molecule on TCR γ lymphocytes is functional. It is associated with and comodulates with TCR γ and it serves as a substrate for protein kinase C-mediated phosphorylation. Anti-T3 monoclonal antibodies induce a rapid increase in cytoplasmic free calcium, indicating that the receptor complex is involved in signal transduction and triggering of TCR γ lymphocytes.

The initial events in antigen-specific recognition and activation of human T lymphocytes are mediated by the polypeptides of the T3–T-cell receptor (T3–TCR) complex. On the vast majority of human peripheral blood lymphocytes (PBL), this complex consists of clonally variable TCR α and β glycopeptides, noncovalently associated with invariant T3 (CD3) glycopeptides of 25–28 kDa (T3 γ) and 20 kDa (T3 δ), and a nonglycosylated peptide of 19 kDa (T3 ϵ) (1–3). Recently, we and others have characterized human T lymphocytes that express T3 polypeptides but not TCR α and β polypeptides (4–7). Such cells express distinct T3-associated peptides, which, based upon immunoreactivity with TCR γ -specific antipeptide sera, include the product of the TCR γ -chain gene (8, 9). Biochemical analysis has shown that TCR γ peptides may vary in size and disulfide linkage and may be expressed in conjunction with additional T3-associated peptides (TCR δ).

Notably, initial studies of TCR γ lymphocytes revealed arrays of T3 polypeptides distinct from those typically observed on TCR $\alpha\beta$ cells (4, 5). Since the T3 molecule on TCR $\alpha\beta$ lymphocytes is presumed to play a central role in T-cell activation (10), its unique structure on TCR γ lymphocytes might have important functional implications as well. The present study was conducted to elucidate the structure and function of the T3 polypeptides on human TCR γ lymphocytes. It is demonstrated that differential glycosylation of the T3 δ chain is a common feature of cells expressing a TCR γ peptide. However, the T3 glycoprotein appears to be functional in mediating the activation of TCR γ lymphocytes by means of a mechanism analogous to that characterized for TCR $\alpha\beta$ lymphocytes.

MATERIALS AND METHODS

Cells and Antibodies. TCR $\alpha\beta$ lymphocytes were HPB-MLT (11), a T-leukemia cell line, and 2B5 (J.M. and M.B.B., unpublished), an interleukin 2 (IL-2)-dependent adult peripheral blood T-cell clone. TCR γ lymphocytes were PBL C1, an IL-2-dependent adult peripheral blood T-cell clone that expresses a 40-kDa, disulfide-linked TCR γ peptide (7); IDP2, an IL-2-dependent T-cell line derived from the peripheral blood of an immunodeficiency patient, that expresses a 55-kDa, nondisulfide-linked TCR γ peptide (4); and PEER, a T-leukemia cell line that expresses a 55-kDa, nondisulfide-linked TCR γ peptide (6, 7). Antibodies were UCHT1 [anti-T3 monoclonal antibody (mAb); a gift from P. Beverley, University College, London] (12); OKT3 (anti-T3 mAb; Ortho Diagnostics) (13); W6/32 (anti-HLA-A, -B, -C mAb) (14); anti-H (anti-HLA heavy chain serum) (15); anti-C γ (TCR γ -specific antipeptide serum; a gift from J. Seidman, Harvard Medical School) (4); 5E9 (anti-human transferrin receptor mAb; a gift from M. Hemler, Dana-Farber Cancer Institute) (16); and 187.1 (anti-mouse κ chain mAb) (17).

Labeled Cell Extracts. Labeling of viable lymphocytes by lactoperoxidase-catalyzed iodination, neuraminidase digestion of intact, labeled cells, and preparation of Nonidet P-40 lysates were as described (18), except that iodination and neuraminidase digestion were routinely performed at 23°C unless otherwise noted. Lysis of iodinated cells under denaturing conditions (boiling NaDodSO₄) was as described (7). Cells were pulse-labeled for 1 hr with [³⁵S]methionine as described (7) and labeled with [³²P]orthophosphate as described (19).

Immunoprecipitation and Gel Electrophoresis. Immunoprecipitation was carried out as described (20), and endoglycosidase H (endo H; Miles) digestion of immunoprecipitated species was performed as in an earlier study (15). Gel electrophoresis was performed on 10.0% NaDodSO₄/polyacrylamide gels or on two-dimensional gels employing pH 3.5–10.0 nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and either 12.5% or 15.0% NaDodSO₄/PAGE in the second dimension (18). Radiolabeled species were visualized by autoradiography using an intensifying screen or by fluorography (21).

Cytoplasmic Calcium ([Ca²⁺]_i) Determination. Cells (2–4 × 10⁶ per ml) were incubated with 5 μ M acetoxymethyl ester of indo-1 (Molecular Probes, Junction City, OR) for 45 min at 37°C on a shaker platform. Indo-1-loaded cells were washed twice, resuspended in Hanks' balanced salt solution (M.A. Bioproducts, Walkersville, MD) containing 2% heat-inactivated fetal bovine serum, and stored in the dark until

analysis. $[Ca^{2+}]_i$ was monitored by flow cytometry using a FACS I analyzer (Becton Dickinson) as described (22, 23).

RESULTS

Unusual Arrays of T3 Polypeptides on TCR γ Lymphocytes.

Examples of TCR $\alpha\beta$ and TCR γ lymphocytes were labeled by means of lactoperoxidase-catalyzed iodination, and, following detergent lysis and immunoprecipitation using anti-T3 mAb UCHT1, T3 polypeptides were resolved by NaDodSO₄/PAGE (Fig. 1). TCR $\alpha\beta$ cells, such as the HPB-MLT T-leukemia line and peripheral blood clone 2B5, displayed the typical pattern of T3 polypeptides—namely, diffuse bands of approximately 25 kDa and 20 kDa. The 25-kDa band represents the T3 γ chain, whereas the 20-kDa band includes the T3 δ and ϵ chains (2, 3). A strikingly different pattern was observed on TCR γ cells. The peripheral blood-derived T-cell clone PBL C1 and the IDP2 cell line displayed arrays of T3 polypeptides similar to each other but different from those on TCR $\alpha\beta$ cells. Similar arrays of T3 polypeptides have been observed on thymic-derived T-cell clone CII (5) and on the IDP1 cell line (4). The TCR γ T-cell tumor PEER displayed a polypeptide pattern that was more difficult to characterize, possibly due to its lower level of T3 expression.

Differential Glycosylation of T3 δ . The T3 molecules on the TCR $\alpha\beta$ cell line HPB-MLT and the TCR γ cell line IDP2 were compared in further detail by two-dimensional gel electrophoresis (Fig. 2 A and B). The T3 polypeptides on HPB-MLT are resolved into a series of spots representing the glycosylated T3 γ and δ chains as well as a single spot representing the nonglycosylated T3 ϵ chain. By comparison (using the nonglycosylated T3 ϵ peptide as an invariant reference point), the IDP2 T3 γ series of spots is weighted more to the basic end of the gel and the IDP2 T3 δ spots are more acidic and of slightly greater apparent molecular mass than their HPB-MLT counterparts.

Similar comparison of T3 peptides from neuraminidase-treated cells revealed the T3 γ patterns to be identical (Fig. 2 C and D). Thus, the difference in T3 γ noted above indicates that HPB-MLT T3 γ is more highly sialylated than its IDP2 counterpart. However, although the T3 δ patterns from neuraminidase-treated cells were identical by NEPHGE, they were still distinct by NaDodSO₄/PAGE (compare T3 δ to T3 ϵ , Fig. 2 C and D). The nature of this difference was revealed by digestion of immunoprecipitates with endo H. Mature, cell-surface T3 δ has been shown to carry one high-mannose and one complex carbohydrate on HPB-MLT cells (2, 3, 24, 25). Consistent with the presence of a single

FIG. 1. Distinct T3 peptides on TCR $\alpha\beta$ and TCR γ lymphocytes. Immunoprecipitates from detergent lysates of cell-surface iodinated lymphocytes using either control mAb p3X63Ag8 (lanes 1, 3, 5, 7, and 9) or anti-T3 mAb UCHT1 (lanes 2, 4, 6, 8, and 10) were analyzed by NaDodSO₄/PAGE. TCR $\alpha\beta$ cells examined were HPB-MLT and 2B5. TCR γ cells examined were PBL C1, IDP2, and PEER.

FIG. 2. Differential glycosylation of T3 peptides on TCR $\alpha\beta$ and TCR γ lymphocytes. Immunoprecipitates from detergent lysates of cell-surface iodinated HPB-MLT (A, C, and E) and IDP2 (B, D, and F) using anti-T3 mAb UCHT1 were analyzed by two-dimensional gel electrophoresis. (A and B) Untreated cells. (C and D) Neuraminidase-treated cells, mock-digested immunoprecipitates (no endo H). (E and F) Neuraminidase-treated cells, endo H-digested immunoprecipitates. Dashed lines serve as an invariant reference with which to compare mobilities. Mock-digested and untreated immunoprecipitates were identical (data not shown).

high-mannose oligosaccharide, endo H digested the HPB-MLT T3 δ polypeptide to a form 3 kDa smaller than the fully glycosylated form (Fig. 2E). However, IDP2 T3 δ was almost completely resistant to this treatment, indicating that in this cell line T3 δ oligosaccharides were almost exclusively converted to the complex form (Fig. 2F). This is likely to be a general result for T3 molecules associated with TCR γ , because a similar result has been obtained for T3 δ on PEER (data not shown), which, as seen in Fig. 1, is difficult to classify by its NaDodSO₄/PAGE pattern alone.

To determine whether identical T3 γ , δ , and ϵ peptide backbones were synthesized by HPB-MLT and IDP2 cells, these molecules were examined in cells metabolically pulse-labeled with [³⁵S]methionine. Under these labeling conditions, the glycosylated T3 γ and δ polypeptides would be expected to carry largely high-mannose, precursor oligosaccharides, which can be completely removed by endo H digestion. The glycosylated (Fig. 3 A and B) and endo H-digested, nonglycosylated (Fig. 3 C and D) T3 precursor polypeptides were identical by two-dimensional gel analysis. In other experiments, endoglycosidase F digestion of immunoprecipitates from surface-iodinated HPB-MLT and IDP2 cells revealed the polypeptide backbones of the respective mature, cell-surface T3 molecules to be identical as well (data not shown). Together, these results suggest that HPB-MLT and IDP2 cells synthesize identical T3 γ , δ , and ϵ peptides and that, although the T3 γ and δ peptides receive identical precursor oligosaccharides in the endoplasmic reticulum, subsequent T3 oligosaccharide processing in TCR γ lymphocytes is distinct from that in TCR $\alpha\beta$ lymphocytes. We have no data regarding the comparative structures of a newly described component of the human T3 molecule (26),

FIG. 3. Identical glycosylated and nonglycosylated T3 polypeptide precursors in TCR $\alpha\beta$ and TCR γ lymphocytes. Immunoprecipitates from detergent lysates of metabolically pulse-labeled HPB-MLT (A and C) and IDP2 (B and D) using anti-T3 mAb UCHT1 were analyzed by two-dimensional gel electrophoresis. (A and B) Mock-digested immunoprecipitates (no endo H). (C and D) Endo H-digested immunoprecipitates.

since it is difficult to visualize using the techniques employed in this study.

T3 Triggering Increases Intracellular Free Calcium Concentration. The initial events in the activation of TCR $\alpha\beta$ lymphocytes involve a rapid rise in intracellular free calcium concentration ($[Ca^{2+}]_i$), phosphatidylinositol phosphate breakdown, and the activation of protein kinase C (27–32). T3 appears to be of central importance in initiating these events, since anti-T3 mAb alone may induce increases in $[Ca^{2+}]_i$ and phosphatidylinositol phosphate breakdown. Given the selective structural differences in TCR γ -associated T3 polypeptides, we wished to determine whether these T3 peptides were functional in mediating T-lymphocyte triggering. TCR γ lymphocytes IDP2, PBL C1, and PEER were loaded with the Ca^{2+} -sensitive fluorescent indicator indo-1 (22), and antibody- and lectin-induced changes in $[Ca^{2+}]_i$ were analyzed by flow cytometry (Fig. 4). As measured by changes in the indo-1 violet/blue emission ratio, neither mock incubation (data not shown) nor a control antibody (Fig. 4 A, D, and G) induced a change in $[Ca^{2+}]_i$, whereas anti-T3 mAb OKT3 (Fig. 4 B, E, and H) and Con A (Fig. 4 C, F, and I) induced a marked and rapid increase in $[Ca^{2+}]_i$. Thus, the T3 peptides on TCR γ lymphocytes appear to function in the initial stages of the activation pathway by means of a mechanism analogous to that characterized for T3–TCR $\alpha\beta$.

Phosphorylation of the T3 γ Chain. Activators of protein kinase C induce phosphorylation of T3 γ (and to a lesser extent T3 δ) in the T3–TCR $\alpha\beta$ complex on HPB-MLT (19). In addition, a number of activation-related phosphorylation events have been described for murine T3 peptides (33, 34). Phosphorylation of HPB-MLT T3 γ appears to control the levels of surface T3–TCR $\alpha\beta$ (18, 19), by modulating the endocytosis and recycling of this receptor (18). T3 γ phosphorylation has been visualized directly by phorbol ester treatment of [^{32}P]orthophosphate-loaded cells. Alternatively, an acidic and a basic form of T3 γ (denoted T3 γ “a” and “b,” respectively) have been detected by two-dimensional gel analysis of immunoprecipitates from surface-iodinated and neuraminidase-treated cells, if cells have been maintained at 0°C through the iodination and neuraminidase steps. T3 γ a was converted to the b form by alkaline phosphatase

FIG. 4. Anti-T3 mAb and lectin increase $[Ca^{2+}]_i$. Indo-1-loaded IDP2 (A–C), PBL C1 (D–F), and PEER (G–I) cells were treated with IgG2a control mAb W6/32 (1:400 dilution of ascites fluid) (A, D, and G), IgG2a mAb OKT3 (1:400 dilution of ascites fluid) (B, E, and H), or Con A (10 μ g/ml) (C, F, and I). Relative $[Ca^{2+}]_i$ was determined as the ratio of 405 nm/480 nm emission and is expressed as a contour histogram plotted against the volume of the cells analyzed. Contours represent 3% increments in cell number, from 0 to 15%. A–F display the results of 2500 events collected at 3 min after the addition of mAb or lectin. G–I display the results of 12,000 events collected over the first 5 min after addition of mAb or lectin. An increase in the ratio of fluorescence emission >2 standard deviations above baseline was considered a significant rise in $[Ca^{2+}]_i$ (region above the horizontal line), and the fraction of cells displaying a significant rise in $[Ca^{2+}]_i$ is noted within each panel.

treatment, suggesting that the former was phosphorylated, whereas the latter was not (18).

Because of the potential importance of T3 phosphorylation in mediating or regulating T3 function, we assessed the basal and phorbol ester-induced levels of T3 γ phosphorylation in IDP2 cells. Only one iodinated T3 γ species, corresponding to the nonphosphorylated form, could be detected in untreated cells (Fig. 5A). However, treatment of cells with phorbol dibutyrate at 50 ng/ml resulted in a dramatic shift to the phosphorylated, T3 γ a form (Fig. 5B). Consistent with this result, a species with an electrophoretic mobility identical to that of T3 γ a was observed in immunoprecipitates from [^{32}P]orthophosphate-loaded, phorbol ester-induced cells (Fig. 5C). Thus, the T3 γ peptide on TCR γ lymphocytes, like that on TCR $\alpha\beta$ lymphocytes, may serve as a substrate for protein kinase C-mediated phosphorylation.

Comodulation of T3 and TCR γ from the Cell Surface. T3 and TCR $\alpha\beta$ peptides can be rapidly comodulated from the cell surface by the binding of anti-TCR or anti-T3 mAb (1, 35, 36). Receptor modulation may be of functional relevance, since it can be induced by high doses of antigen and can result in antigen unresponsiveness (35, 37–39). To determine whether T3 and TCR γ can be rapidly modulated from the surface of IDP2 cells were incubated with anti-T3 mAb

FIG. 5. Phorbol ester induces T3 γ phosphorylation. IDP2 cells either mock-incubated (A) or incubated for 30 min in the presence of phorbol dibutyrate at 50 ng/ml (B) were surface iodinated at 0°C. Alternatively, cells loaded with [32 P]orthophosphate were incubated for 30 min in the presence of phorbol dibutyrate at 50 ng/ml (C). Cells were neuraminidase treated at 0°C, detergent lysates were prepared, and immunoprecipitations using anti-T3 mAb UCHT1 were analyzed by two-dimensional gel electrophoresis.

UCHT1 for either 4 or 18 hr at 37°C. As determined by flow cytometry (Fig. 6 A–D), the vast majority of cell-surface T3 was modulated within 4 hr. An assessment of comodulation of the TCR γ peptide could not be made by flow cytometry due to the lack of antibodies that could recognize the native peptide on the cell surface. However, rapid comodulation could be demonstrated by cell-surface iodination followed by immunoprecipitation. After lysis of iodinated cells under

denaturing conditions, immunoprecipitation with a TCR γ -specific antipeptide serum (anti-C γ) demonstrated the loss of the vast majority of cell-surface iodinated TCR γ peptide after 4 hr of modulation (Fig. 6E, lanes 9–11). In sharp contrast, immunoprecipitation from denatured lysates showed no modulation of the class I HLA heavy chain (lanes 12–14), and immunoprecipitation from native lysates showed no modulation of either the HLA heavy chain- β_2 -microglobulin heterodimer (lanes 5–7) or the transferrin receptor (lanes 2–4). Thus, modulation of T3 from the cell surface with anti-T3 mAb UCHT1 induced the specific comodulation of the TCR γ peptide.

DISCUSSION

The T3 polypeptides on TCR γ lymphocytes are structurally distinct from those on TCR $\alpha\beta$ lymphocytes. Our results indicate that the T3 γ , δ , and ϵ polypeptide backbones on TCR γ lymphocytes are indistinguishable from their respective counterparts on TCR $\alpha\beta$ lymphocytes. However, differential glycosylation appears to be a general feature distinguishing the T3 molecules on these cells. Whereas only one of the two immature, high-mannose oligosaccharides on the T3 δ chain is typically processed to the complex form in TCR $\alpha\beta$ lymphocytes, both are converted to the complex form in TCR γ lymphocytes. This structural difference is shared by lymphocytes that express TCR γ peptides varying in size and disulfide linkage and thus appears to be a general property of cells that express TCR γ .

Efficient processing of both T3 δ oligosaccharides in TCR γ lymphocytes may reflect increased accessibility of these oligosaccharides to the processing enzymes of the endoplasmic reticulum and the Golgi complex. Differential accessibility of T3 δ oligosaccharides may in turn reflect a quaternary structure of the receptor complex in TCR γ lymphocytes that is distinct from that in TCR $\alpha\beta$ lymphocytes. Hence, the

FIG. 6. T3 and TCR γ peptides comodulate from the cell surface. IDP2 cells were incubated for 4 hr or 18 hr at 37°C in the presence of a 1:500 dilution of UCHT1 ascites fluid. Modulated and fresh unmodulated viable cells were isolated by Ficoll/Hypaque density gradient centrifugation, were stained at 0°C with either control mAb p3X63Ag8 or anti-T3 mAb UCHT1, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG, and were analyzed on an Ortho cytofluorograph. (A) Zero-hour modulation, control staining. (B) Zero-hour modulation, UCHT1 staining. (C) Four-hour modulation, UCHT1 staining. (D) Eighteen-hour modulation, UCHT1 staining. Alternatively, viable unmodulated and modulated cells were incubated for 1 hr at 0°C in the presence of a 1:500 dilution of UCHT1 and were surface iodinated at 0°C. Labeled cells were then lysed under either native (lanes 1–7) or denaturing (lanes 8–14) conditions. Immunoprecipitations were performed using control mAb P3X63Ag8 (E, lane 1), anti-human transferrin receptor mAb 5E9 (lanes 2–4), anti-HLA-A, -B, -C mAb W6/32 (lanes 5–7), control normal rabbit serum (E, lane 8), TCR γ -specific antipeptide serum anti-C γ (lanes 9–11), and anti-HLA heavy chain serum anti-H (lanes 12–14) and were analyzed by 10% NaDodSO $_4$ /PAGE.

processing difference might imply that the interaction of TCR γ (and δ) polypeptides with T3 is distinct from the interaction of TCR α and β peptides with T3. However, differential TCR-T3 interactions may not be properties of the mature cell-surface receptors but, rather, may be characteristic of a discrete stage of receptor biosynthesis. For example, significantly different kinetics of association of TCR γ versus TCR $\alpha\beta$ with T3 could dramatically affect oligosaccharide accessibility during biosynthesis.

Despite these structural differences, the T3 molecule on TCR γ lymphocytes is functional. This T3 molecule is associated with and comodulates with TCR γ and serves as a substrate for protein kinase C-mediated phosphorylation. Importantly, anti-T3 mAb induce rapid increases in $[Ca^{2+}]_i$ in lymphocytes expressing disulfide-linked and nondisulfide-linked forms of the TCR γ peptide. This indicates that both of these receptor complexes are functionally involved in signal transduction and triggering of TCR γ lymphocytes. These results are supported by other studies of TCR γ lymphocytes, showing that anti-T3 mAb can induce IL-2 secretion, proliferation, and cytolysis (5, 7). Thus, the T3 polypeptides on TCR γ lymphocytes appear to be functionally analogous to their counterparts on TCR $\alpha\beta$ lymphocytes. However, the requirements for specific triggering of TCR $\alpha\beta$ and TCR γ lymphocytes may be distinct, in light of the fact that several examples of the latter have recently been shown capable of lysing targets in a major histocompatibility complex-unrestricted fashion (7).

Note Added in Proof. Additional examples of human TCR γ T lymphocytes have been reported since this manuscript was submitted (40, 41).

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