Modification of the T Cell Antigen Receptor (TCR) Complex by UDP-glucose:Glycoprotein Glucosyltransferase

TCR FOLDING IS FINALIZED CONVERGENT WITH FORMATION OF αβδεγζ COMPLEXES

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Most T lymphocytes express on their surfaces a multisubunit receptor complex, the T cell antigen receptor (TCR) containing α, β, γ, δ, ε, and ζ molecules, that has been widely studied as a model system for protein quality control. Although the parameters of TCR assembly are relatively well established, little information exists regarding the stage(s) of TCR oligomerization where folding of TCR proteins is completed. Here we evaluated the modification of TCR glycoproteins by the endoplasmic reticulum folding sensor enzyme UDP-glucosyl:glycoprotein glucosyltransferase (GT) as a unique and sensitive indicator of how TCR subunits assembled into multisubunit complexes are perceived by the endoplasmic reticulum quality control system. These results demonstrate that all TCR subunits containing N-glycans were modified by GT and that TCR proteins were differentially reglucosylated during their assembly with partner TCR chains. Importantly, these data show that GT modification of most TCR subunits persisted until assembly of CD3δβ chains and formation of CD3-associated, disulfide-linked αβ heterodimers. These studies provide a novel evaluation of the folding status of TCR glycoproteins during their assembly into multisubunit complexes and are consistent with the concept that TCR folding is finalized convergent with formation of αβδεγζ complexes.

The antigen receptor expressed on most T lymphocytes is the multisubunit αβ T cell receptor complex (TCR),1 important for recognition of major histocompatibility complex molecules containing bound peptides (1). The αβ/TCR is composed of six distinct proteins: clonotypic TCRα and β-molecules and invariant CD3γ, δ, ε, and ζ chains (1). TCR assembly is initiated in the endoplasmic reticulum (ER) and occurs via the ordered pairing of: (i) CD3γ, δ, ε, and ζ chains into partial complexes of δε and γε components; (ii) association of clonotypic proteins with CD3 chains to form αδε and βγε intermediate complexes; (iii) joining of αδε and βγε molecules to create incomplete αβδεγζ complexes, within which disulfide linkage of α and β chains occurs; and finally, (iv) addition of ζ homodimers to form complete αβδεγζζ complexes (2, 3). In most T cell types, intracellular transport and expression of TCR proteins is tightly regulated by their assembly status. Unassembled and partially assembled TCR proteins are retained within the ER and disposed of by poorly understood mechanisms involving retrograde transport to the cytosol and degradation by proteasomes (4–6). Incomplete (αβδεγζζ) and complete (αβδεγζζζζ) TCR complexes egress from the ER to the Golgi; however, incomplete TCR complexes are sorted to lysosomes where they are degraded. Only complete TCR complexes efficiently traffic to the cell surface (1).

Four TCR subunits are post-translationally modified by addition of oligosaccharides TCRα (3 N-glycans), TCRβ (4 N-glycans), CD3δ (3 N-glycans), and CD3γ (1 N-glycan) (1). N-Glycan chains on newly translated proteins have the structure Glc3Man9GlcNAc2 and are sequentially processed by glucosidase I and II enzymes to form monoglucosylated Glc3Man9GlcNAc2 species, important for interaction with the endogenous lectins calnexin and calreticulin that function in the quality control system of protein folding (7–10); the final, innermost Glc residue is removed by glucosidase II (gII) before or after chaperone disassembly. Fully trimmed (Glcα) proteins that persist in a malformed state are modified by UDP-glucose:glycoprotein glucosyltransferase (GT), which transfers a single Glc residue, (re)creating monoglucosylated (Glcα) species that can (re)enter the calnexin, calreticulin assembly pathway (9). GT is proposed to be a major sensor of protein folding in the ER (11–13) and will only add back Glc residues removed by gII if a glycoprotein has not yet acquired its proper tertiary structure (14). The deglucosylation/reglucosylation cycle continues until correct conformation is achieved (9, 14).

GT modification of incompletely folded proteins involves interaction with both polypeptide and glycan determinants, including recognition of hydrophobic amino acids and interestingly, the innermost GlcNAc residue of the glycan chain (the site of attachment of oligosaccharide to protein) (15). Both recognition elements must be covalently linked to effectively catalyze Glc transfer (15) and accessible to GT modification, which for certain glycoproteins may be concealed by molecular chaperone association in vivo, particularly under conditions of extreme ER stress (14). The size of the glycan chain, e.g. the oligomannose core, is also important for the efficiency of reglucosylation; Mannα-GlcNAc2 glycans are reglucosylated much more efficiently than shorter Mannβ-GlcNAc2 glycans (12, 16). As recently demonstrated in mutant BW cell types synthesizing truncated GlcαMan9GlcNAc2 N-glycans, TCRα molecules having shortened oligosaccharides were reglucosylated much less efficiently than TCRα molecules having normal size glycans, which was correlated with TCRα instability (16).

To evaluate the folding status of TCR glycoproteins as a function of their assembly into multisubunit complexes in the ER, we studied the GT modification of TCR proteins in 2B4 T

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1 The abbreviations used are: TCR, T cell antigen receptor; ER, endoplasmic reticulum; GT, UDP-glucose:glycoprotein glucosyltransferase; mAb, monoclonal antibody; dmj, deoxymannojirimycin; chx, cycloheximide; PAGE, polyacrylamide gel electrophoresis; EH, endoglycosidase H.
hybridoma cells. These studies show that all TCR subunits bearing N-glycan chains were modified by GT and that TCR proteins were differentially reglucosylated during their assembly into multisubunit complexes. Furthermore, these data demonstrate that reglucosylation of most TCR subunits was extinguished following CD3αβ assembly and formation of CD3-associated disulfide-linked αβ heterodimers, indicating that TCR folding is finalized convergent with formation of αββεγε complexes.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—2B4 T hybridoma cells and the TCR-deficient 2B4 variant 21.2.2 were maintained by weekly passage in RPMI 1640 medium containing 5% fetal calf serum at 37 °C in 5% CO₂ (17–19). The following monoclonal antibodies (mAb) were used in this study: H57-597, specific for TCRγδ proteins (20); A2B4 specific for 2B4 TCRα proteins (18); 145-2C11 specific for CD3ε; and 2C11, which recognizes both murine CD3y and CD3δ proteins (22); the following antiserum was used: R9, specific for CD3ε molecules (23). Deoxymannojirimycin (dmj) was purchased from Roche Molecular Biochemicals and was used at a final concentration of 75 μg/ml. [3H]Galactose Labeling and Biotinylation of Proteins—Metabolic pulse-labeling with [3H]galactose was performed as described previously (16). Briefly, cells were incubated in glucose-free RPMI 1640 medium (Life Technologies, Inc.) containing 10% dialyzed fetal calf serum, 5 mM sodium pyruvate (Life Technologies, Inc.), and 1 mM cycloheximide (chx) for 3 min at 37 °C in 5% CO₂; cells were centrifuged and resuspended in similar medium containing 0.5 mM [3H]galactose (ICN, Irvine, CA) and labeled for 15–45 min at 37 °C in 5% CO₂. Effectiveness of chx treatment in blocking new protein synthesis was verified by parallel experiments using [35S]methionine (data not shown). In experiments using dmj, cells were cultured overnight in medium containing 75 μg/ml dmj at 37 °C in 5% CO₂; cell viability was identical in medium- and dmj-treated cultures (data not shown). Biotinylation of cell surface proteins was performed as described previously (24).

**Cell Lysis, Immunoprecipitation, Gel Electrophoresis, and Immunoblotting**—Cells were solubilized in 1% digitonin (Wako, Kyoto, Japan) lysis buffer (20 mM Tris, 150 mM NaCl, plus protease inhibitors) at 1 × 10⁶ cells/ml for 20 min at 4 °C. Cell lysates were clarified by centrifugation to remove insoluble material and immunoprecipitated with the appropriate antibodies preabsorbed to protein A-Sepharose beads as described previously (16). Sequential immunoprecipitation, one- and two-dimensional SDS-PAGE gel electrophoresis, and immunoblotting were performed according to previously published methods (16, 25).

**RESULTS**

Recently we examined the reglucosylation of unassembled TCRα and -β proteins in BW thymoma cells using [3H]galactose as a radioactive tracer of Glc residues (16). Here we extended these studies in 2B4 T hybridoma cells to approximate at which stage(s) of TCR complex formation folding of individual TCR glycoproteins is completed, with the rationale that GT modification (reglucosylation) will cease upon attainment of proper conformation. As shown in Fig. 1, [3H]galactose may be incorporated into N-linked oligosaccharides on glycoproteins via three major pathways: (i) conversion into UDP-[3H]galactose, the sugar donor for galactosyltransferase enzymes that 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 GT that transfers Glc residues to high mannose glycans on incompletely folded glycoproteins in the ER; and (iii) conversion of UDP-[3H]glucose into dephospho-[3H]glucose, which is incorporated into nascent GlcMan,GlcNAc₄ glycans that are cotranslationally added to newly synthesized polypeptides in the ER (16, 26–28). In the current study, cycloheximide was included in all experiments to inhibit incorporation of [3H]galactose into newly translated proteins, thereby restricting radiolabeling to galactosylation and reglucosylation routes (Fig. 1) (16). 2B4 T hybridoma cells were used, which have served as a model cell type for TCR assembly in numerous studies (1).

**FIG. 1. Radiolabeling of glycoproteins with [3H]galactose.** Diagram illustrating the routes of incorporation of [3H]galactose into glycoproteins containing N-linked oligosaccharides and sensitivity of labeling to chx and dmj. +, incorporation via this route; −, no incorporation via this route. See text for details.

**TCR Subunits Bearing N-Glycans Are Substrates for GT**—As shown in Fig. 2A, multiple TCR subunits were detected in anti-CD3ε precipitates of [3H]galactose-labeled 2B4 T cells, including CD3δ and -γ glycoproteins and clonotypic TCRα and -β proteins (Fig. 2A); as expected, nonglycosylated CD3ε and TCRγδ molecules were not visualized (Fig. 2A). Because anti-CD3ε precipitates contain a mixture of TCR components at various stages of their assembly superimposed upon one another, sequential precipitation techniques were used to separate more completely assembled TCR proteins (capable of becoming galacosylated in the Golgi) from partially assembled and unassembled TCR subunits (retained in the ER) (25). As demonstrated, when supernatants from anti-CD3ε precipitates were sequentially precipitated with anti-TCRβ mAb, radiolabeled TCRβ proteins were detected (Fig. 2A), representing unassembled TCRβ proteins modified via the reglucosylation pathway. Consistent with incorporation of [3H]glucose into glycan chains on ER-localized TCRβ proteins, the radioactive signal on reglucosylated TCRβ proteins was sensitive to digestion with endoglycosidase H (EH), specific for immature oligosaccharides (data not shown). The vast majority of radiolabeled CD3γ glycoproteins associated with CD3ε were not simultaneously assembling with TCRβ but existed in partial complexes of CD3δε components (Fig. 2A, anti-TCRβ → anti-CD3ε precipitates, respectively), indicating that most radiolabeled CD3γ chains associated with CD3ε were modified by GT. More than half of the CD3ε-associated CD3δ chains were assembled with TCRβ (Fig. 2A), with remaining CD3δ proteins existing in partial δε complexes containing reglucosylated N-glycans; unassembled CD3δ glycoproteins were also modified by GT as shown by sequential immunoprecipitation of anti-CD3ε precipitates with anti-CD3δ Ab to capture "free," unassembled CD3δ chains (Fig. 2A). Reglucosylation of partially assembled and free CD3γ and -δ glycoproteins was verified in 21.2.2 cells (Fig. 2B), a TCRβ-deficient 2B4 variant that cannot assemble CD3 chains into a form capable of ER exit (29). Similar to our results in parental 2B4 cells, significantly more radiolabeled CD3γ proteins were associated with CD3ε than CD3δ proteins in 21.2.2 cells (Fig. 2B). In agreement with previous studies showing that nascent glycoproteins undergo multiple cycles of deglycosylation and reglucosylation in the ER (9, 16), Glc residues did not persist on CD3ε-associated γ proteins in 21.2.2 cells (Fig. 2C), and CD3γ proteins were effectively radiolabeled during a secondary pulse period with [3H]galactose (Fig. 2C). Taken together, these results demonstrate that glycosylated TCR subunits were substrates for GT, including invariant
sates of 2B4 T cells labeled with [3H]galactose for 30 min were sequen-
EH-digested TCR A
Fig. 3
cosylated (unassembled) TCR 32 and see below). Interestingly, increased amounts of reglu-
mature (galactosylated) glycans in the Golgi (30); thus, only
precludes conversion of immature, high mannose glycans to
molecules. In addition, these data show that both “free” and assembled CD3γ and
-δ molecules contained reglucosylated glycans.
Reglucosylation and Disulfide Linkage of Clonotypic TCRa and -β Proteins—To determine the contribution of reglucosyla-
tion in [3H]galactose radiolabeling of TCR proteins, studies were performed using the mannosidase inhibitor dmj, which
 prevents conversion of immature, high mannose glycans to mature (galactosylated) glycans in the Golgi (30); thus, only
reglucosylated glycoproteins are visualized in such experi-
ments (Fig. 1). As shown in Fig. 3A, markedly fewer radiola-
beled TCR proteins were present in anti-TCRa (A2B4) immu-
noprecipitates of dmj-treated cells relative to media-treated
cells with TCRa and -β chains being completely absent and only TCRa proteins detected (Fig. 3A). Consistent
with restriction of radiolabeling to immature N-glycans, TCRa
proteins in dmj lysates migrated with increased mobility com-
pared with TCRa proteins from control lysates (Fig. 3A) and
unlike control TCRa molecules, disappeared completely follow-
ing EH digestion (Fig. 3A). Note that increased mobility of
EH-digested TCRa-associated TCRa proteins in control lysates
results from the fact that several N-glycans on TCRa proteins
remain in the immature high mannose form, even on surface-
expressed molecules, which is also true for CD3δ proteins (31,
32 and see below). Interestingly, increased amounts of reglu-
cosylated (unassembled) TCRa proteins existed in dmj lysates
relative to control lysates (Fig. 3A), which was accompanied by
augmented survival of newly synthesized TCRa proteins.

These results were specific in that the half-life and reglucosyla-
tion of unassembled TCRa molecules were relatively unaf-
fected; similar results were observed in BW thymoma cells
(data not shown). Biochemical analysis of surface-labeled mole-
cules showed that the vast majority of TCR a glycoproteins
expressed on dmj-treated cells contained immature, EH-sensi-
tive oligosaccharides (Fig. 3B), demonstrating the effectiveness
of dmj in blocking maturation of N-oligosaccharides in these
studies and showing that dmj treatment did not perturb TCR assembly. Taken together, these results show that most radiola-
labeled TCRa and CD3γ and -δ glycoproteins associated with
2B4 TCRa represent galactosylated species and not reglucosy-
lated TCRa molecules. We conclude that TCRa proteins assem-
bled into TCRa and -β heterodimers are ineffectively modified
by GT (and thus no longer perceived by the ER quality control
system as incompletely folded) and relatedly, that reglucosyla-
tion of TCRa and CD3γ and -δ proteins is terminated following
their association with TCRa molecules. In addition, these data
show that inhibition of mannosidase activity resulted in en-
hanced reglucosylation of unassembled TCRa proteins.

Reglucosylated TCRa and -β Proteins Are Assembled with

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2 K. P. Kearse, unpublished observations.
**CD3 Subunits**—Assembly of TCRα and -β proteins into disulfide-linked heterodimers is preceded by the association of monomeric TCRα and TCRβ proteins with CD3 components in the form of αδε and βγε intermediates, which join to form αβδεγε complexes (3). Thus, we next wished to determine whether CD3-associated TCRα and -β proteins were modified by GT. As shown in Fig. 4, both monomeric and dimeric radiolabeled TCRα and -β proteins were detected in association with CD3 chains in lysates of [3H]galactose-labeled 2B4 T cells (Fig. 4). Because monomeric TCRαs and -β proteins are restricted to the ER (1, 3), these data indicate that reglucosylated (incompletely folded) TCRα and -β proteins exist in association with CD3 in 2B4 T cells, most likely as CD3α and CD3β intermediates because our previous results showed that few, if any, reglucosylated TCRβ proteins were associated with TCRα chains. To determine the contribution of GT modification to [3H]galactose radiolabeling of CD3-associated TCRα and -β proteins, dmj treatment was utilized to restrict radiolabeling to the reglucosylation pathway as before. In agreement with our previous findings that CD3γ and -δ glycoproteins assembled into partial δε, γε complexes were modified by GT, CD3γ and -δ chains were effectively labeled in dmj-treated cells (Fig. 5A). As noted earlier, reglucosylation of unassembled TCRβ chains, captured in sequential precipitates with anti-TCRβ mAb, was enhanced in dmj-treated cells relative to media-treated cells (Fig. 5A). Importantly, these data show that reduced amounts of TCRα and -β proteins were associated with CD3 chains in dmj-treated cells compared with media-treated cells (Fig. 5A), which was expected as our previous results showed that a significant portion of radiolabeled TCRα and -β proteins were assembled into disulfide-linked heterodimers modified by galactosylation. Analysis on two-dimensional nonreducing × reducing (NR × R) gels showed that relatively few radiolabeled TCRα and -β dimers were detected in anti-CD3 precipitates of dmj-treated cells (Fig. 5B), unlike CD3 chains, which were readily visible (Fig. 5B). Interestingly, radiolabeled TCRα proteins existed as both disulfide-linked and monomeric species whereas the vast majority of TCRβ molecules were present as non-disulfide-linked monomers (Fig. 5B). Formation of TCRα and -β heterodimers was not precluded in dmj-treated cells as shown by immunoblotting of CD3 precipitates with anti-TCRα mAb (Fig. 6); dimeric TCRβ proteins in media-treated groups existed as two species: an upper band representing mature (EH-resistant) proteins and a lower band migrating parallel with non-disulfide-linked TCRα monomers containing immature (EH-sensitive) glycan (Fig. 6). As demonstrated, only immature TCRα proteins were present in lysates of dmj-treated cells (Fig. 6). These results corroborate our previous findings that TCRβ subunits associated with TCRα proteins were ineffectively modified by GT and that reglucosylated (incompletely folded) CD3-associated monomeric TCRα and -β proteins exist in normal (untreated) cells. Taken together, these studies demonstrate that reglucosylation of most TCR components is extinguished following the CD3α and -β assembly and formation of disulfide-linked TCRα and -β heterodimers, indicating that TCR folding is finalized convergent with formation of αβδεγε complexes.

**DISCUSSION**

The current report has examined the modification of TCR glycoproteins by the ER folding sensor enzyme GT and provides the first example where GT modification of a multisubunit protein complex has been studied. The data in the current report significantly extend previous studies on TCR processing in splenic T lymphocytes, which showed that significant Glc trimming of newly synthesized CD3δ and TCRα glycoproteins takes place prior to association with partner TCR chains (33), based on their comigration with calnexin-associated glycoformas following digestion with jack bean α-mannosidase. Indeed, the current study utilizes a sensitive radiolabeling method which specifically identifies TCR subunits containing monoglucosylated N-glycans generated via the reglucosylation pathway. The current report establishes that all TCR subunits containing N-glycans are substrates for GT and evaluated reglucosylation as a function of TCR assembly, previously examined only on unassembled TCRα and -β proteins expressed in BW thymoma cells, which do not efficiently assemble TCR complexes due to deficient CD3δ synthesis (16).

The results in this study suggest a scheme in which reglucosylation of invariant CD3γ and -δ subunits persists until their association with clonotypic TCRα and -β chains, and GT modification of TCRβ proteins is terminated following the assembly of αβδεγε complexes and formation of disulfide-linked TCRα and -β heterodimers.

Interestingly, unlike CD3-associated TCRβ molecules, which were ineffectively modified by GT following disulfide linkage with TCRα proteins, reglucosylated TCRα molecules existed as both CD3-associated monomers and dimers. Although the exact significance of these findings remain to be determined, these data suggest that folding of TCRα may be one of the final steps of ER quality control that precedes TCR egress to the Golgi. It is conceivable that folding of the TCR complex occurs concomitantly with the ordered assembly of TCR subunits and that GT recognition motifs become progressively “masked” as
TCR oligomerization proceeds, similar to ER retention and lysosomal targeting information contained within the polypeptide sequences of certain TCR subunits (1, 34–36). Consistent with this idea, the results in the current study provide evidence that most TCR glycoproteins are no longer perceived by the ER quality control system as incompletely folded following the assembly of αβεγεγ TCR complexes, which, interestingly, is the stage at which TCR complexes become competent for ER exit. It is possible that reglucosylation of higher ordered TCR complexes ceases due to relocalization from the ER to the Golgi complex; however, we favor the idea that assembly, folding, and intracellular transport of TCR proteins are closely coupled events, similar to what has been observed for other multimeric immune protein complexes, i.e. major histocompatibility complex molecules (37–39). Indeed, previous studies have demonstrated that protein reglucosylation is not static but proceeds in a rapid, cyclic fashion in concert with Glc removal by glucosidase II enzymes (9, 16).

Interestingly, we found that reglucosylation of “free” TCRβ proteins was increased under conditions of manniosidase blockade, which was specific in that GT modification of TCRα proteins was relatively unaffected. Because the efficiency of GT modification is inversely correlated with asparagine-xylosylation (12, 16), and dmj inhibits the activity of certain ER mannosidase enzymes (40, 41), it is reasonable that increased reglucosylation of unassembled TCRβ proteins in dmj-treated cells results from persistence of Man residues on N-glycan chains. However, it was also noted that the stability of newly synthesized TCRβ molecules was enhanced under these conditions, similar to what has been described for CD3δ proteins by Weissman and colleagues (6). Thus, the relationship between increased reglucosylation and increased survival of TCR proteins under conditions of manniosidase blockade remains to be determined. Moreover, despite the fact that GT modification of certain TCR subunits was enhanced by prevention of Man removal, relatively few reglucosylated TCR proteins assembled into higher ordered TCR complexes were detected under these conditions, indicating that GT modification (folding) of TCR subunits is tightly regulated.

Finally, it is unknown to what extent specific N-glycans on TCR proteins containing multiple oligosaccharides may be differentially modified by GT enzymes. Recent studies by Dessen et al. (42) demonstrate that N-acetylglucosamine residues interact with neighboring amino acids of proteins in native conformations, which may be one of the major mechanisms by which GT modification of newly synthesized proteins is regulated (14, 43). The data in the current report suggest that determinants that signify malformed molecules may persist on TCRα proteins compared with other TCR subunits, an idea that is consistent with previous findings that TCRα survival is uniquely sensitive to perturbations in the ER quality control system (16, 27, 44). Identification of polypeptide and N-glycan domains important for GT recognition of TCR glycoproteins should provide valuable information regarding the molecular basis of GT modification and the regulation of quality control mechanisms that monitor the presence of unassembled and incompletely folded TCR proteins in the ER.

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