Reglucosylation of N-Linked Glycans Is Critical for Calnexin Assembly with T Cell Receptor (TCR) α Proteins but Not TCRβ Proteins*

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Association of calnexin with newly synthesized glycoproteins involves recognition of monoglucosylated glycans, generated in the endoplasmic reticulum via initial removal of two glucose (Glc) residues from immature glycan chains by glucosidase enzymes (Glc trimming), or addition of a single Glc residue to fully trimmed glycans by glucosyltransferase enzymes (reglucosylation). While it has been established that creation of monoglucosylated glycans is important for chaperone binding, it is unknown if most proteins require both deglucosylation and reglucosylation for calnexin assembly or if initial Glc trimming is sufficient. Here, we studied the deglucosylation and reglucosylation of two related glycoproteins, the α and β subunits of the T cell receptor (TCR) complex, and their assembly with calnexin in BW thymoma cells. Our data demonstrate that TCRβ glycoproteins undergo multiple cycles of Glc removal and addition within the endoplasmic reticulum and that numerous reglucosylated proteins assemble with calnexin, including TCRα/β glycoproteins. Importantly, the current study shows that TCRβ proteins, but not TCRα proteins, effectively associate with calnexin under conditions of functional Glc trimming but impaired reglucosylation. These data demonstrate that reglucosylated proteins associate with lectin-like chaperones in vivo and provide evidence that reglucosylation is of differential importance for the association of individual, indeed similar, glycoproteins with calnexin.

Upon insertion into the lumen of the endoplasmic reticulum (ER), many polypeptides are modified by addition of oligosaccharide chains to asparagine residues, a cellular process termed N-linked glycosylation (1). Immature glycan chains on nascent polypeptides have the structure Glc3Man9GlcNAc2 (Glc = glucose; Man = mannose; GlcNAc = N-acetyl glucosamine) and are initially processed by the sequential action of two ER enzymes, glucosidase I and glucosidase II (2). Within the last few years, it has become apparent that processing of Glc residues, or Glc trimming, is an important step in the association of many glycoproteins with the molecular chaperone calnexin (3–7) and, more recently, calreticulin (8–10). Calnexin and calreticulin are transmembrane and lumenal ER proteins, respectively, that are two known members of a family of endogenous lectin-like proteins that recognize partially trimmed, monoglucosylated (GlcManGlcNAc) glycan chains on newly synthesized glycoproteins (8, 9, 11).

Stable interaction of calnexin and calreticulin with newly synthesized glycoproteins is believed to occur by a two-step process involving initial binding of monoglucosylated glycan chains, followed by protein:protein interactions, which stabilize these associations (12, 13). Monoglucosylated GlcManGlcNAc glycan chains may be generated via two mechanisms: cotranslational processing of immature GlcManGlcNAc glycan chains by glucosidase I and glucosidase II enzymes (the Glc trimming pathway), or reglucosylation of fully trimmed ManαGlcNAc2 glycans by UDP-glucose:glycoprotein-dependent glucosyltransferase enzymes (the reglucosylation or salvage pathway) (4, 14–17). Recent studies demonstrate that glucosyltransferase effectively reglucosylates denatured but not native glycoproteins in vitro (18–20), suggesting that reglucosylation plays an important role in the ER quality control system. Indeed, it has been proposed that glucosyltransferase functions as a major sensor for incompletely folded proteins in the ER and that glycosylation of nascent proteins stops upon adoption of the correct conformation (16, 17, 19).

Studies using cell-free assays have determined that the size of the glycan chain, e.g. the Man core, is a critical factor in their modification by glucosyltransferase enzymes; glycoproteins containing Manα-GlcNAc2 glycans are reglucosylated much more efficiently than those having Manβ-GlcNAc2 glycans, which are poorly recognized by glucosyltransferase (16). In contrast, although Man core size is important for interaction of oligosaccharide chains with calnexin (which associates preferentially with GlcManGlcNAc2 glycans), it is not crucial, as calnexin also binds smaller GlcManGlcNAc2 species with significant efficiency (12). Spiro and colleagues (10) recently reported similar findings regarding calreticulin association with glycoproteins. Like calnexin and calreticulin chaperones, glucosyltransferase is believed to interact with both glycan and protein domains on incompletely folded proteins; this interaction, interestingly, involves recognition of hydrophobic stretches on the polypeptide backbone and the innermost GlcNAc residue on the oligosaccharide chain (19). Hebert et al. (15) recently provided evidence that viral hemagglutinin glycoproteins synthesized in cell-free systems are processed by glucosidase I and II and glucosyltransferase enzymes and interact with calnexin. However, because no inhibitors of glucosyltransferase currently exist, the relative importance of the reglucosylation pathway for calnexin assembly remains to be determined. Indeed, it has never been demonstrated that reglucosylated proteins associate with lectin-like chaperones in

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This work is dedicated to Flora Ann Kirby Casteel.
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The abbreviations used are: ER, endoplasmic reticulum; TCR, T cell receptor; Ab, antibody; mAb, monoclonal antibody; Chx, cycloheximide; Cas, castanospermine; Man, mannosamine; PAGE, polyacrylamide gel electrophoresis; NPHGE, nonequilibrium pH gradient gel electrophoresis.
intact cells of any type (21).

Glc trimming and calnexin association are initial molecular events in the translation of T cell antigen receptor (TCR) proteins, occurring coincident with or immediately after their translocation into the ER and preceding their assembly into multisubunit protein complexes (14, 22). Clonotypic TCRα and TCRβ polypeptides are members of the immunoglobulin gene superfamily and share several structural features, including conserved cysteine residues and post-translational modification by a similar number of N-linked glycan chains (6, 23). Interestingly, oligosaccharide processing and calnexin association is critical for stabilizing nascent TCRα proteins within the ER, but is not required for the stability of analogous TCRβ proteins (6). This apparent paradox may relate to the suggestion that, unlike the TCRβ transmembrane domain, the transmembrane region of TCRα does not function efficiently as a stop transfer region within the lipid bilayer of the ER and may rely on calnexin association for membrane stabilization (6, 24).

To further our understanding of the role of glucose processing and chaperone association in the quality control system of the ER, we studied the reglucosylation of TCRα/β proteins and their assembly with calnexin in BW thymoma cells. These data demonstrate that TCRα/β proteins undergo cycles of deglucosylation and reglucosylation in the ER and show that numerous reglucosylated proteins assemble with calnexin in intact cells, including TCRα/β proteins. Finally, the results in the current study indicate that reglucosylation is critical for calnexin assembly with TCRα proteins but not TCRβ proteins.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—BW parental cells, BWPHAR2.7 cells (25), and BWE cells (26) were maintained by weekly passage in RPMI 1640 medium containing 5% fetal calf serum at 37°C in 5% CO2. BWPHAR2.7 cells and BWE cells were generously provided by Drs. Rosalind Kornfeld and Carolyn M. Knoll (Washington University, St. Louis, MO) and Drs. Stuart Kornfeld and Carolyn M. Knoll (Washington University, St. Louis, MO), respectively. Castanospermine (Cas) and mannansamine (Msn) were purchased from Calbiochem and Sigma, respectively.

**Metabolic Labeling and Mannansamine/Castanospermine Treatment**—Metabolic pulse-labeling of BW cells with [35S]methionine was performed as described previously (22). Briefly, cells were incubated in methionine-free RPMI 1640 medium (Biofluids, Rockville, MD) containing 10% fetal calf serum and 1 mCi/ml [35S]methionine (Trans[35S]-label, ICN, Irvine, CA) for 30 min at 37°C in 5% CO2. For [3H]glucose labeling, cells were incubated in Glc-free RPMI 1640 medium (Life Technologies, Inc.) containing 10% dialyzed fetal calf serum, 5 mM sodium pyruvate (Life Technologies, Inc.), and 0.5 mM [6-3H]glucose (ICN) for 15–30 min at 37°C in 5% CO2. Where indicated, [3H]glucose labeling was performed in the presence of cycloheximide (Chx; Sigma). For these studies, cells were incubated in RPMI 1640 medium containing 5% fetal calf serum and 1 mM Chx for 2 min at 37°C in 5% CO2, centrifuged, and resuspended in Glc-free medium containing [3H]glucose and 1 mM Chx; radiolabeling was then performed as described above. Effectiveness of Chx treatment in blocking new protein synthesis was verified by parallel experiments using [35S]methionine. In experiments using Msn, cells were cultured overnight in medium containing 10 mM Msn at 37°C in 5% CO2; cell viability was identical in medium- and Msn-treated cell cultures (data not shown). In experiments using both Man and Cas, Cas was included (100 μg/ml final concentration) during the final 3 h of culture.

**Cell Lysis and Immunoprecipitation**—Cells were solubilized in 1% Nonidet P-40 (Calbiochem) lysis buffer (20 mM Tris, 300 mM NaCl, plus protease inhibitors) or 1% digitonin (Wako, Kyoto, Japan) lysis buffer (20 mM Tris, 150 mM NaCl, plus protease inhibitors) at 1 x 10⁵ cells/ml for 20 min at 4°C. Cell lysates were clarified by centrifugation to remove insoluble material and immunoprecipitated with the appropriate antibodies pre-absorbed to protein A-Sepharose beads; sequential immunoprecipitation and immunoprecipitation/release/recapture techniques were performed as described previously (22). The following monoclonal antibodies (mAb) were used in this study: H28–710, specific for TCRα (27), and H57–597, specific for TCRβ (28); the following antiserum were used: SPA-860 anti-calnexin Ab (Stressgen Biotechnologies Corp., Victoria, BC, Canada), anti-calreticulin Ab (Affinity Bioreagents Inc., Neshanic Station, NJ), and anti-glucosyltransferase Abs directed against both native and denatured glucosyltransferase (kindly provided by Dr. Armando Parodi, Buenos Aires, Argentina).

**Gel Electrophoresis and Immunoblotting**—One- and two-dimensional SDS-PAGE gel electrophoresis were performed as described previously (29). Gels containing [3H]glucose-labeled material were processed for autoradiography using dimethyl sulfoxide (Sigma), saturated with 2,5-diphenyloxazole (Aldrich). Gels containing [3H]glucosylated material were processed for autoradiography using ENHANCE (DuPont). Immunoblotting experiments were performed as described previously (29).

**RESULTS**

**Deglucosylation and Reglucosylation of TCRα/β Proteins in BW Cells**—To study reglucosylation of TCRα/β proteins in BW cells, [3H]galactose was utilized as a radioactive tracer of Glc residues on oligosaccharide chains (30, 31). As illustrated in Fig. 1, exogenous [3H]galactose internalized by cells can radio-labeled glycoproteins containing N-linked oligosaccharides via three major pathways: (i) conversion into UDP-[3H]glactose, the sugar donor for galactosyltransferases enzymes which transfer galactose residues to mature, complex type oligosaccharides in the trans-Golgi; (ii) epimerization of UDP-[3H]galactose to UDP-[3H]glucose, the sugar donor for UDP-glucose:glycoprotein-dependent glucosyltransferase enzymes that transfer Glc residues to high mannose glycans on incompletely folded glycoproteins in the ER; and (iii) conversion of UDP-[3H]glucose into dolicholphospho-[3H]glucose, which is incorporated into nascent Glc₃Man₉GlcNAc₂ glycans that are cotranslationally added to newly synthesized polypeptides in...
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Fig. 2. Reglucosylation of TCRα/β proteins in BW cells. BW cells were cultured in the presence of medium or Cas (100 μg/ml) for 1 h at 37 °C and radiolabeled with [35S]methionine or [3H]galactose for 30 min at 37 °C. Where indicated, cells were pretreated with 1 mM Chx for 3 min prior to radiolabeling; the presence of Chx and/or Cas was maintained during the radiolabeling procedure. Cells were solubilized in 1% Nonidet P-40 and lysates immunoprecipitated with anti-TCRβ mAb; precipitates were analyzed on SDS-PAGE gels under reducing conditions. Effectiveness of Chx treatment in inhibiting de novo protein synthesis was verified by parallel [35S]methionine-labeling experiments, which showed that >99% of protein synthesis in BW cells was inhibited under these conditions (data not shown). The position of TCRβ proteins is indicated.

![Image](83x634 to 273x729)

Fig. 3. Reglucosylated glycans do not persist on TCRα/β proteins in BW cells. A, BW cells were radiolabeled with [3H]galactose for 30 min at 37 °C and chased in medium without radiolabel at 37 °C for the time period indicated in the absence or presence of Cas. Note that the presence of Chx (1 mM) was maintained throughout the entire experiment and that Cas was present only during the chase period. Cells were solubilized in 1% Nonidet P-40 and lysates sequentially immunoprecipitated with anti-TCRβ mAb, followed by anti-TCRα mAb; precipitates were analyzed as in Fig. 2A. B, BW cells were radiolabeled with [3H]galactose for 15 min at 37 °C, chased in medium without radiolabel for 15 and 30 min at 37 °C, and at the conclusion of the 30-min chase period, labeled a second time with [3H]galactose for 15 min at 37 °C; the presence of Chx (1 mM) was maintained throughout the experiment. TCRα and TCRβ proteins were immunoprecipitated from lysates and analyzed as in A. Efficacy of Chx treatment was verified by parallel [35S]methionine-labeling experiments (data not shown).

![Image](313x448 to 558x729)

The ER (Fig. 1) (1, 32, 33). In our studies, radiolabeling of TCRα/β proteins with [3H]galactose was restricted to newly translated and pre-existent proteins localized within the ER, since TCRα/β glycoproteins made in BW cells are retained within the ER as unassembled chains (6).

Initially, BW cells were cultured in medium or the glucosidase inhibitor Cas (34) and radiolabeled with [35S]methionine or [3H]galactose. Cells were solubilized in Nonidet P-40 and lysates immunoprecipitated with anti-TCRβ mAb. As expected, the vast majority of TCRβ proteins synthesized in Cas-treated [35S]methionine-labeled groups showed decreased migration on SDS-PAGE gels relative to TCRβ proteins in medium-treated groups (Fig. 2, compare lanes 1 and 2), due to blockade of co-translational removal of Glc residues from nascent glycan chains. TCRβ proteins in medium-treated [3H]galactose-labeled groups showed mobility similar to that of TCRβ proteins in medium-treated [35S]methionine-labeled groups (Fig. 2, compare lanes 1 and 3); interestingly, however, two TCRβ species were visible in Cas-treated groups of [3H]galactose-labeled cells: an upper band corresponding to newly synthesized TCRβ proteins containing untrimmed glycan chains (Fig. 2, lane 4), analogous to the species observed in [35S]methionine-labeled Cas groups (Fig. 2, lane 2), and a lower band with mobility similar to that of TCRβ proteins in medium-treated groups (Fig. 2, compare lanes 3 and 4), which was not observed in [35S]methionine-labeled Cas groups (Fig. 2, lane 2). We reasoned that the lower TCRβ band in [3H]galactose-labeled cells represented pre-existent TCRβ proteins that had undergone Glc trimming prior to the addition of Cas, but contained radioactive Glc residues added via the reglucosylation pathway. To confirm this idea, [3H]galactose-labeling experiments were performed in the presence of the de novo protein synthesis inhibitor Chx. Chx treatment effectively inhibited >99% of de novo protein synthesis in BW cells as determined by parallel [35S]methionine-labeling experiments (data not shown). Radiolabeling of TCRβ proteins with [3H]galactose in BW groups without Cas was not significantly affected by Chx (Fig. 2, compare lanes 3 and 5), and importantly, the upper TCRβ species in Cas-treated [3H]galactose-labeled groups was completely sensitive to Chx, whereas the lower species was not (Fig. 2, compare lanes 4 and 6). These results verify that the upper and lower TCRβ species in [3H]galactose-labeled groups represent newly synthesized and pre-existent TCRβ proteins, respectively. Consistent with conversion of [3H]galactose into [3H]glucose, which is incorporated into glycan chains on pre-existent proteins within the ER, the radioactive signal on TCRβ glycoproteins in medium/Chx-treated groups (the species shown in Fig. 2, lane 5) was sensitive to digestion with endoglycosidase H, which cleaves immature glycan chains, but resistant to digestion with jack bean mannosidase (data not shown), specific for fully trimmed glycan species devoid of Glc residues (22).

Next, we evaluated if Glc residues added via the reglucosylation pathway persisted on nascent TCRα/β proteins in BW cells. BW cells were radiolabeled with [3H]galactose in the presence of Chx for 30 min and chased in nonradioactive medium containing Chx for various time periods in the presence or absence of Cas. Cells were solubilized in Nonidet P-40 and lysates sequentially immunoprecipitated with anti-TCRβ mAb, followed by anti-TCRα mAb, to capture TCRβ and TCRα proteins in BW lysates, respectively, which exist in this cell type as unassembled, free proteins due to low synthesis of CD3 chains (6). As shown in Fig. 3A, the relative amounts of radioactively labeled TCRα/β proteins visualized in chase groups was significantly decreased relative to pulse groups (Fig. 3A, lanes 1–4), indicative of removal of radioactively labeled glycans from TCRα/β proteins during the chase period by glucosidase enzymes. Indeed, inclusion of Cas during the chase period prevented the disappearance of radioactively labeled TCRα/β proteins (Fig. 3A, compare lanes 2–4 and 5–7). These results further support our conclusion that
Intermediate in the terminal stages of glycan biosynthesis (1, duetodeficientformation of dolichol-P-mannose (26, 36), a key element [35S]methionine labeling experiments; data not shown). Protein synthesis was completely abrogated (verified by parallel [35S]methionine labeling experiments; data not shown). These results show that TCR/β glycoproteins undergo multiple cycles of deglucosylation and reglucosylation in the ER, a finding that is consistent with current models of protein folding and quality control (15). The molecular basis for differential radiolabeling of TCRα and TCRβ proteins in secondary pulse periods remains to be determined, but does not result from differences in protein survival, as the half-lives of TCRα/β chains are quite comparable in BW cells (6), even during extended chase periods (data not shown); rather, these results most likely reflect accelerated folding of TCRα proteins relative to TCRβ proteins in BW cells.

Assembly of Reglucosylated TCRα/β Proteins with Calnexin—To determine if reglucosylated TCRα/β proteins were associated with calnexin and calreticulin, digitonin lysates of [3H]galactose-labeled BW cells were precipitated with anti-calnexin and anti-calreticulin Abs and analyzed on SDS-PAGE gels. As shown in Fig. 4A, numerous reglucosylated proteins were associated with calnexin in BW lysates (Fig. 4A, lane 1), including proteins that comigrated with clonotypic TCRα/β proteins (Fig. 4A, lanes 3 and 4). The identity of reglucosylated TCR proteins in anti-calnexin precipitates was confirmed by analysis on two-dimensional NEPHGE/SDS-PAGE gels (Fig. 4B) and immunoprecipitation/release/recapture experiments and preclearing studies (data not shown). Interestingly, with the exception of an unknown 70-kDa protein, which coprecipitated equivalently with calnexin and calreticulin molecules in BW lysates (Fig. 4A, asterisk), markedly fewer reglucosylated proteins existed in anti-calreticulin precipitates compared to anti-calnexin precipitates (Fig. 4A, compare lanes 1 and 2). The significance of this finding is unclear but may reflect transient interaction of glycoproteins with calreticulin relative to calnexin, which we have recently observed regarding the association of nascent TCRα/β proteins with calnexin, calreticulin chaperones in BW cells (35). Importantly, these results demonstrate that numerous reglucosylated proteins associate with calnexin in BW cells, including TCRα/β proteins.

Synthesis and Processing of TCRα/β Proteins Containing Truncated Glycan Chains—It has been shown that efficiency of reglucosylation is a function of glycan chain length (Man core size) (16). Therefore, we evaluated processing of TCRα/β proteins in BW cell types that synthesize truncated glycan chains. For these studies, mutant BW cells were utilized, BWE, that synthesize glycoproteins with short Glc3Man5GlcNAc2 glycans due to deficient formation of dolichol-P-mannose (26, 36), a key intermediate in the terminal stages of glycan biosynthesis (1, 34). In addition, parental BW cells were treated with Msn, a chain terminator of glycan elongation resulting in transfer of truncated Glc3Man5GlcNAc2 glycans to glycoproteins (37, 38).

BW and BWE cells were cultured in medium or Msn and where indicated, the glucosidase inhibitor Cas was included in cultures to inhibit removal of Glc residues from nascent glycan.
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chains. As shown in Fig. 5A, mobility of TCRα/β proteins synthesized in Msn-treated BW cells and untreated BWE cells was increased relative to TCRα/β proteins made in untreated BW cells (Fig. 5A, compare lanes 1, 3, and 5) and migration of TCRα/β proteins from Msn-treated BW cells was slightly retarded compared to TCRα/β proteins from untreated BWE cells (Fig. 5A, compare lanes 3 and 5); migration of TCRα/β proteins in BWE cells was relatively unaffected by Msn treatment, as expected (Fig. 5A, compare lanes 5 and 7). TCRα/β proteins in Cas groups migrated with decreased mobility relative to their respective control groups without Cas (Fig. 5A, compare even and odd numbered lanes), indicating that TCRα/β proteins containing truncated glycan chains are processed by Glc trimming enzymes. The Glc trimming status of truncated glycan chains was also compared in parental BW cells and glucosidase II-deficient BWPHAR2.7 cells (6, 25); TCRα/β proteins synthesized in Msn-treated parent BW cells migrated with increased mobility relative to those made in Msn-treated BWPHAR2.7 cells (Fig. 5B, compare lanes 3 and 4), indicating that TCRα/β proteins having truncated glycans are effectively processed to at least the monoglycosylated stage. Taken together, these data are consistent with the synthesis of TCRα/β proteins containing Man₉ core glycans in untreated BW cells, Man₅–₇ core glycans in Msn-treated BW cells, and Man₉ core glycans in both untreated and Msn-treated BWE cells; the data show that TCRα/β proteins having truncated glycans are effectively processed by the Glc trimming pathway.

Reglucosylation of TCRα/β proteins containing truncated glycan chains was studied in [3H]galactose labeling experiments performed in the presence of cycloheximide. As shown in Fig. 6A, reglucosylation of TCRα/β proteins was markedly decreased in mutant BW cells relative to parental BW cells (Fig. 6A), which did not result from decreased glucosyltransferase expression as determined by immunoblotting (Fig. 6B). Similar results were obtained regarding reglucosylation of TCRα/β proteins and glucosyltransferase expression in medium- versus Msn-treated parental BW cells (data not shown). These data demonstrate that reglucosylation of TCRα/β proteins having truncated glycans is severely impaired relative to those having standard length glycan chains.

Association of TCRα/β Proteins Containing Truncated Glycan Chains with Calnexin—Having determined that TCRα/β proteins containing truncated glycan chains are effectively processed by the Glc trimming pathway but not the reglucosylation pathway, we next examined their assembly with calnexin in [35S]methionine-labeling experiments. As shown in Fig. 7A, numerous cellular proteins were assembled with calnexin in both BW and BWE cells, including TCRα/β proteins (Fig. 7A, top panels); interestingly, however, calnexin assembly with nascent TCRα proteins was markedly reduced in BWE cells compared to parental BW cells (Fig. 7A, top panels), despite the abundant presence of TCRα proteins in BWE lysates (Fig. 7A, bottom panels). Decreased assembly of TCRα proteins with calnexin in mutant BWE cells versus parental BW cells was confirmed by immunoprecipitation/release/recapture studies (Fig. 7B). Assembly of nascent proteins with calnexin in BWE cells was dependent on Glc trimming as it was inhibited by pretreatment with Cas (data not shown). Similar to what was

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**Fig. 5.** Synthesis and processing of TCRα/β proteins containing truncated glycan chains. A, BW and BWE cells were cultured in medium or 10 mM Msn for 10–12 h at 37°C in 5% CO₂ and radiolabeled with [35S]methionine for 30 min. Where indicated, castanospermine was included in the last 3 h of culture. The presence of Msn and/or Cas was maintained during the radiolabeling period. Cells were solubilized in 1% Nonidet P-40 and lysates sequentially immunoprecipitated with anti-TCRβ mAb, followed by anti-TCRα mAb; precipitates were analyzed on SDS-PAGE gels under reducing conditions. The positions of TCRα/β proteins are indicated. The proteins migrating slightly above TCRα/β proteins in BW cells treated with Msn and BWE cells are unknown, but do not represent glycoproteins, as indicated by their insensitivity to glycosidase digestion (data not shown). B, parental BW cells and glucosidase II-deficient BWPHAR2.7 cells were cultured in medium or 10 mM Msn for 10–12 h at 37°C in 5% CO₂, and radiolabeled with [35S]methionine for 30 min; the presence of Msn was maintained during the radiolabeling period. TCRα and TCRβ proteins were immunoprecipitated from lysates and analyzed as in A. Note that TCRα/β proteins made in BWPHAR2.7 cells migrate with decreased mobility relative to TCRα/β proteins synthesized in parental BW cells due to persistence of diglucosylated glycans on TCRα/β polypeptides. Lanes 1 and 3 contain untreated parent BW cells and Msn-treated parent BW cells, respectively; lanes 2 and 4 contain untreated BWPHAR2.7 cells and Msn-treated BWPHAR2.7 cells, respectively.
observed in BWE cells, nascent TCRβ proteins, but not TCRα proteins, were efficiently assembled with calnexin in Msn-treated BW cells (Fig. 8A), which was verified by immunoblotting of anti-TCRα and anti-TCRβ precipitates with anti-calnexin Ab (Fig. 8B). Taken together, these results demonstrate that Glc trimming is sufficient for calnexin assembly with TCRβ proteins, and numerous other unidentified cellular proteins, but that both deglucosylation and reglucosylation are required for effective formation of calnexin-TCRα protein complexes.

Stability of TCRα/β Proteins Containing Truncated Glycan Chains—Finally, the stability of newly synthesized TCRα/β proteins containing truncated glycan chains was examined in [35S]methionine-labeling experiments. As expected, the vast majority of both TCRα and TCRβ proteins synthesized during a 30-min pulse in untreated parent BW cells were stable during a 30-min chase period (Fig. 9). In contrast, TCRα proteins made in Msn-treated BW cells showed relatively limited survival (Fig. 9), whereas stability of TCRβ proteins was unaffected by Msn treatment (Fig. 9). Similar results were observed in untreated BWE cells in that TCRβ proteins, but not TCRα proteins, were inherently unstable (Fig. 9). Consistent with these findings, significantly fewer TCRα proteins existed in lysates of untreated BWE cells compared to lysates of untreated BW cells as determined by immunoblotting with anti-TCRα mAb (data not shown). These data show that TCRα proteins containing truncated glycans have limited survival within the ER compared to TCRα proteins having standard length glycan chains, and that stability of TCRβ proteins is unaffected by glycan core size.

DISCUSSION

In the current report we examined the deglucosylation and reglucosylation of TCRα/β proteins and their assembly with calnexin in BW thymoma cells. The results in this study show that reglucosylated TCRα/β proteins and other unidentified reglucosylated proteins associate with calnexin in vivo, providing the first evidence that reglucosylated proteins interact with calnexin chaperones in intact cells of any type (15, 21). Interestingly, we found that significantly fewer reglucosylated proteins were associated with calreticulin compared to calnexin, which might reflect transient interactions of nascent glycoproteins with calreticulin relative to calnexin (8). In support of this idea, we find that association of TCRα/β proteins with calreticulin is significantly transitory relative to their assembly with calnexin (35). However, we have also considered the possibility that calreticulin may preferentially associate with glycoproteins bearing monoglucosylated glycans generated via the Glc trimming pathway, which raises the interesting suggestion that proteins containing monoglucosylated species created by reglucosylation may not be conformationally identical to those derived via initial deglucosylation of nascent glycans.

The data in the current study are of important significance regarding the role of glucosidase enzymes in removal of Glc residues from reglucosylated proteins, as it has been proposed that endomannosidase enzymes localized within the intermediate compartment between the trans ER and the cis Golgi might also perform this function, providing a distal, more stringent level of quality control (10, 39). Our finding that removal of Glc residues from reglucosylated TCRα/β proteins was effectively blocked by castanospermine, an inhibitor of glucosidase I and glucosidase II enzymes (34), but not endomannosidase activity (40, 41), indicates that glucosidase II is responsible for deglucosylation of the vast majority of reglucosylated TCRα/β proteins in BW thymoma cells.

In agreement with current models of protein folding and quality control, our data indicate that TCRα/β proteins undergo multiple cycles of Glc removal and addition. Interestingly, we found that comparable amounts of TCRβ proteins were reglucosylated in consecutive labeling experiments, suggesting that no significant improvement in the folding status of TCRβ proteins had occurred during a 30-min lag period. Regarding this issue, it is noteworthy to mention that similar amounts of reglucosylated TCRβ proteins were associated with calnexin in consecutive labeling experiments in BW cells (data not shown), raising the consideration as to whether calnexin assembly with reglucosylated TCRβ glycoproteins actually promotes their folding into the correct conformation, or if calnexin functions solely to retain incompletely folded glycoproteins in the ER.

Truncated Glc3Man1GlcNAc2 glycan chains are generated via the alternative pathway of glycoprotein biosynthesis, which is utilized by many cell types following glucose starvation or ATP depletion (42–44). Glucosylated Man9,GlcNAc2 glycans are effectively transferred to proteins in the ER and can be processed to complex-type oligosaccharides in the Golgi (26, 45). The current study shows that numerous molecules interact with calnexin in cell types synthesizing proteins with truncated glycans, providing evidence that quality control mechanisms that regulate egress of properly folded proteins from the ER to the Golgi complex are sustained under conditions where truncated glycan chains are added to newly synthesized proteins. These data are in agreement with recent findings by Spiro and co-workers, demonstrating that smaller size Glc3Man9,GlcNAc2 glycans effectively interact with calreticulin in cell-free assays (10). The present study shows that reglucosylation of TCRα/β glycoproteins is markedly decreased in BW cell types synthesizing truncated Manα5–7 core glycans compared to BW cells making typical Manα7 core glycans. While decreased reglucosylation of TCRα proteins is confounded by the differential stability of TCRα proteins under these conditions, decreased re-
glucosylation of TCRβ chains in BW cells making truncated glycans must result from differences in the recognition of TCRβ proteins by glucosyltransferase enzymes in these cell types, as the stability of TCRβ proteins is unaffected by glycan chain length. Indeed, our findings are in clear agreement with previously published results, showing that glycopeptides containing truncated glycans are extremely poor substrates for glucosyltransferase in vitro (16).

Our findings that reglucosylated TCRβ proteins were assembled with calnexin even though reglucosylation was not necessary for formation of calnexin-TCRβ protein complexes suggest that two populations of TCRβ proteins exist within the ER of BW cells: TCRβ proteins that effectively assemble with calnexin immediately after Glc trimming and those that require...
Reglucosylation of N-Linked Glycans in TCRα Calnexin Assembly

**Fig. 9. Stability of TCRαβ proteins containing truncated glycans.** BW and BWE cells were cultured in medium (med) or MnS as indicated, pulse-labeled with [35S]methionine for 30 min, and chased in medium containing excess unlabeled methionine for 30 min. The protein that is more pronounced in BWE-medium groups relative to others is unknown but is not a glycoprotein as determined by glycosidase digestion studies (data not shown).

multiple rounds of deglucosylation and reglucosylation for chaperone interaction. Interaction of TCRαβ proteins with calnexin was not significantly decreased under conditions of impaired reglucosylation, however, indicating that most TCRαβ proteins associate with calnexin following initial removal of Glc residues from nascent glycan chains. We would also note that our data are consistent with the idea that TCRαβ proteins are reglucosylated while assembled into calnexin-TCRαβ protein complexes. We think this is possible because oligosaccharides are not required to maintain calnexin-protein interactions and are readily accessible to macromolecular probes such as lectins and glycosidases (12, 13, 22, 46). Moreover, TCRαβ proteins contain multiple glycan chains, all of which are most likely not involved in calnexin binding. Experiments designed to explore these issues are currently in progress.

Finally, the current study demonstrates that survival of newly synthesized TCRα proteins in the ER is severely limited under conditions where reglucosylation is severely impaired. These findings importantly extend previous results that TCRα proteins containing full-length glycans are rapidly degraded under conditions where Glc residues persist on glycan chains and calnexin association is impaired (6), by showing that TCRα proteins are specifically degraded under conditions where numerous cellular proteins, including TCRαβ proteins, effectively assemble with calnexin. Impaired calnexin assembly with TCR proteins containing truncated glycans might also be explained by the failure of glucosidase enzymes to remove Glc residues from nascent oligosaccharides, as glucosidase I and II enzymes have been shown to deglucosylate Glc1–2Manα–2GlcNAc2 glycans less efficiently than Glc1–3Manα–2GlcNAc2 glycans in vitro (47). However, as noted in the current report, Glc trimming of TCRαβ proteins to monoglucosylated species was not significantly impaired in BW cell types synthesizing truncated glycans. Whether or not removal of the final, innermost Glc residue occurs more slowly on TCRαβ proteins containing truncated glycans than those having standard size glycans is unknown; however, we would point that this would favor the opportunity for calnexin binding, not decrease it (12). The ineffective of the TCRαβ transmembrane domain to function as a stop transfer region (24) may relate to the reason why TCRαβ proteins containing truncated glycan chains do not effectively associate with calnexin as TCRαβ proteins might require numerous rounds of deglucosylation and reglucosylation to stably assemble with calnexin in the lipid bilayer of the ER. Alternatively, reglucosylation of an unknown accessory protein may be necessary for stable interaction of TCRαβ proteins with calnexin molecules. These issues are currently under investi-