Calnexin Associates with Monomeric and Oligomeric (Disulfide-linked) CD3δ Proteins in Murine T Lymphocytes

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The antigen-binding receptor expressed on most T lymphocytes consists of disulfide-linked clonotypic αβ heterodimers noncovalently associated with monomeric CD3γδζε proteins and disulfide-linked ζζ homodimers, collectively referred to as the T cell antigen receptor (TCR) complex. Here, we examined and compared the disulfide linkage status of newly synthesized TCR proteins in murine CD4+CD8+ thymocytes and splenic T cells. These studies demonstrate that CD3δ proteins exist as both monomeric and oligomeric (disulfide-linked) species that differentially assemble with CD3ε subunits in CD4+CD8+ thymocytes and splenic T cells. Interestingly, unlike previous results on glucose trimming and TCR assembly of CD3ε proteins in murine T cells (Van Leeuwen, J. E. M., and K. P. Kearse (1996) J. Biol. Chem. 271, 9660–9665), we found that glucose residues were not invariably removed from CD3δ glycoproteins prior to their assembly with CD3ε subunits in CD4+CD8+ thymocytes. Finally, these studies show that calnexin associates with both monomeric and disulfide-linked CD3δ proteins in murine T cells. The data in the current report demonstrate that CD3δ proteins exist as both monomeric and disulfide-linked molecules in murine T cells that differentially associate with partner TCR chains in CD4+CD8+ thymocytes and splenic T cells. These results are consistent with the concept that folding and assembly of CD3δ proteins is a function of their oxidation state.

Most T lymphocytes express on their surfaces a multisubunit complex consisting of clonotypic αβ proteins associated with invariant CD3γδζε and ζ chains, designated the T cell antigen receptor (TCR) complex (1–3). Assembly of the TCR occurs in the endoplasmic reticulum (ER) and proceeds in a highly ordered manner involving: (i) formation of noncovalently associated pairs of δε and γε proteins, (ii) assembly of αβ proteins with δε,γε pairs to form αδε and βγε intermediate complexes, (iii) joining of αδε and βγε chains followed by disulfide bonding of CD3-associated αβ proteins to yield incomplete αβδεγε complexes, and finally (iv) addition of disulfide-linked ζζ homodimers to form the complete αβδεγεζζ TCR complex (1–3).

In general, the surface expression of TCR proteins is tightly associated with their assembly status (1, 3). Individual, unassembled TCR proteins and partially assembled TCR complexes containing two or three TCR subunits do not effectively exit the ER. Both incomplete αβδεγεζζ and complete αβδεγεζζζ TCR egress from the ER to the Golgi complex; however, only complete TCR complexes are efficiently transported to the cell surface (1–3). Unlike mature CD4+ and CD8+ (single positive) T cells, which fundamentally express complete αβδεγεζζζ TCR (3), immature CD4+CD8+ (double-positive) thymocytes express both complete TCR and partial complexes of CD3δε. CD3ε proteins associated with calnexin (4–6), referred to as clonotypic independent complexes (4, 5). The molecular basis for clonotypic independent complex expression is unknown but is postulated to result from inefficient ER retention mechanisms in CD4+CD8+ thymocytes that do not persist in mature T cells (5, 7).

Previous studies by Jin et al. reported that a small subfraction of CD3ε proteins exists as disulfide-linked dimers in human T lymphocytes (8), which assemble with TCRβ subunits; CD3ε dimers were likewise observed to be present in murine thymocytes, although their assembly status was not evaluated (8). Disulfide-linked heterodimers of CD3γε proteins have also been described in REX variant human T cell lines, which fail to express TCRα or TCRαβ molecules (9). More recently, Huppa and Ploegh demonstrated that human CD3ε molecules translated in vitro in the absence of other TCR proteins have a tendency to form disulfide-linked homooligomers, which assemble with the molecular chaperone calnexin (10). Cotranslation of CD3δ or CD3γ proteins was sufficient to maintain CD3ε proteins in a principally monomeric phase, however, suggesting that CD3δ and CD3γ may guide the folding of CD3ε proteins during the initial stages of their biosynthesis (10). In the current study we evaluated the disulfide linkage status of newly synthesized TCR proteins in murine CD4+CD8+ thymocytes and splenic T cells. These studies show that newly synthesized CD3δ proteins exist as both monomeric and disulfide-linked molecules that differentially assemble with CD3ε molecules in CD4+CD8+ thymocytes and splenic T cells. In addition, these data document that calnexin associates with both monomeric and oligomeric (disulfide-linked) CD3δ proteins in murine T lymphocytes.

EXPERIMENTAL PROCEDURES

Animals, Cell Preparation, and Reagents—C57BL/6 (B6) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). CD4+CD8+ thymocytes were isolated by their adherence to plastic plates coated with anti-CD8 mAb (83-12-5) and were typically >95% CD4+CD8− as described (3). Splenic T cells were purified by incubation of single cell suspensions of spleen cells on tissue culture plates coated with rabbit anti-mouse immunoglobulin, (Organ-Technika-Cappel, Malvern, PA) for 60 min at 37 °C, followed by isolation of nonadherent cells. The resultant cell populations were typically 80–95% CD3− as determined by flow cytometry analysis. Antibodies—The following mAb were used in this study: 145–2C11, specific for CD3ε proteins associated with CD3δ or CD3γ chains (11); HMT 3.1, which recognizes CD3ε proteins irrespective of their assembly status.

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§ The abbreviations used are: TCR, T cell antigen receptor; ER, endoplasmic reticulum; mAb, monoclonal antibody; EH, endoglycosidase H; JB, jack bean mannosidase; PAGE, polyacrylamide gel electrophoresis; Ab, antibody.
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RESULTS

Disulfide-linked 26-kDa Proteins Differentially Associate with TCR Subunits in CD4⁺CD8⁺ Thymocytes and Splenic T Cells—As shown in Fig. 1A, analysis of anti-CD3ε immunoprecipitates of radiolabeled CD4⁺CD8⁺ thymocytes on two-dimensional nonreducing × reducing (NR × R) gels shows expected nondisulfide-linked monomeric CD3γ,δ and CD3ε proteins that migrate on and slightly above the diagonal, respectively (Fig. 1A, top panel), and disulfide-linked TCRβ heterodimers and ζ homodimers, which migrate below the diagonal (Fig. 1A, top panel). Interestingly, a dimeric species of unknown identity was also present in such precipitates, migrating at approximately 26 kDa following reduction of disulfide-linked bonds (Fig. 1A, top panel, arrow). Disulfide-linked 26-kDa proteins were isolated from CD4⁺CD8⁺ thymocyte lysates using two different anti-CD3ε mAbs of distinct specificity, 145-2C11 and HMT3.1 (Fig. 1, A, top panel, and B, top panel, respectively) (see “Experimental Procedures”), and by CD3δ-specific Ab (Figs. 1B, bottom panel) but not anti-TCRβ mAb (Fig. 1A, bottom panel) or anti-ζ antisemum (data not shown). Unlike CD4⁺CD8⁺ thymocytes, disulfide-linked 26-kDa proteins were not observed in anti-CD3ε precipitates of radiolabeled splenic T cells (Fig. 2, A and B) but were clearly present in anti-CD3δ precipitates of splenic T cells (Fig. 2B, bottom panel). Disulfide linkage of 26-kDa proteins did not result from artificial formation of disulfide bonds during cell lysis because identical results were observed when cells were solubilized in lysis buffer containing excess (75 mM) iodoacetamide (data not shown). Taken together, these data demonstrate that disulfide-linked 26-kDa proteins differentially associate with CD3ε and TCRβ subunits in CD4⁺CD8⁺ thymocytes and splenic T cells.

CD3δ Proteins Are Assembled into Disulfide-linked Dimers in Murine T Cells—Because their molecular mass is similar to that of CD3 components, we reasoned that disulfide-linked 26-kDa proteins might represent newly synthesized CD3δ proteins, CD3ε proteins, or both. To determine whether CD3δ,ε proteins were assembled into disulfide-linked dimers in CD4⁺CD8⁺ thymocytes, anti-CD3δ precipitates of CD4⁺CD8⁺ thymocytes were analyzed on two-dimensional NR × R gels and immunoblotted with antisemur specific for CD3δ and CD3ε molecules. As shown in Fig. 3, CD3δ proteins existed as both monomeric and disulfide-linked molecules in CD4⁺CD8⁺ thymocytes (Fig. 3, top panel). In contrast, CD3ε proteins were present exclusively as nondisulfide-linked monomers (Fig. 3, bottom panel). Identical results were obtained in immunoblot experiments of anti-CD3ε precipitates of CD4⁺CD8⁺ thymocytes (data not shown). These results show that CD3δ proteins exist as both monomeric and disulfide-linked molecules in CD4⁺CD8⁺ thymocytes.
CD3δ and CD3ε proteins are easily distinguished from each other in that CD3δ is post-translationally modified by the addition of three N-linked oligosaccharide chains unlike CD3ε, which does not contain N-glycans (1, 17). To confirm that CD3δ glycoproteins were disulfide-linked in murine T cells, digitonin lysates of radiolabeled CD4⁺CD8⁻ thymocytes were immunoprecipitated with anti-CD3δ Ab, CD3δ precipitates were boiled in SDS to release bound material, and CD3δ proteins were recaptured by precipitation with anti-CD3δ Ab. Precipitates were digested with Endo H glycosidase (specific for cleavage of immature N-linked glycans) and analyzed on one-dimensional SDS-PAGE gels under nonreducing conditions. Most CD3δ proteins radiolabeled during a 30-min pulse period migrated as monomeric 26-kDa proteins (Fig. 4, first lane), which fell to 17 kDa following removal of N-linked glycan chains, as expected (Fig. 4, second lane). Importantly, these data show that remaining CD3δ molecules existed as disulfide-linked proteins that migrated at approximately 52 kDa in mock treated samples (Fig. 4, first lane) and at 34 kDa following glycosidase digestion (Fig. 4, second lane); these results were confirmed by immunoblotting experiments using anti-CD3δ Ab (data not shown). These data are consistent with the assembly of CD3δ glycoproteins into disulfide-linked dimers that are composed of CD3δ proteins linked to itself (CD3δ-x) or to another molecule of similar size (CD3δ-y), which like CD3δ, must also contain N-glycans as the magnitude of decrease in molecular mass following deglycosylation is greater than would be expected for CD3δ associated with a nonglycosylated protein.

Glc Trimming and Calnexin Assembly of CD3δ Molecules in CD4⁺CD8⁻ Thymocytes—Immature N-glycan chains on newly synthesized glycoproteins having the structure Glc₃Man₇GlcNAc₂ are initially processed by the sequential action of glucosidase I and glucosidase II enzymes in the ER, creating monoglucosylated Glc₂Man₆GlcNAc₂ glycans important for interaction with the lectin-like chaperone calnexin (18–22). Calnexin is proposed to function in the quality control of folding and assembly of numerous newly synthesized glycoproteins, including TCRαβ and CD3δ,γ subunits (23–25). Previous studies on the processing of TCR glycoproteins in splenic T cells show that Glc residues are removed from newly synthesized CD3δ molecules prior to their assembly with other TCR subunits and that calnexin associates exclusively with unassembled, “free” CD3δ proteins containing incompletely trimmed glycan chains (24). To determine whether CD3δ proteins were similarly processed in CD4⁺CD8⁻ thymocytes, cells were pulse-labeled with [³⁵S]methionine for 30 min and solubilized in 1% digitonin, and lysates were immunoprecipitated with anti-CD3δ Ab to purify total CD3δ proteins; alternatively, lysates were sequentially immunoprecipitated with anti-TCRβ mAb to isolate CD3δ proteins assembled into complete αβδγεζζ and incomplete αβδεγε

Fig. 2. Disulfide-linked 26-kDa proteins are not assembled with CD3ε molecules in splenic T cells. A, CD4⁺CD8⁺ thymocytes and splenic T cells were radiolabeled with [³⁵S]methionine for 30 min and solubilized in 1% digitonin, and lysates were immunoprecipitated with anti-CD3ε mAb (145–2C11) mAb. Precipitates were analyzed on two-dimensional NR × R SDS-PAGE gels. The positions of TCR proteins and disulfide-linked 26-kDa proteins (arrow) are indicated. B, same as in A, except that splenic T cell lysates were immunoprecipitated with anti-CD3ε mAb (145–2C11) and anti-CD3δ Ab (R9).

Fig. 3. CD3δ proteins are assembled into disulfide-linked dimers in murine T cells. Anti-CD3δ immunoprecipitates of digitonin lysates of CD4⁺CD8⁺ thymocytes were analyzed on two-dimensional NR × R SDS-PAGE gels and immunoblotted with anti-CD3δ or anti-CD3ε Ab as indicated. The positions of monomeric and disulfide-linked CD3δ proteins are indicated. Note that anti-CD3ε proteins existed exclusively as nondisulfide-linked, monomeric proteins in these studies.
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**Fig. 4.** A subset of newly synthesized CD3δ glycoproteins exists as disulfide-linked dimers in CD4+CD8+ thymocytes. Digitonin lysates of [35S]methionine-radiolabeled CD4+CD8+ thymocytes were immunoprecipitated with anti-CD3δ Ab, precipitates were boiled in 1% SDS to release bound material, and CD3δ proteins were specifically recaptured by precipitation with anti-CD3δ Ab. Recapture precipitates were digested with EH glycosidases as indicated and analyzed on 13% SDS-PAGE gels under nonreducing conditions. The positions of monomeric and disulfide-linked CD3δ proteins are marked. An asterisk indicates a nonglycosylated molecule that nonspecifically coprecipitates with CD3δ proteins, believed to be actin; this molecule is not disulfide-linked to CD3δ proteins as determined by analysis on two-dimensional nonreducing × reducing gels (see Fig. 6).

TCR complexes, followed by precipitation with anti-CD3ε mAb to capture CD3ε chains present in partial complexes of CD3ε components and finally precipitation with anti-CD3δ Ab to purify remaining unassembled, free CD3δ chains. Precipitates were boiled in SDS to release bound material, CD3δ proteins were specifically recaptured with anti-CD3δ Ab, and recapture precipitates were digested with JB and EH glycosidases. JB digestion is useful for evaluating the Glc trimming status of newly synthesized glycoproteins because it removes eight mannoses from fully trimmed N-glycan chains devoid of Glc residues (Manα-5GlcNAc2) but only five mannoses from incompletely trimmed N-glycans containing one to three Glc saccharides (Glcα-3Manα-5GlcNAc2) (19, 24). In contrast, EH removes all but a single GlcNAc from N-glycan chains irrespective of their Glc content (26). Similar to what was previously observed in splenic T cells (24), CD3δ proteins synthesized in CD4+CD8+ thymocytes existed in four major glycoforms (A–D), indicative of CD3δ proteins containing three (A), two (B), one (C), and zero (D) incompletely trimmed glycan chains, respectively (Fig. 5A, left-hand side). Interestingly, however, unlike splenic T cells, CD3δ proteins containing incompletely trimmed N-glycans in CD4+CD8+ thymocytes were present as both free, unassembled chains and as assembled molecules associated with CD3ε proteins (Fig. 5, A and B). In contrast, CD3δ proteins associated with TCRβ were totally devoid of Glc residues as shown by their complete sensitivity to JB digestion (Fig. 5, A and B). Taken together, these data show that CD3δ glycoforms are similarly generated in immature CD4+CD8+ thymocytes and splenic T cells and that CD3δ proteins containing incompletely trimmed N-glycans exist as both free and assembled TCR subunits in CD4+CD8+ thymocytes.

Next, the assembly of newly synthesized CD3δ proteins with the molecular chaperone calnexin was examined. Metabolically labeled CD3δ proteins coprecipitated with calnexin in CD4+CD8+ thymocytes that, as expected, contained incompletely trimmed glycan chains that were partially resistant to JB digestion (Fig. 5A, middle lanes). As similarly noted for other T cell types (25), CD3δ proteins synthesized in CD4+CD8+ thymocytes did not associate with the calnexin-related molecule, calreticulin (data not shown). To determine the disulfide linkage status of CD3δ proteins associated with calnexin, digitonin lysates of radiolabeled CD4+CD8+ thymocytes were immunoprecipitated with anti-calnexin Ab or were sequentially immunoprecipitated with anti-TCRβ mAb, followed by anti-CD3ε mAb, and finally were immunoprecipitated with anti-CD3δ Ab. Precipitated material was released by boiling in SDS and CD3δ proteins specifically recaptured by precipitation with anti-CD3δ Ab; recapture precipitates were digested with JB and EH glycosidases as indicated. The positions of CD3δ glycoforms (A–D) and Endo-H-sensitive, deglycosylated CD3δ proteins (CD3δ EH5) are marked. B, same as in A except that CD4+CD8+ thymocyte lysates were sequentially immunoprecipitated with anti-TCRβ mAb, followed by immunoprecipitation with anti-CD3ε mAb and finally with anti-CD3δ Ab.

**DISCUSSION**

In the current report we evaluated the disulfide linkage status of newly synthesized TCR proteins in CD4+CD8+ thymocytes and splenic T cells. These studies show that: (i) CD3δ proteins exist as both monomeric and oligomeric (disulfide-linked) species in murine T cells; (ii) disulfide-linked CD3δ proteins differentially assemble with CD3ε and TCRβ subunits
in CD4+CD8+ thymocytes and splenic T cells; (iii) unlike CD3\(\varepsilon\) processing in splenic T cells, Glc residues are not invariably removed from CD3\(\varepsilon\) glycoproteins prior to their assembly with CD3\(\varepsilon\) chains in CD4+CD8+ thymocytes; and (iv) calnexin associates with both monomeric and disulfide-linked CD3\(\varepsilon\) proteins in murine T cells.

Previous studies by Jin et al. reported that a fraction of CD3\(\varepsilon\) proteins exist as disulfide-linked dimers in murine T cells, including thymocytes (8). The data in the current study show that CD3\(\varepsilon\) proteins were present as disulfide-linked dimers in both CD4+CD8+ thymocytes and splenic T cells, but no evidence was found for disulfide linkage of CD3\(\varepsilon\) molecules in either cell type. The reason for these apparent discrepancies are unclear but may result from the fact that identification of disulfide-linked CD3\(\varepsilon\) proteins in previous studies relied on their detection by rabbit antiserum directed against murine CD3\(\varepsilon\) (8), which may detect unique epitopes not recognized by the anti-CD3\(\varepsilon\) mAbs used in our study. Regardless, the current study clearly demonstrates that CD3\(\varepsilon\) glycoproteins were assembled into disulfide-linked dimers in murine T lymphocytes using several different approaches, including immunoblotting, immunoprecipitation/release/recapture experiments, and glycosidase digestion studies. Our results are consistent with the assembly of CD3\(\varepsilon\) chains into homodimers containing CD3\(\varepsilon\) molecules, although such a6,e intermediates remain to be directly demonstrated in primary murine T cells (3). Thus, it is possible that TCR\(\alpha\) association with CD3\(\varepsilon\) or CD3\(\varepsilon\) proteins retards assembly of disulfide-linked CD3\(\varepsilon\) proteins with CD3\(\varepsilon\) molecules, although such a6,e intermediates remain to be directly demonstrated in primary murine T cells (3). In both CD4+CD8+ thymocytes and splenic T cells, dimeric CD3\(\varepsilon\) proteins were precluded from incorporation into complete TCR complexes as evidenced by their failure to coprecipitate with TCR\(\alpha\), \(\beta\), and CD3\(\gamma\) proteins (this study), indicating that quality control mechanisms exist in both cell types that control assembly of dimeric CD3\(\varepsilon\) proteins into higher ordered TCR complexes.

Finally, our results that newly synthesized CD3\(\varepsilon\) chains bearing incompletely trimmed oligosaccharides were assembled with CD3\(\varepsilon\) subunits in CD4+CD8+ thymocytes are in agreement with previous reports that Glc-containing CD3\(\varepsilon\) proteins are expressed on the surfaces of immature thymocytes in association with CD3\(\varepsilon\) molecules (5, 7). Importantly, however, the data in the current study provide the first assessment of the efficiency of Glc removal from newly synthesized CD3\(\varepsilon\) proteins in CD4+CD8+ thymocytes and show that CD3\(\varepsilon\) glycoforms are effectively generated in CD4+CD8+ thymocytes as in splenic T cells (24).

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