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Calnexin Associates Exclusively with Individual CD3δ and T Cell Antigen Receptor (TCR) α Proteins Containing Incompletely Trimmed Glycans That Are Not Assembled into Multisubunit TCR Complexes*

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Most T lymphocytes express on their surfaces an oligomeric protein complex consisting of clonotypic αβ polypeptides associated with invariant CD3-γδε and ζ chains, designated the T cell antigen receptor (TCR) complex. Assembly and intracellular transport of nascent TCR proteins is believed to be assisted by their interaction with the molecular chaperone calnexin, which for certain molecules functions as a lectin for monoglucosylated glycans. However, as most of our knowledge about calnexin-TCR protein associations has been obtained under conditions of limited TCR assembly, the role of calnexin in the formation of nascent TCR complexes is unclear. Here, we studied the role of glucose (Glc) trimming and calnexin association in the oligomerization of TCRα and CD3δ glycoproteins in murine splenic T lymphocytes, a model cell type for efficient assembly of complete TCR complexes. We show that removal of Glc residues from both CD3δ proteins and TCRα proteins occurred prior to their association with any other TCR components and that calnexin specifically interacted with unassembled TCRα and CD3δ proteins containing incompletely trimmed oligosaccharides. Interestingly, we found that removal of Glc residues from glycan chains was necessary for efficient association of calnexin with TCRα glycoproteins but not with CD3δ glycoproteins. These studies define Glc trimming and calnexin association as initial molecular events in the translation of CD3δ and TCRα proteins, occurring coincident with or immediately after their translocation into the endoplasmic reticulum and preceding the ordered pairing of TCR chains. In addition, these data document that calnexin assembly with CD3δ and TCRα glycoproteins involves both glycan-dependent and glycan-independent mechanisms.

Assembly of the T cell antigen receptor (TCR)[1] complex occurs within the endoplasmic reticulum (ER) and proceeds in a highly ordered manner by: (i) formation of noncovalently associated pairs of δε and γε proteins, (ii) association of individual clonotypic α, β polypeptides with δε and γε pairs to form intermediate αδε and βγε protein complexes, (iii) rapid pairing of αδε and βγε chains and disulfide-bonding of CD3-associated αβ proteins to yield incomplete αβδεγε TCR complexes, and finally (iv) addition of ζζ homodimers to form complete, fully assembled αβδεγεζζ TCR complexes (1–4). Egress of TCR proteins from the ER is directly related to their assembly status; most unassembled TCR polypeptides and partial TCR complexes are retained within the ER. Only TCR proteins assembled into incomplete αβδεγε TCR complexes or complete αβδεγεζζ TCR complexes effectively transit from the ER to the Golgi system (1).

The molecular chaperone calnexin is a nonglycosylated resident ER transmembrane protein that associates with numerous oligomeric protein complexes within the ER, including β_1 and α_6 integrins (5), major histocompatibility class I (6, 7) and class II molecules (8), and the antigen receptors expressed on T and B lymphocytes (7, 9, 10). Regarding the TCR complex, four individual TCR proteins have been shown to associate with calnexin, including clonotypic TCRα and TCRβ polypeptides (7, 10) and CD3δ and CD3ε chains (7, 9, 11). Association between calnexin and ζ proteins has never been observed (12). While convincing evidence exists for association of calnexin with individual, unassembled TCR proteins, much less is known about the assembly of calnexin with incompletely assembled TCR complexes containing multiple TCR subunits. Indeed, the assembly status of most nascent TCR proteins associated with calnexin has not been rigorously evaluated (7, 9). Recently, Wiest and co-workers reported that calnexin is expressed on the surfaces of immature thymocytes in association with CD3γε and CD3δε pairs (13, 14). Expression of such clonotype-independent complexes appears to be developmentally regulated as they were not detected on the surfaces of mature T cells (14). Unlike mature T cells, immature thymocytes do not efficiently assemble CD3 components into complete αβδεγεζζ TCR complexes (15), leading to the suggestion that calnexin association with partial TCR complexes is exaggerated in cell types that are deficient in formation of complete TCR complexes (14).

A growing body of evidence signifies that removal of glucose (Glc) residues from nascent oligosaccharide chains is important for initial association of glycoproteins with calnexin (10, 16, 17). Removal of Glc residues from immature $Glc_3Man_9GlcNAc_2$ species (Glc, glucose; Man, mannose; GlcNAc, *N*-acetyl glucosamine) is accomplished by the sequential action of ER glucosidase I and glucosidase II enzymes, which remove the outermost and two innermost Glc residues, respectively (18), a process referred to as Glc trimming. Recent studies by several laboratories indicate that calnexin recognizes glycan chains on nascent glycoproteins bearing monoglucosylated ($Glc_1Man_9GlcNAc_2$) saccharides (10, 16–19), indicating that both glucosidase I and glucosidase II activities are necessary for creation of glycan substrates for calnexin binding (20). Oligo-

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[1] The abbreviations used are: TCR, T cell antigen receptor; Endo H, endoglycosidase H; ER, endoplasmic reticulum; Glc, glucose; GlcNAc, *N*-acetyl glucosamine; JB, jack bean mannosidase; Man, mannose; Ab, antibody; mAb, monoclonal Ab; Cas, castanospermine; PAGE, polyacrylamide gel electrophoresis.

saccharide chains are not strictly required for calnexin association, however, as several nonglycosylated molecules interact stably with calnexin, including recombinant multidrug resistance P glycoprotein lacking *N*-linked addition sites (21) and the CD3ϵ subunit of the TCR complex (11).

The role of Glc trimming and calnexin association in the oligomerization of TCR proteins within the ER is poorly understood. Indeed, it is unknown at which stage(s) of TCR assembly Glc residues are removed from oligosaccharide chains on nascent TCR glycoproteins in any cell type. Regarding the role of calnexin in TCR assembly, it has been suggested that calnexin interacts with individual newly synthesized TCR proteins to facilitate their folding within the ER and to prevent their escape to the Golgi compartment (7, 9, 10, 12). Moreover, since calnexin has been shown to associate with all TCR components except ζ, it has also been proposed that calnexin functions in the oligomerization of TCR proteins into incomplete αβδϵγϵ TCR complexes (9, 12). To determine at which stage(s) of TCR assembly Glc trimming and calnexin association occurs and to see if calnexin does, in fact, participate in the oligomerization of TCR subunits within the ER, we studied the role of Glc trimming and calnexin association in the assembly of TCRα and CD3δ glycoproteins into TCR complexes in murine splenic T cells. Our studies show that removal of Glc residues from nascent glycan chains on both CD3δ and TCRα proteins occurs prior to their association with other TCR proteins and that calnexin associates exclusively with unassembled CD3δ and TCRα proteins containing incompletely trimmed glycans. Moreover, we demonstrate that Glc trimming is required for effective interaction of calnexin with TCRα proteins, but not with CD3δ proteins, and report that removal of oligosaccharides does not affect the stability of existing protein complexes of calnexin and TCRα, CD3δ proteins. The implications of these findings on our current knowledge of TCR assembly are discussed.

EXPERIMENTAL PROCEDURES

Animals, Cell Preparation, and Reagents—C57BL/6 (B6) mice 6–8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). T cell receptor transgenic Vα11$^+$ mice (22) were generously provided by Dr. Stephen Hedrick. Splenic T cells were obtained by incubation of single cell suspensions of spleen cells on tissue culture plates coated with rabbit anti-mouse immunoglobulin (Ig) (Organon-Technika-Cappel, Malvern, PA) for 60 min at 37 °C, followed by isolation of non-adherent cells. The resulting cell populations were typically 85–90% CD3$^+$, as determined by flow cytometry analysis. Castanospermine (Cas) was obtained from Calbiochem (La Jolla, CA) and was used at a final concentration of 100 μg/ml.

Metabolic Labeling, Cell Lysis, and Immunoprecipitation—Metabolic pulse-labeling of splenic T cells was performed as described (4). Briefly, cells were incubated in methionine-free RPMI 1640 medium (Biofluids, Rockville, MD) containing 10% fetal calf serum and 1 mCi/ml [^{35}S]methionine (Tran^{35}S-label) (ICN, Irvine, CA) for 30 min at 37 °C in 5% CO_2. In experiments using Cas, cells were preincubated with medium or Cas for 30 min at 37 °C prior to metabolic labeling. The presence of Cas was maintained throughout the pulse and chase periods. Cells were lysed by solubilization in 1% digitonin (Wako, Kyoto, Japan) lysis buffer (20 mM Tris, 150 mM NaCl, plus protease inhibitors) at 1×10^8 cells/ml for 25 min at 4 °C. Cell lysates were clarified by centrifugation to remove insoluble material. Sequential immunoprecipitation and release/recapture procedures were performed as described previously (4).

Antibodies—The following monoclonal antibodies (mAb) were used in this study: 145-2C11, which is specific for CD3ϵ proteins associated with CD3γ or CD3δ chains (23); HMT3.1, which recognizes unassembled and assembled CD3ϵ proteins (24); H28-710, which is specific for TCRα (25); H57-597, which is specific for TCRβ (26), and RR8–1 anti-TCR Vα11 mAb (Pharmingen). The following antisera were used: R9 anti-CD3δ Ab (27) and SPA-860 anti-calnexin Ab (Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada).

Glycosidase Digestions and Gel Electrophoresis—Digestion with Endoglycosidase H (Endo H) was performed by resuspending precipitates in glycosidase digestion buffer (75 mM sodium phosphate, pH 6.1, 75 mM EDTA, 0.1% Nonidet P-40) containing 10 milliunits of Endo H (Genzyme, Cambridge, MA); digestion with jack bean mannosidase (JB) (Oxford Glycosystems, Rosedale, NY) was performed according to the manufacturer's instructions. Samples were placed at 37 °C for 12–16 h and digestions stopped by addition of 3 × electrophoresis sample buffer. For experiments examining the effects of oligosaccharide release on association of TCR glycoproteins with calnexin, lysates were precipitated with anti-calnexin Abs; precipitates were either mock-treated or digested with Endo H, washed, boiled in 1% SDS, and TCR proteins recaptured with anti-TCR Abs as described above. SDS-PAGE gel electrophoresis was performed as described previously (15).

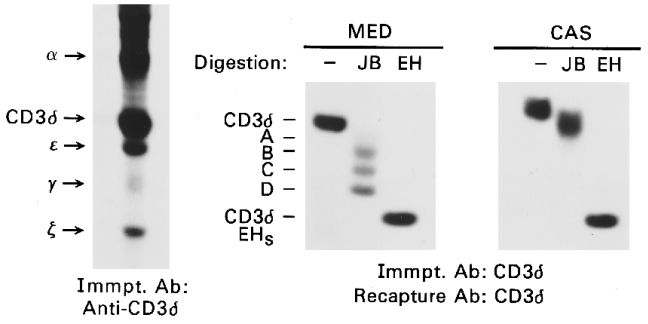

FIG. 1. **CD3δ glycoforms reflect differential processing of individual glycan chains on CD3δ molecules.** Digitonin lysates of metabolically pulse-labeled splenic T cells were immunoprecipitated with anti-CD3δ Ab and analyzed on SDS-PAGE gels under reducing conditions. The positions of radiolabeled α, δ, ϵ, γ, and ζ proteins are indicated (*left-hand side*). In the experiments shown on the *right*, CD3δ proteins were precipitated from lysates of splenic T cells treated with medium (*MED*) or castanospermine (*CAS*); precipitates were boiled in 1% SDS to release precipitated material, and CD3δ proteins recaptured with anti-CD3δ Abs; recaptured CD3δ chains were either mock-treated or digested with jack bean mannosidase (*JB*) or Endo H (*EH*). The positions of CD3δ glycoforms (*A–D*) and Endo H-sensitive CD3δ glycoproteins (*CD3δ EH*$_S$) are indicated.

RESULTS

Differential Glucose Trimming of Individual Glycan Chains on CD3δ Proteins Results in the Generation of Glycoforms—In our initial studies, the Glc trimming status of nascent CD3δ glycoproteins synthesized in splenic T cells was examined. For these experiments, splenic T cells were metabolically pulse-labeled for 30 min with [^{35}S]methionine, solubilized in 1% digitonin, and lysates immunoprecipitated with anti-CD3δ Ab. Radiolabeled TCRα, CD3ϵ, CD3γ, and ζ chains coprecipitated with CD3δ chains in splenic T cell lysates (Fig. 1, *left-hand side*), reflecting their assembly into TCR complexes. For specific isolation of CD3δ proteins, precipitates were boiled in 1% SDS, Nonidet P-40 detergent added, and released material immunoprecipitated with anti-CD3δ Ab to recapture CD3δ chains (4). The Glc trimming status of glycan chains on CD3δ proteins was evaluated by digestion with jack bean mannosidase (JB), which removes eight mannose residues from fully trimmed ($Man_9GlcNAc_2$) oligosaccharides, but only five mannoses from incompletely trimmed ($Glc_{1-3}Man_9GlcNAc_2$) glycan chains (17). Because CD3δ proteins are modified by addition of 3 *N*-linked sugar chains, 4 glycoforms of CD3δ can exist, reflecting the presence of 0–3 incompletely trimmed saccharide chains on CD3δ proteins. Indeed, as shown in Fig. 1, JB digestion of CD3δ proteins revealed the existence of one minor (*A*) and three major (*B–D*) CD3δ glycoforms in splenic T cell lysates (Fig. 1, *middle*), denoting CD3δ proteins with 3 (*A*), 2 (*B*), 1 (*C*), and 0 (*D*) incompletely trimmed glycan chains, respectively. In contrast, digestion with Endo H, which removes all but a single GlcNAc residue from both incompletely and fully trimmed glycan chains, generated a single CD3δ species, which migrated just below the D glycoform (Fig. 1, *CD3δ EH*$_S$). Existence of CD3δ glycoforms in splenic T cells was dependent on glucosidase activity, as they were not present in lysates of splenic T

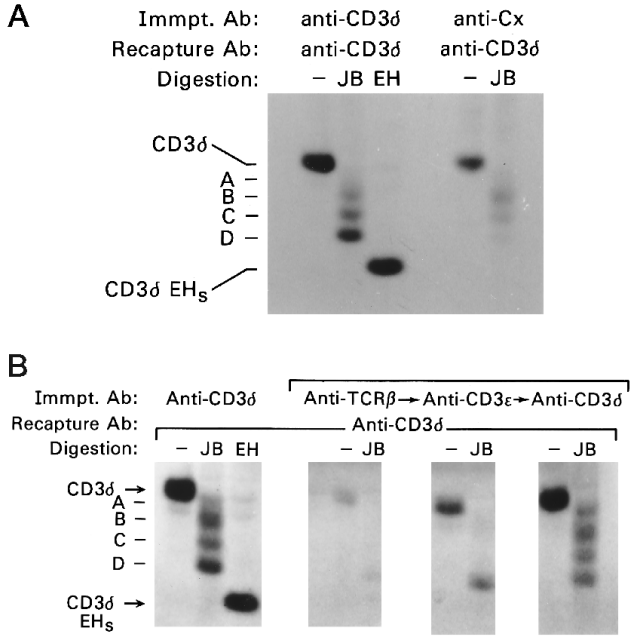

FIG. 2. **Calnexin associates specifically with unassembled CD3δ proteins containing incompletely trimmed glycan chains.** *A*, digitonin lysates of radiolabeled splenic T cells were immunoprecipitated with anti-CD3δ Ab (*left-hand side*) or with anti-calnexin Ab (*right-hand side*). Precipitated material was released by boiling in SDS, CD3δ proteins recaptured by precipitation with anti-CD3δ Ab, and precipitates digested with glycosidases as indicated. The positions of CD3δ glycoforms (*A–D*) and Endo H sensitive CD3δ glycan chains (*CD3δ EH*$_S$) are marked. *B*, digitonin lysates of splenic T cells metabolically pulse-labeled for 30 min were immunoprecipitated with anti-CD3δ Ab, or sequentially precipitated with anti-TCRβ Ab, followed by anti-CD3ε mAb, and finally with anti-CD3δ Ab. Material was released from precipitates by boiling in SDS, CD3δ proteins were recaptured by precipitation with anti-CD3δ Ab, and precipitates digested with glycosidases as indicated. The positions of CD3δ glycoforms (*A–D*) and Endo H-sensitive CD3δ glycoproteins (*CD3δ EH*$_S$) are marked.

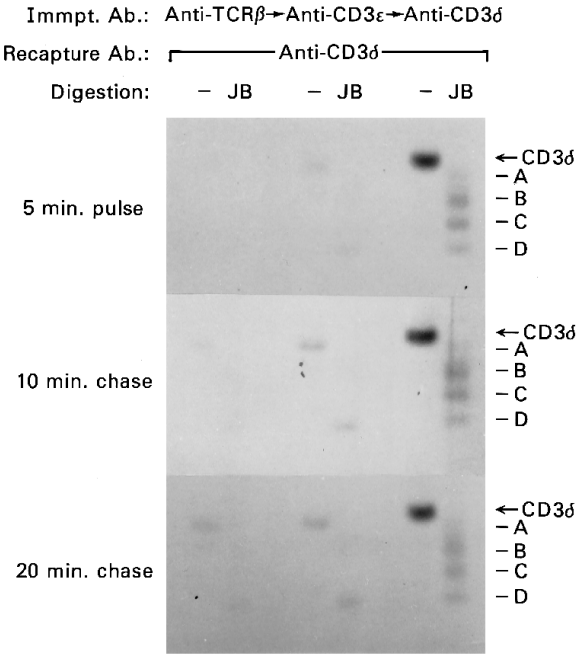

FIG. 3. **Glucose trimming and assembly of newly synthesized CD3δ glycoproteins in splenic T cells.** Same as in Fig. 2*B*, except that cells were pulsed for only 5 min and chased for the time period indicated. The positions of CD3δ glycoproteins and CD3δ glycoforms (*A–D*) are marked.

cells treated with the glucosidase inhibitor castanospermine (18) (Fig. 1, *right-hand side*). Taken together, these results show that distinct glycoforms of CD3δ proteins are generated in splenic T cells that reflect the differential removal of Glc residues from individual glycan chains.

Calnexin Associates Specifically with Unassembled CD3δ Proteins Containing Incompletely Trimmed Glycan Chains—To determine the Glc trimming status of CD3δ proteins associated with calnexin, lysates of radiolabeled splenic T cells were precipitated with anti-calnexin Ab, precipitated material was released by boiling, and CD3δ proteins recaptured by precipitation with anti-CD3δ Ab; recaptured CD3δ proteins were digested with JB enzymes. As shown in Fig. 2*A*, CD3δ proteins associated with calnexin in splenic T cell lysates contained incompletely trimmed glycan chains, with most existing in the B glycoform, and to a lesser extent, the C glycoform (Fig. 2*A*, *right-hand side*).

To determine if calnexin remained associated with CD3δ proteins following their assembly with other TCR proteins and to ascertain at which stage(s) of TCR assembly Glc residues are completely removed from CD3δ proteins, a series of sequential immunoprecipitation studies was performed to examine the Glc trimming status of CD3δ proteins containing incompletely trimmed and fully trimmed glycan chains. For these experiments, digitonin lysates of radiolabeled splenic T cells were precipitated with anti-CD3δ antiserum to capture total CD3δ proteins, or were sequentially immunoprecipitated with: (i) anti-TCRβ mAb to isolate CD3δ proteins existing in complete αβδεγεζζ TCR and incomplete αβδεγε TCR complexes, followed by (ii) anti-CD3ε Ab to capture CD3δ proteins existing in intermediate αδε and partial δε protein complexes, and finally (iii) anti-CD3δ Ab to isolate free, unassembled CD3δ proteins. CD3δ glycoproteins were recaptured from precipitates and their Glc trimming status evaluated by JB digestion. As previously established, four distinct glycoforms of CD3δ chains existed in splenic T cell lysates (Fig. 2*B*, *left-hand side*, *A–D*). Clearly, both incompletely trimmed and fully trimmed CD3δ glycoforms were present in the pool of unassembled CD3δ proteins (Fig. 2*B*, *right-hand side*, *A–D*). In contrast, CD3δ proteins associated with CD3ε and TCRβ molecules contained glycan chains that were devoid of Glc residues as they were completely susceptible to JB digestion (Fig. 2*B*, *middle*, *D*). Chase studies showed that Glc residues were completely removed from CD3δ glycoproteins within several hours of their synthesis in splenic T cells, which correlated with their assembly into TCR complexes and transport to the Golgi system (data not shown).

Taken together, these results demonstrate that calnexin associates with unassembled CD3δ proteins containing incompletely trimmed oligosaccharide chains. Moreover, since CD3δ proteins assembled with CD3ε containing complexes contained fully trimmed glycan chains, these studies indicate that Glc trimming of CD3δ glycoproteins occurs prior to their assembly with other TCR proteins.

To confirm these results, we wished to assess the Glc trimming status of CD3δ proteins at an earlier time point prior to their association with CD3ε and TCRβ proteins. For these studies, splenic T cells were pulse-labeled for only 5 min and chased for 10 and 20 min. Sequential precipitations of assembled and unassembled CD3δ chains and release/recapture procedures were performed as described in Fig. 2*B*. As expected, the vast majority of CD3δ proteins existing at the end of the short pulse period were not associated with CD3ε chains, but existed as free, unassembled CD3δ chains (Fig. 3, *top*). During the chase period, nascent CD3δ proteins assembled with CD3ε chains (Fig. 3, *middle*), and successively with TCRβ molecules (Fig. 3, *bottom*). These results show that the first detectable

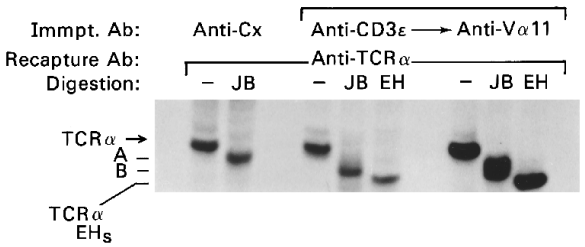

FIG. 4. **Calnexin associates with unassembled TCRα proteins containing incompletely trimmed oligosaccharides.** Digitonin lysates of radiolabeled splenic T cells were immunoprecipitated with anti-calnexin Ab, or sequentially precipitated with anti-CD3ϵ mAb, followed by anti-Vα11 mAb. Precipitated material was released by boiling in SDS, TCRα proteins recaptured by precipitation with anti-TCRα mAb, and samples digested with glycosidases as indicated. Precipitates were analyzed on 13% SDS-PAGE gels under reducing conditions. The positions of TCRα glycoforms (*A* and *B*) and Endo H-sensitive TCRα glycoproteins (*TCRα EH$_S$*) are marked. Note that higher percentage acrylamide gels reveal additional TCRα glycoforms that are compressed between the A and B glycoforms displayed here (data not shown); because resolution of other TCRα glycoforms was not optimal on such gels, however, material was analyzed on 13% gels for these studies.

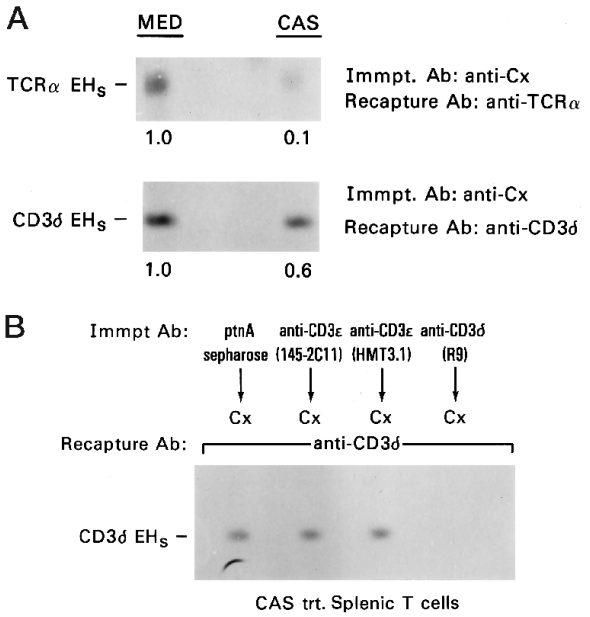

FIG. 5. **Glc trimming is required for effective association of TCRα proteins, but not CD3δ proteins, with calnexin.** *A*, splenic T cells were treated with medium (*MED*) or castanospermine (*CAS*), metabolically pulse-labeled, lysed in digitonin, and precipitated with anti-calnexin Ab. The presence of Cas was maintained throughout the pulse period. Calnexin precipitates were boiled in SDS, TCR proteins recaptured, and precipitates were digested with Endo H. The relative amount of TCR proteins present in Endo H-digested samples was quantitated by densitometry scanning, with the amount of calnexin-associated TCR proteins in medium groups set at 1.0. Multiple exposures of autoradiographs were scanned to ensure linearity. *B*, digitonin lysates of Cas-treated splenic T cells were incubated with three rounds each of protein A-Sepharose (*control*), 145-2C11 anti-CD3ϵ mAb, HMT3.1 anti-CD3ϵ mAb, or anti-CD3δ Ab. Material was then sequentially precipitated with anti-calnexin Ab, precipitates boiled in SDS, and CD3δ proteins recaptured with anti-CD3δ Ab. For quantitative purposes, precipitates were digested with Endo H. The position of digested CD3δ proteins (*CD3δ EH$_S$*) is indicated.

CD3δ proteins assembled with other TCR proteins in our studies contained fully trimmed glycan chains. Note that most nascent CD3δ proteins synthesized during a 5 min pulse period were susceptible to JB digestion (Fig. 3, *top*, *A–D*), indicating that processing of nascent CD3δ proteins by ER glucosidase enzymes occurs coincident with or immediately after their translation and insertion into the ER lumen (28, 29).

Calnexin Associates with Unassembled TCRα Proteins Containing Incompletely Trimmed Oligosaccharides—Next, we examined the Glc trimming status of TCRα proteins associated with calnexin and CD3 proteins in splenic T cells. Since the TCRα protein is clonotypic and thus quite heterogeneous, transgenic mice expressing a single rearranged TCRα gene product, the Vα11 protein, were used for these experiments (22). Splenic T cells of Vα11$^+$ transgenic mice were pulse-labeled for 30 min, solubilized in 1% digitonin, and lysates immunoprecipitated with anti-calnexin Ab; alternatively, lysates were sequentially precipitated with anti-CD3ϵ mAb to isolate CD3-associated TCRα proteins, followed by anti-Vα11 mAb to capture unassembled TCRα proteins. Precipitated material was released by boiling in SDS and recaptured TCRα proteins were digested with JB or Endo H glycosidases. Unassembled TCRα proteins existed in two major glycoforms in splenic T cell lysates, marked *A* and *B*, which represent "free" TCRα proteins containing incompletely trimmed and fully trimmed glycan chains, respectively (Fig. 4, *right-hand side*). As demonstrated, TCRα proteins associated with calnexin contained incompletely trimmed glycans (Fig. 4, *left-hand side*, *A*), whereas TCRα proteins assembled with CD3ϵ chains possessed fully trimmed oligosaccharide chains (Fig. 4, *middle*, *B*). Thus, similar to what was observed for CD3δ proteins, these results show that calnexin associated specifically with unassembled TCRα proteins containing incompletely trimmed *N*-linked sugar chains, and that Glc residues were removed from glycan chains on nascent TCRα proteins prior to their assembly with CD3ϵ proteins. Although transgenic mice were used in these experiments, it should be realized that similar conclusions were reached in studies using normal, nontransgenic mice (data not shown). It should also be appreciated that radiolabeled TCRβ proteins are not visible in these studies as formation of CD3-associated disulfide-linked $\alpha\beta$ heterodimers involves pairing of newly synthesized TCRα proteins with preexisting nonradiolabeled TCRβ proteins in murine T cells (15).

Requirement for Oligosaccharide Chains for Association of Calnexin with TCRα and CD3δ Glycoproteins—To assess the importance of Glc trimming in the initial association of nascent TCRα and CD3δ proteins with calnexin, studies were performed on splenic T cells treated with the glucosidase inhibitor Cas. As shown in Fig. 5*A*, Glc trimming was required for efficient association of nascent TCRα proteins with calnexin as only trace amounts (approximately 10%) of TCRα proteins were recaptured from anti-calnexin precipitates of Cas-treated splenic T cells relative to untreated splenic T cells (Fig. 5*A*, *top*). Surprisingly, we found that association of calnexin with CD3δ chains was only modestly affected by blockade of glucosidase activity, as significant amounts of CD3δ proteins were associated with calnexin in Cas-treated splenic T cells (Fig. 5*A*, *bottom*). These data indicate that Glc trimming is required for effective interaction of TCRα proteins, but not CD3δ proteins, with calnexin in splenic T cells.

We reasoned that CD3δ proteins might associate directly with calnexin molecules in Cas-treated splenic T cells, or indirectly, via pairing with CD3ϵ proteins (11). To examine this issue, we evaluated the ability of various anti-CD3ϵ mAbs to preclear calnexin-associated CD3δ proteins from splenic T cell lysates. As demonstrated in Fig. 5*B*, precipitation of Cas-treated splenic T cell lysates with anti-CD3δ Abs effectively removed calnexin-associated CD3δ chains, as expected (Fig. 5*B*, compare *lanes 1* and *4*). In contrast, precipitation with two different anti-CD3ϵ mAbs failed to preclear calnexin-associated CD3δ proteins from lysates of Cas-treated splenic T cells (Fig. 5*B*, compare *lanes 1*, *2*, and *3*). Thus, we conclude that CD3δ proteins associate directly with calnexin molecules in Cas-

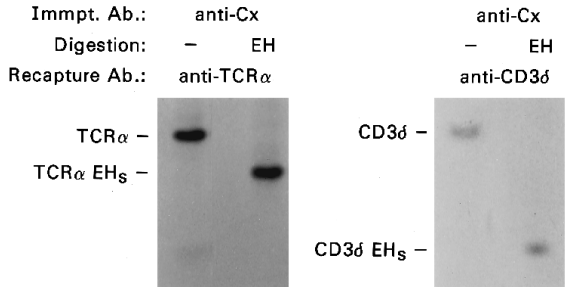

FIG. 6. **Oligosaccharide chains are not necessary to maintain stable association of calnexin with TCRα and CD3δ glycoproteins.** Digitonin lysates of radiolabeled splenic T cells were immunoprecipitated with anti-calnexin Ab; precipitates were resuspended in Endo H digestion buffer in the presence or absence of Endo H and incubated at 37 °C for 14 h. Following digestion, precipitates were washed in PBS; supernatants containing material released by Endo H digestion were removed, pellets (precipitates) were boiled in SDS, and TCR proteins recaptured by precipitation with anti-TCR specific Abs. The positions of CD3δ and TCRα proteins are indicated. Note that negligible amounts of TCRα and CD3δ proteins were recovered from supernatants of mock-treated and Endo H-digested calnexin precipitates (data not shown).

treated splenic T cells. While these experiments do not formally exclude the possibility that anti-CD3ϵ mAbs do not effectively recognize calnexin-associated $\delta\epsilon$ protein complexes in Cas-treated splenic T cells, it should be noted that these mAbs are capable of precipitating calnexin-associated CD3$\delta\epsilon$ proteins formed in immature T cells (14).

Finally, we wished to evaluate whether *N*-linked glycan chains on TCRα and CD3δ glycoproteins were required for maintaining stable interaction with calnexin. Calnexin precipitates of radiolabeled splenic T cell lysates were either mock-treated or digested with Endo H, and association of TCR glycoproteins with calnexin before and after deglycosylation was compared. As demonstrated, equivalent amounts of TCRα and CD3δ proteins were recaptured from mock-treated and Endo H-digested anti-calnexin precipitates (Fig. 6), showing that once formed, complexes of calnexin and individual CD3δ and TCRα proteins do not require oligosaccharide chains to maintain their association.

DISCUSSION

The quality control system of the ER ensures that properly folded, fully assembled protein complexes are expressed on the cell surface. The molecular chaperone calnexin is believed to assist in the oligomerization of nascent TCR proteins within the ER and to play a role in regulating the transport of assembled TCR complexes from the ER to the Golgi compartment. In the current study, we evaluated the role of Glc trimming and calnexin association in the oligomerization of CD3δ and TCRα proteins in splenic T lymphocytes. Our results show that: (i) removal of Glc residues from nascent CD3δ proteins and TCRα proteins occurs prior to their association with partner TCR chains within the ER; (ii) calnexin associates specifically with unassembled CD3δ proteins and unassembled TCRα proteins containing incompletely trimmed glycan chains; (iii) Glc trimming is required for effective association of TCRα proteins, but not CD3δ proteins, with calnexin; and (iv) oligosaccharide chains are important in the initial assembly of TCRα-calnexin protein complexes, but that once formed, are not required to maintain their association. Taken together, these data effectively rule out the postulate that calnexin functions as a scaffold for assembly of nascent TCR complexes and show that calnexin assembly with TCR glycoproteins involves both glycan-dependent and glycan-independent mechanisms.

Recent studies suggest that association of nascent glycoproteins with calnexin proceeds in a two-step fashion involving initial binding of monoglucosylated glycans by calnexin, followed by protein-protein interactions which stabilize these associations (19, 30). In agreement with this model, we found that inhibition of glucose trimming markedly impaired association of nascent TCRα proteins with calnexin in splenic T cells and, similar to what has been reported for major histocompatibility class I proteins (19) and major histocompatibility class II proteins (31), removal of oligosaccharide chains from existing calnexin-TCRα complexes did not affect the stability of their interaction. Interestingly, the transmembrane domain of all of these proteins has been implicated in maintaining their association with calnexin (31, 32), suggesting that calnexin interactions may be stabilized within the lipid bilayer of the ER. The finding that blockade of Glc trimming severely limits the assembly of nascent TCRα proteins with calnexin in splenic T cells is in agreement with previous studies showing that association of nascent TCRα proteins with calnexin is impaired in glucosidase II-deficient BW PHAR2.7 thymoma cells and in Cas-treated wild type BW thymoma cells (10). Importantly, the current study extends these findings by showing that, unlike calnexin association with TCRα proteins, assembly of calnexin with CD3δ proteins was only modestly affected by inhibition of Glc trimming. Thus, CD3δ represents the second TCR component that has been described to interact with calnexin in a glycan-independent manner, with CD3ϵ being the first (11). Because CD3δ and CD3ϵ proteins are structurally homologous (1), it is reasonable to speculate that CD3δ and CD3ϵ molecules share a common region that mediates their association with calnexin. It remains to be determined why some CD3δ chains synthesized in Cas-treated splenic T cells failed to associate with calnexin in our studies. The presence of monoglucosylated glycans on CD3δ proteins, although not absolutely required, may increase the efficiency with which CD3δ proteins interact with calnexin. Alternatively, as persistence of Glc residues on oligosaccharide chains has been observed to result in increased association of CD3δ chains with CD3ϵ molecules (4), it is conceivable that enhanced assembly of CD3δ chains into $\delta\epsilon$ pairs in Cas-treated splenic T cells precludes their association with calnexin.

The role of calnexin in the oligomerization of TCR proteins has been controversial as most studies on calnexin association with TCR proteins have been performed in cell types that are markedly impaired or completely deficient in the assembly of complete $\alpha\beta\delta\epsilon\gamma\epsilon\zeta\zeta$ TCR complexes (10, 14). Studies on calnexin interaction with TCR proteins are further complicated by the fact that multiple TCR proteins coprecipitate with calnexin, which may or may not be assembled with each other into oligomeric TCR complexes (7, 9). To overcome these problems, we performed studies in murine splenic T lymphocytes, which efficiently assemble complete $\alpha\beta\delta\epsilon\gamma\epsilon\zeta\zeta$ TCR complexes (15) and, most importantly, implemented a precipitation/recapture/glycosidase digestion protocol that allowed us to rigorously identify the Glc trimming status of TCRα and CD3δ proteins associated with calnexin and partner TCR chains. Indeed, our results demonstrate that calnexin associates exclusively with unassembled CD3δ and TCRα proteins containing incompletely trimmed oligosaccharide chains in splenic T cells and show that processing of oligosaccharides on CD3δ and TCRα glycoproteins by ER glucosidase enzymes precedes their assembly into intermediate $\alpha\delta\epsilon$ and incomplete $\alpha\beta\delta\epsilon\gamma\epsilon$ TCR complexes (4). Thus, we believe that calnexin assists in the folding of newly synthesized, individual TCR proteins within the ER but do not think that calnexin plays a significant role in the oligomerization of multiple TCR protein subunits. It remains possible that calnexin release occurs coincident with assembly of individual TCR proteins with partner chains and that such assembly

facilitates displacement of calnexin; importantly, however, our data show that calnexin does not remain associated with TCRα and CD3δ proteins assembled into multisubunit TCR complexes in splenic T cells. Finally, it is noteworthy to mention that our results on calnexin association with unassembled TCR components in splenic T cells parallel recent findings on the assembly of IgM complexes in B cells, which showed that calnexin associated with free IgM heavy chains but not with IgM chains assembled with Igα proteins (33).

In summary, the current report has evaluated the role of Glc trimming and calnexin association in the assembly of CD3δ and TCRα proteins into TCR complexes in splenic T cells. These studies define Glc trimming and calnexin association as initial molecular events in the assembly cascade of TCR glycoproteins that precede the ordered pairing of TCR chains within the ER.

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