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A Novel Cysteine-rich Domain of Sep15 Mediates the Interaction with UDP-glucose:Glycoprotein Glucosyltransferase*[S]

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Selenium is an essential trace element with potent cancer prevention activity in mammals. The 15-kDa selenoprotein (Sep15) has been implicated in the chemopreventive effect of dietary selenium. Although the precise function of Sep15 remains elusive, Sep15 co-purifies with UDP-glucose:glycoprotein glucosyltransferase (GT), an essential regulator of quality control mechanisms within the endoplasmic reticulum. Recent studies identified two GT and two Sep15 homologues in mammals. We characterize interactions between these protein families in this report. Sep15 and GT form a tight 1:1 complex, and these interactions are conserved between mammals and fruit flies. In mammalian cells, Sep15 co-immunoprecipitates with both GT isozymes. In contrast, a Sep15 homologue, designated selenoprotein M (SelM), does not form a complex with GT. Sequence analysis of members of the Sep15 family identified a novel N-terminal cysteine-rich domain in Sep15 that is absent in SelM. This domain contains six conserved cysteine residues that form two C*xx*C motifs that do not coordinate metal ions. If this domain is deleted or the cysteines are mutated, Sep15 no longer forms a complex with GT. Conversely, if the cysteine-rich domain of Sep15 is fused to the N-terminus of SelM, the resulting chimera is capable of binding GT. These data indicate that the cysteine-rich domain of Sep15 exclusively mediates protein-protein interactions with GT.

Selenium is present in 25 human proteins in the form of selenocysteine, which is the 21st natural amino acid in the genetic code and is encoded by the UGA codon (1, 2). Recent studies provide strong evidence that dietary selenium supplements reduce the incidence of cancer in animal models and human clinical trials (3–5). Low molecular weight selenium compounds and selenium-containing proteins have been implicated in this chemopreventive effect. However, the mechanism by which selenium suppresses tumor development remains to be established.

One candidate protein that may mediate the chemopreventive effects of selenium is the 15-kDa selenoprotein (Sep15)[3] (6, 7). The Sep15 gene is located at position p31 on human chromosome 1. This region has been shown to undergo loss of heterozygosity, which is frequently associated with tumor progression, suggesting a potential tumor suppressor role for this selenoprotein (8, 9). In addition, two polymorphisms found at positions 811 (C/T) and 1125 (G/A), the latter of which is located within the selenocysteine insertion sequence element, have been identified within the 3′-untranslated region of the human Sep15 gene. The nucleotide identity at these polymorphic sites has been shown to influence the efficiency of selenocysteine incorporation in a selenium-dependent manner (10).

The precise biological function of Sep15 has not been firmly established; however, this selenoprotein co-purifies with the essential regulator of the calnexin cycle designated UDP-glucose:glycoprotein glucosyltransferase (GT) (11). The calnexin cycle is a quality control pathway localized to the endoplasmic reticulum that specifically assists in the folding of *N*-linked glycoproteins (12). Once a newly synthesized protein enters the endoplasmic reticulum, a glycan core of 14-oligosaccharides ($Glc_3Man_9GlcNAc_2$) is covalently linked to appropriate asparagine residues. *N*-Linked glycoproteins are immediately recognized by glucosidase I and II, the first components of the calnexin cycle, which sequentially trim the two outermost glucose moieties from the glycan core (13). Once processed, membrane-bound calnexin and its lumenal homologue calreticulin bind to the glycan (14–17). This triggers the binding of ERp57 (a lumenal protein disulfide isomerase) to calnexin and calreticulin and accelerates folding by catalyzing disulfide bond exchange (18–22). Regardless of the folded status of the bound glycoprotein, glucosidase II cleaves the remaining glucose residue and the complex disassociates, releasing the glycoprotein. If the glycoprotein is properly folded, it is rapidly exported from the endoplasmic reticulum by vesicular transport to the Golgi complex. Misfolded proteins are re-glucosylated by GT, a 175-kDa lumenal enzyme that catalyzes the transfer of a glucose moiety from UDP-glucose to the distal mannose of the glycan core (23). This event creates a retention signal that re-initiates the calnexin cycle.

In this quality control pathway, GT functions as the folding sensor that assesses the folded status of glycoproteins. Although we do not understand how GT senses structural fidelity, several important clues have been revealed. (i) Sensing involves the innermost (and normally buried) N-GlcNAc glycosidic bond of the glycoprotein (24, 25). (ii) GT preferentially monoglucosylates partially folded late folding intermediates as compared with their fully denatured counterparts (26–28). (iii) GT is capable of distinguishing folded from misfolded domains within a single polypeptide (29, 30). (iv) Localized folding defects in otherwise correctly folded glycoproteins or individual domains are recognized by

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures, Refs. 1–3, and Figs. S1–S3.

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[3] The abbreviations used are: Sep15, 15-kDa selenoprotein; GT, UDP-glucose:glycoprotein glucosyltransferase; SelM, selenoprotein M; PC, protein C.

GT (30, 31). (v) GT monitors the tertiary and quaternary structures of glycoproteins (32).

Given the sequence diversity of glycoproteins, it is unlikely that the recognition mechanism used by GT involves a specific primary structure motif. A more robust mechanism, involving common biophysical properties of misfolded proteins, seems more plausible. Accordingly, the distribution of glycans and their spatial relationship to normally surface-inaccessible hydrophobic residues influence the order in which polypeptide segments achieve their native conformation (12, 27, 28). GT preferentially recognizes hydrophobic motifs proximal to glycosylation sites in glycopeptides (33). The efficiency of glucosylation can be positively correlated with the spatial proximity of site-directed mutations to each glycan, lending support to the idea that normally buried hydrophobic residues found adjacent to glycans mediate interactions with GT (31).

Our initial findings of the interaction between mammalian Sep15 and GT (11) raised the possibility that Sep15 might be a misfolded glycoprotein. Although Sep15 does not contain an endoplasmic reticulum retention sequence, it is neither secreted or glycosylated, suggesting that this selenoprotein might function as a co-factor that assists GT in assessing structural fidelity. Sep15 deficiencies observed in tumors may also impair GT-mediated regulation of the calnexin cycle.

In this report, we characterize interactions between members of Sep15 and GT protein families. Isothermal titration calorimetry was used to demonstrate that Sep15 forms a 1:1 complex with GT with an apparent K_d value of 20 nM. Moreover, co-immunoprecipitation revealed that Sep15 binds to both human GT isozymes. Although selenoprotein M (SelM) shares significant sequence homology with Sep15 and has also been localized to the endoplasmic reticulum, it does not form a complex with GT. The interaction between Sep15 and GT is exclusively mediated by a novel cysteine-rich domain located at the N-terminus of Sep15. This conserved domain is present in other functional Sep15 homologues and contains six conserved cysteine residues that do not coordinate metal ions. When the cysteine-rich domain of Sep15 is removed, it no longer forms a complex with GT. Site-directed mutagenesis was used to demonstrate that the structural integrity of the cysteine-rich domain is an essential factor in the ability of Sep15 to bind GT.

EXPERIMENTAL PROCEDURES

Plasmids—Genes encoding *Mus musculus* Sep15 and SelM were PCR amplified from cDNA clones using the following primers: 5′-CATTAGTATCTAATCTCGAGGGCAGACCGCAGGGAT-3′ and 5′-CTCGAGGGATACTCTAGAGCG-3′ (Sep15) and 5′-ATCTCGAGATGAGCATCCTACTGTCGCCGCCGTC-3′ and 5′-ATGCGGCCGCTACAGGTCGTCGTGTTCTGAAGCTTCCTCG-5′ (SelM). Both PCR products were digested with XhoI and NotI and ligated into the pCI-neo vector (Promega). The QuikChange site-directed mutagenesis kit (Stratagene) was used to mutate these genes. The following mutagenic primers were used to replace naturally occurring selenocysteine residues: U93C (5′-CTTGAAGTCTGCGGATGTAAATTGGGGAGGTTCC-3′ and 5′-GGAACCTCCCCAATTTACATCCGCAGACTTCAAG-3′) (designated Sep15-U93C) and U48C (5′-GACCTGTGGAGGATGTCAGTTGAATCGC-3′ and 5′-GCGATTCAACTGACATCCTCCACAGGTC-3′) (designated SelM-U48C). Expected sequence changes were verified by DNA sequencing. Sep15-U93C served as the template to introduce a C-terminal hexahistidine-tag with the following primers: 5′-CATTAGTATCTAATCTCGAGGGCAGACCGCAGGGAT-3′ and 5′-TCGAATTCTCAGTGGTGGTGGTGGTGGTGTATGCGTTCCAACTTCTCGCTC-3′. The PCR product was digested with XhoI and EcoRI and ligated into the pCI-neo vector. Site-directed mutagenesis of His-tagged Sep15-U93C was performed with the following mutagenic primers: Sep15-C38SU93C (5′-GTTTGCGTCAGAGGCATCCAGAGAGTTGGGTTTC-3′ and 5′-GAAACCCAACTCTCTGGATGCCTCTGACGCAAAC-3′), Sep15-C49SU93C (5′-CCAGCAACTTGCTCTCCAGCTCTTGCGATC-3′ and 5′-GATCGCAAGAGCTGGAGAGCAAGTTGCTGG-3′), Sep15-C52SU93C (5′-CTCTGCAGCTCTTCCGATCTTCTTGGAC-3′ and 5′-GTCCAAGAAGATCGGAAGAGCTGCAGAG-3′), Sep15-C67SU93C (5′-CACTGGACCCTGTTTCCAGAGGGTGCTGTC-3′ and 5′-GACAGCACCCTCTGGAAACAGGGTCCAGTG-3′), Sep15-C70SU93C (5′-GTTTGCAGAGGGTCCTGTCAGGAAGAAG-3′ and 5′-CTTCTTCCTGACAGGACCCTCTGCAAAC-3′), Sep15-C71SU93C (5′-GCAGAGGGTGCTCTCAGGAAGAAGC-3′ and 5′-GCTTCTTCCTGAGAGCACCCTCTGC-3′). Expected sequence changes were verified by DNA sequencing.

A fusion protein of Sep15 and SelM from mouse (designated Sep15::SelM) was generated by fusing the cysteine-rich domain of Sep15 to the N-terminus of SelM. The cysteine-rich domain of Sep15, together with its native signal peptide, was PCR amplified using the following primers: 5′-TATAGACTAGACTCGAGGGCAGACCGCAG-3′ and 5′-GCAGACTTCAAGGATGAATTCTGCATACAGCTTTTTGG-3′. This PCR product was digested with XhoI and EcoRI and ligated into the pCI-neo vector. SelM-U48C was PCR amplified without its signal peptide using the following primers: 5′-CTTGTGGCTCCAGCCGAATTCACCACCAACTACCGACC-3′ and 5′-ATGCGGCCGCTACAGGTCGTCGTGTTCTGAAGCTTCCTCG-3′. The PCR product was digested with EcoRI and NotI and ligated into the pCI-neo vector containing the cysteine-rich domain of Sep15.

Mammalian expression vectors encoding protein C (PC)-tagged human GT1 and GT2 have been described previously (34). The gene encoding Sep15 from *Drosophila melanogaster* was PCR amplified from an expressed sequence tag clone using the following primers: 5′-TGCTCGAGCAAATTGGCGCCGAATTGACGGCCGCCGACTG-3′ and 5′-CAGTTTTGGGTATGGGTACCTTAGATCCTGTTGGTTCGCAGG-3′. The PCR product was digested with EheI and KpnI and ligated into Baculovirus expression vector pFastBac-HTa (Invitrogen). A previously described cDNA clone (35) served as the template to introduce a C-terminal hexahistidine-tag into *Drosophila* GT. To improve secretion from insect cells the native signal sequence was replaced with the honeybee melittin signal peptide. Moreover, the C-terminal endoplasmic reticulum retention sequence was removed. Primer sequences are available upon request.

Cell Growth, Transfection, and Immunoprecipitation—Mouse fibroblast cell line NIH 3T3 was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum to ~80% confluence. Transfections were performed using Lipofectamine and PLUS reagent (Invitrogen) according to manufacturer's instructions. For each 60-mm plate, 2 μg of DNA, 8 μl of PLUS reagent, and 12 μl of Lipofectamine were used. Protein extracts were prepared 48 h following transfection with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, 1% Igepal CA-630 detergent, 0.5% deoxycholate, and EDTA-free complete protease inhibitor mixture tablets (Roche Applied Science). Once cleared of cellular debris, cell lysates were incubated overnight with 50 μl of anti-PC affinity matrix (Roche Applied Science) at 4 °C. Bound proteins were washed twice with 1 ml of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, 1% Igepal CA-630 detergent, and 0.5% deoxycholate and once with 1 ml of 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM $CaCl_2$, 0.1% Igepal CA-630 detergent, and 0.05% deoxycholate. Proteins were eluted with 100 μl of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM

EDTA. Samples were resolved by SDS-PAGE and detected by Western blotting using anti-His-tag, anti-PC, anti-Sep15, and anti-SelM antibodies.

Native Gel Electrophoresis—Recombinant *Drosophila* Sep15 and GT proteins were expressed in Sf9 cells and purified by immobilized metal-affinity chromatography. Complexes of Sep15 and GT were prepared by incubating 5 μg of GT dissolved in 10 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$ with the indicated amounts of Sep15 for 10 min at 37 °C. Samples were subjected to non-denaturing PAGE using Tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. The Sep15-GT complex was visualized by Western blotting with Sep15-specific antibodies.

Isothermal Titration Calorimetry—The apparent association constant for Sep15 binding to GT was determined using isothermal titration calorimetry. Protein solutions of *Drosophila* Sep15 and GT were dialyzed for 2 days against 25 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1 mM $CaCl_2$. The concentrations of both proteins were determined using Beer's law. This experiment consisted of a series of 8-μl injections of Sep15 into a cell containing 1.4 ml of GT equilibrated at 20 °C. Heats associated with Sep15 binding to GT were measured by an isothermal titration calorimeter (Microcal). The apparent association constant derived from these data were determined using software included with the calorimeter.

Metal Ion Analysis—The concentrations of twenty biologically relevant metal ions were determined by inductively coupled argon plasma emission spectrometry at the Chemical Analysis Laboratory, University of Georgia. Protein samples dissolved in 50 mM sodium phosphate (pH 7.0), 300 mM NaCl, and 250 mM imidazole were analyzed. Metal ion concentrations were calculated by subtracting the ion content of the buffer from that of the protein sample.

RESULTS

Sep15, but Not SelM, Interacts with Human GT Homologues—Sep15 is a resident protein of the endoplasmic reticulum that has been previously shown to form a complex with GT when purified from rat and mouse liver (11). Two human GT homologues (GT1 and GT2) have been identified (36). These enzymes are highly homologous and display 83% sequence identity in their proposed C-terminal catalytic domains. However, their N-terminal non-catalytic domains are more diverse (49% sequence identity), and only GT1 displays measurable catalytic activity. To assess whether Sep15 functions as a protein co-factor that binds both GT isozymes, mouse Sep15 was co-expressed with PC-tagged GT1 or GT2 in NIH 3T3 cells. Sep15 co-immunoprecipitated with GT1 and GT2 (Fig. 1*A*), indicating that both human GT isozymes are capable of binding Sep15 *in vivo*. The reduced intensity of the Sep15 signal observed with GT2 is consistent with its lower level of expression as compared with that of GT1.

The recently described selenoprotein SelM (37) shares 31% sequence identity with Sep15 (Fig. 2, *A* and *B*). Both proteins encode a single selenocysteine residue. Primary sequence alignments illustrate that SelM has an elongated C terminus, whereas Sep15 possesses an extended cysteine-rich N terminus. The N-terminal sequence of Sep15 is conserved among functional Sep15 homologues and is conversely absent in sequence homologues of SelM (Fig. 2*C*). As observed with Sep15, selenoprotein SelM is also localized to the endoplasmic reticulum. Given the common intracellular location of Sep15 and SelM and their respective sequence homology, we speculated that SelM might also bind to GT. However, co-immunoprecipitation experiments indicate that mouse SelM does not form a complex with either human GT1 or GT2 (Fig. 1*B*).

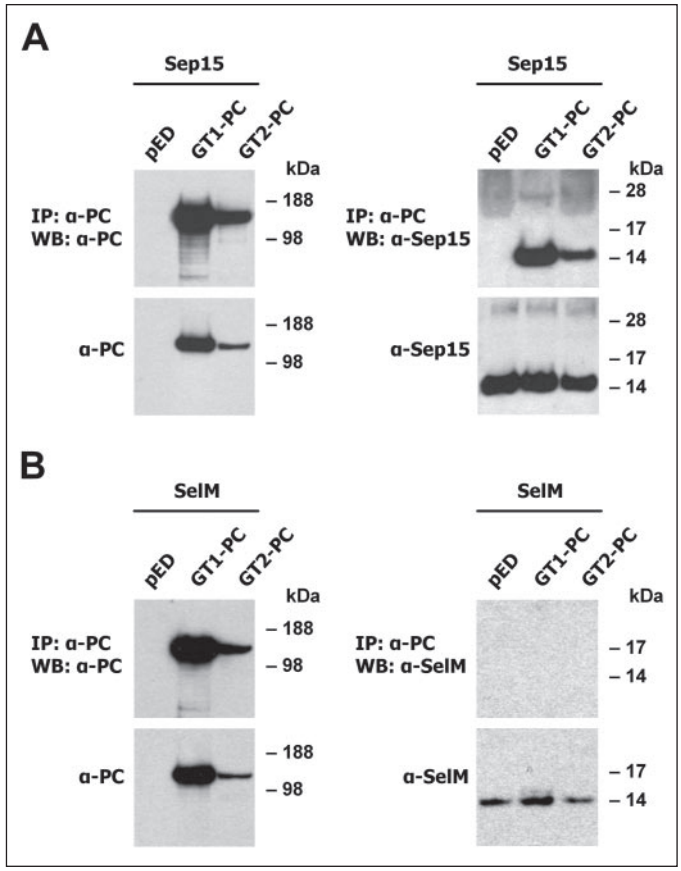

FIGURE 1. **Sep15, but not SelM, forms complexes with GT1 and GT2.** *A*, NIH 3T3 cells were co-transfected with plasmid-encoded mouse Sep15 and the empty control vector pED or plasmids encoding either PC-tagged human GT1 (*GT1-PC*) or PC-tagged human GT2 (*GT2-PC*). Proteins were immunoprecipitated (*IP*) using anti-PC antibodies, resolved by SDS-PAGE, and detected by Western blotting (*WB*) with anti-PC (*upper left panel*) and anti-Sep15 (*upper right panel*) antibodies. In addition, prior to immunoprecipitation cell lysates were analyzed by Western blotting using anti-PC (*lower left panel*) and anti-Sep15 (*lower right panel*) antibodies. *B*, NIH 3T3 cells were co-transfected with plasmid-encoded mouse SelM and the empty control vector pED or plasmids encoding either PC-tagged human GT1 (*GT1-PC*) or PC-tagged human GT2 (*GT2-PC*). Proteins were immunoprecipitated using anti-PC antibodies, resolved by SDS-PAGE, and detected by Western blotting with anti-PC (*upper left panel*) and anti-SelM (*upper right panel*) antibodies. In addition, prior to immunoprecipitation cell lysates were analyzed by Western blotting using anti-PC (*lower left panel*) and anti-SelM (*lower right panel*) antibodies.

Cysteine-rich Domain of Sep15 Mediates the Interaction with GT—The absence of an N-terminal extension in SelM suggested that this region of Sep15 may potentially mediate protein-protein interactions with GT. To test this hypothesis, the cysteine-rich N-terminal extension of mouse Sep15 was fused to the N terminus of mouse SelM. When expressed in NIH 3T3 cells, this mouse protein chimera co-immunoprecipitates with human GT1 (Fig. 3). These data suggest a functional role for the N-terminal extension of Sep15 in the formation of a complex with GT.

Sequence analyses of the N-terminal domain of Sep15 proteins identified six conserved cysteine residues (Fig. 2*C*). Four of these six cysteine residues form a pair of C*xx*C motifs. To establish a structural and/or functional role for these cysteine residues and evaluate their potential involvement in constituting the GT-binding site, all cysteine residues were individually replaced with serine residues. Co-immunoprecipitation experiments demonstrate that formation of the Sep15-GT complex was abolished for each mutant (Fig. 4). These data indicate that the N-terminal extension of Sep15 forms a distinct cysteine-rich domain and that the structural integrity of this domain is required for Sep15 to form a complex with GT.

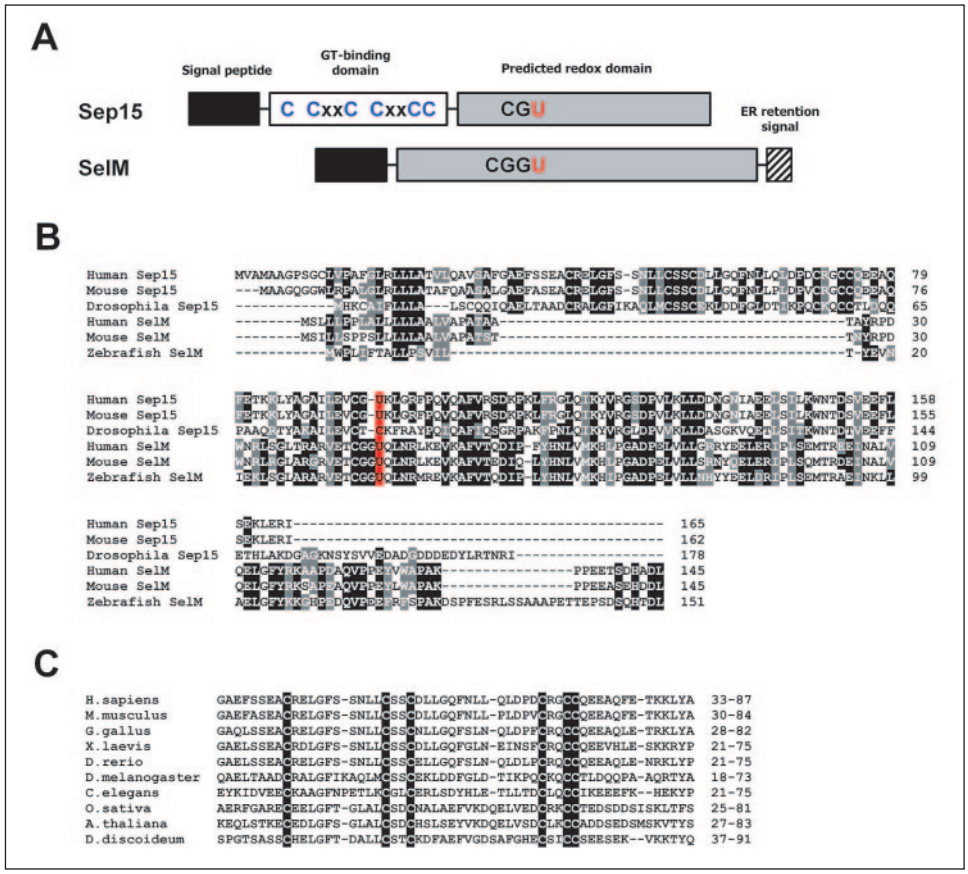

FIGURE 2. **Sequence alignment of members of the Sep15 family.** *A*, schematic illustration of the domain arrangement of Sep15 and SelM. Both selenoproteins encode an N-terminal signal peptide (colored *black*), one selenocysteine (*U*) residue (colored *red*), and a common redox domain (colored *gray*). The N-terminal extension of Sep15, containing six strictly conserved cysteine residues (colored *blue*) has been labeled as the GT-binding domain. *B*, multiple sequence alignment of Sep15 and SelM proteins. Conserved residues are *highlighted*. The genetically encoded selenocysteine residues and the corresponding cysteine residue found in the *Drosophila* Sep15 protein are colored *red*. *C*, multiple sequence alignment of the N-terminal cysteine-rich domain of Sep15 proteins. Conserved cysteine residues are *highlighted*.

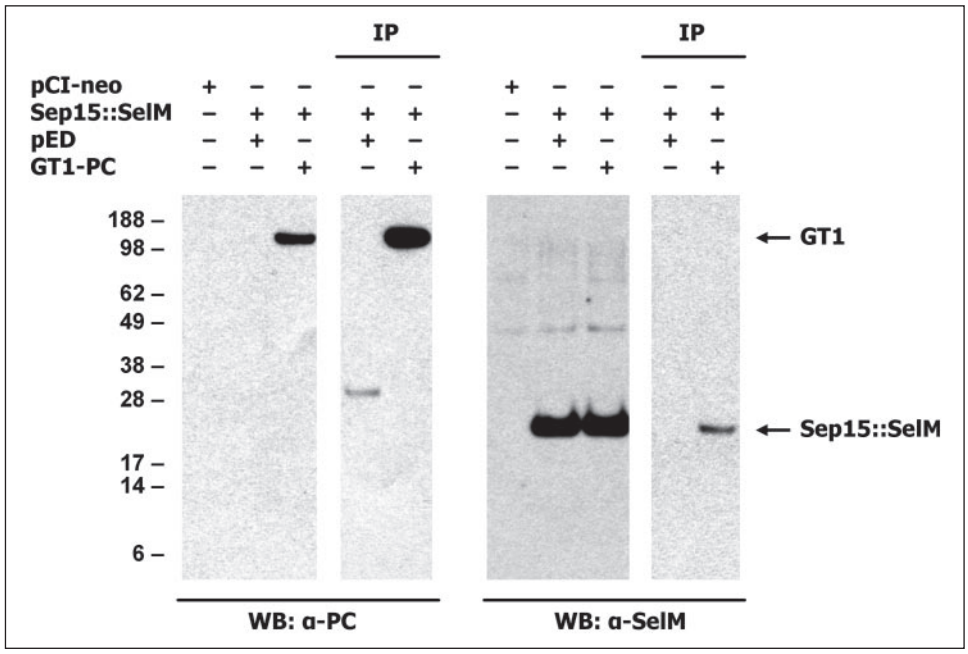

FIGURE 3. **The N-terminal domain of Sep15 mediates the interaction with GT.** NIH 3T3 cells were transfected with the empty control vector pCI-neo or co-transfected with plasmid-encoded mouse Sep15::SelM chimera and the empty control vector pED or plasmid-encoded PC-tagged human GT1 (*GT1-PC*). Samples were immunoprecipitated (*IP*) using anti-PC antibodies. Cell lysates and immunoprecipitated samples were resolved by SDS-PAGE and detected by Western blotting with anti-PC (*left panel*) and anti-SelM (*right panel*) antibodies.

Sep15 and GT Form a 1:1 Complex—One technical obstacle to investigating protein-protein interaction between Sep15 and GT is the naturally occurring selenocysteine residue encoded within the Sep15 gene. Selenocysteine-containing proteins are frequently difficult to express and purify. However, the Sep15 protein of *Drosophila* is not a selenoprotein and encodes two cysteine residues in place of the C*x*U motif (Fig. 2*B*). To examine the binding between *Drosophila* Sep15 and GT, both recombinant proteins were expressed and purified from Sf9 cells.

Formation of the complex between Sep15 and GT was analyzed by polyacrylamide gel electrophoresis under non-denaturing conditions (Fig. 5). Western blotting using antibodies specific for Sep15 detected a low molecular weight band corresponding to unbound Sep15 in addition to a higher molecular weight band that co-migrates with GT. Several minor low molecular weight bands were also detected, indicating that Sep15 may form homomultimers and/or is degraded under these experimental conditions. The band intensity of the Sep15-GT complex

continued to increase as the molar equivalents of Sep15 and GT were increased from 0.2 to 2.0, at which point the amount of unbound and GT-bound Sep15 became equivalent.

The stoichiometry of the Sep15-GT complex was precisely determined by isothermal titration calorimetry (Fig. 6). Sep15 forms a 1:1 complex with GT with an apparent K_d value of 20 nM. The mixed species Sep15::SelM chimera also bound tightly to GT (supplemental Fig. S1). In contrast, no interactions were observed for SelM and Sep15 without its N-terminal cysteine-rich domain. These isothermal titration calorimetry results were confirmed by measuring the intensity of the strongest methyl resonance by one-dimensional ^{15}N-edited proton NMR spectroscopy (supplemental Fig. S2). A decrease in the strongest methyl resonance intensity in the proton NMR spectrum of the protein mixture in comparison to the added strongest methyl resonance intensities of the individual proteins indicates specific protein-protein interactions (38). Consistent with co-immunoprecipitation and isothermal titration calorimetry data, these NMR data indicate that both Sep15 and the Sep15::SelM chimera form complexes with GT. Conversely, SelM and Sep15 without its corresponding cysteine-rich domain do not bind to GT. Taken together these complementary techniques demonstrate that the cysteine-rich domain of Sep15 mediates that formation of a tight 1:1 complex with GT.

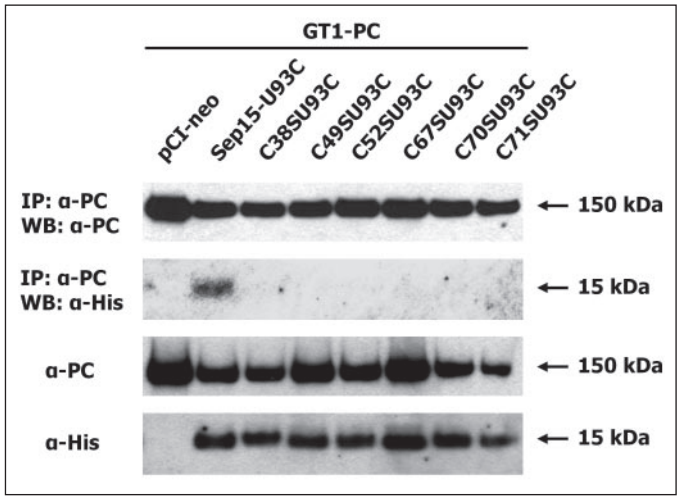

FIGURE 4. **Binding of Sep15 to GT is dependent upon the structural integrity of the cysteine-rich domain.** NIH 3T3 cells were co-transfected with plasmid-encoded PC-tagged human GT1 (*GT1-PC*) and the empty control vector pCI-neo or plasmid-encoded His-tagged mouse Sep15-U93C or Sep15-C38SU93C, Sep15-C49SU93C, Sep15-C52SU93C, Sep15-C67SU93C, Sep15-C70SU93C, and Sep15-C71SU93C mutants. Samples were immunoprecipitated (*IP*) using anti-PC antibodies, resolved by SDS-PAGE, and detected by Western blotting (*WB*) with anti-PC (*first panel*) and anti-His (*second panel*) antibodies. Cell lysates were also directly probed using anti-PC (*third panel*) and anti-His antibodies (*fourth panel*).

Formation of the Sep15-GT Complex Is Not Mediated by Coordinated Metal Ions—Four of the six conserved cysteine residues in the cysteine-rich domain of Sep15 form two C*xx*C motifs, which are typically involved in metal ion coordination. To establish whether the cysteine-rich domain of Sep15 does coordinate metal ions, we further analyzed *Drosophila* Sep15. When expressed in bacterial or insect cells, purified Sep15 protein was colorless. Moreover, UV-visible spectrophotometric analysis revealed only a single peak centered at 280 nm, indicating the absence of certain co-factors and iron-sulfur clusters. Sep15 protein samples were also analyzed for the presence of twenty biologically relevant metal ions by inductively coupled argon plasma emission spectrometry. Only Co^{2+} was detected (0.24 molar equivalents), which is most likely non-specifically bound during protein purification by immobilized metal-affinity chromatography using cobalt-based resin. These data suggest that the cysteine-rich domain itself does not coordinate metal ions.

It has been previously reported that the catalytic activity of GT is dependent upon the presence of divalent cations (preferably Ca^{2+}) (39). Although metal ions were not detected in Sep15 protein preparations, it is possible that metal ions mediate the interaction between Sep15 and GT. To address this possibility, complex formation between Sep15 and GT was analyzed by polyacrylamide gel electrophoresis under non-denaturing conditions in the absence and presence of 1 mM Ca^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}. However, the Sep15-GT complex was observed independently of the presence of metal ions (data not shown). These results indicate that formation of the Sep15-GT complex is not mediated by coordinated metal ions.

Sep15 Does Not Affect Glucosylation of Thyroglobulin—The association of Sep15 with GT suggests that this selenoprotein may modulate the enzymatic activity of GT and/or be involved in assessing structural fidelity. To test this hypothesis, GT activity in the presence of Sep15 was analyzed by monitoring the amount of incorporated [^{14}C]glucose into denatured thyroglobulin. The presence of Sep15 did not affect the GT activity toward this protein (supplemental Fig. S3). These results indicate that, at least in the case of thyroglobulin, Sep15 does not modulate GT activity and is not required for glucosylation under these experimental conditions.

DISCUSSION

Selenoproteins have not been previously shown to participate in the quality control networks of the endoplasmic reticulum. The observation that Sep15 co-purifies with the essential regulator of the calnexin cycle

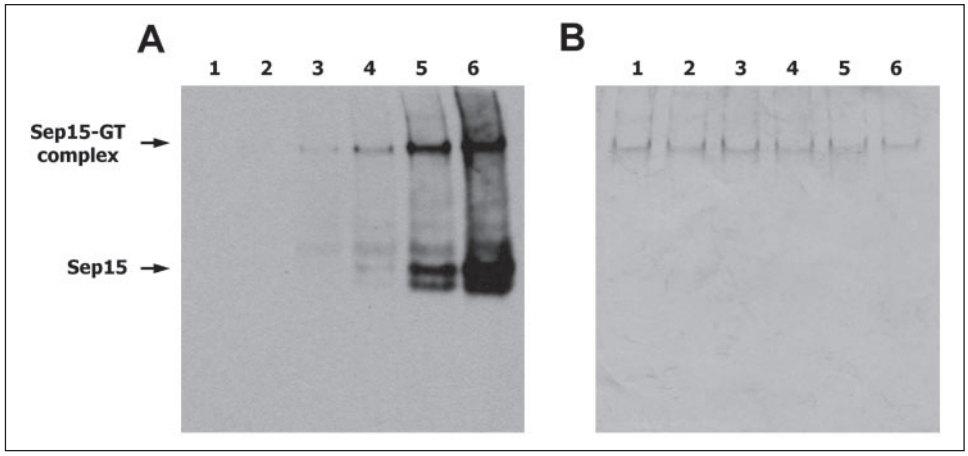

FIGURE 5. **Stoichiometry of the Sep15-GT complex from *D. melanogaster*.** *A*, Western blot of the Sep15-GT complex using antibodies specific for *Drosophila* Sep15. *B*, Coomassie Blue staining of the corresponding native gel. Samples were resolved by PAGE under non-denaturing conditions. For both panels: *lane 1*, GT; *lanes 2–6*, GT pre-incubated with 5:1 (*lane 2*), 2:1 (*lane 3*), 1:1 (*lane 4*), 1:2 (*lane 5*), and 1:5 (*lane 6*) molar equivalents of Sep15 to GT. Complexes of Sep15 and GT were prepared by incubating of 5 μg of GT contained in 10 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$ with 0.11, 0.28, 0.56, 1.12, or 2.8 μg of Sep15 for 10 min at 37 °C.

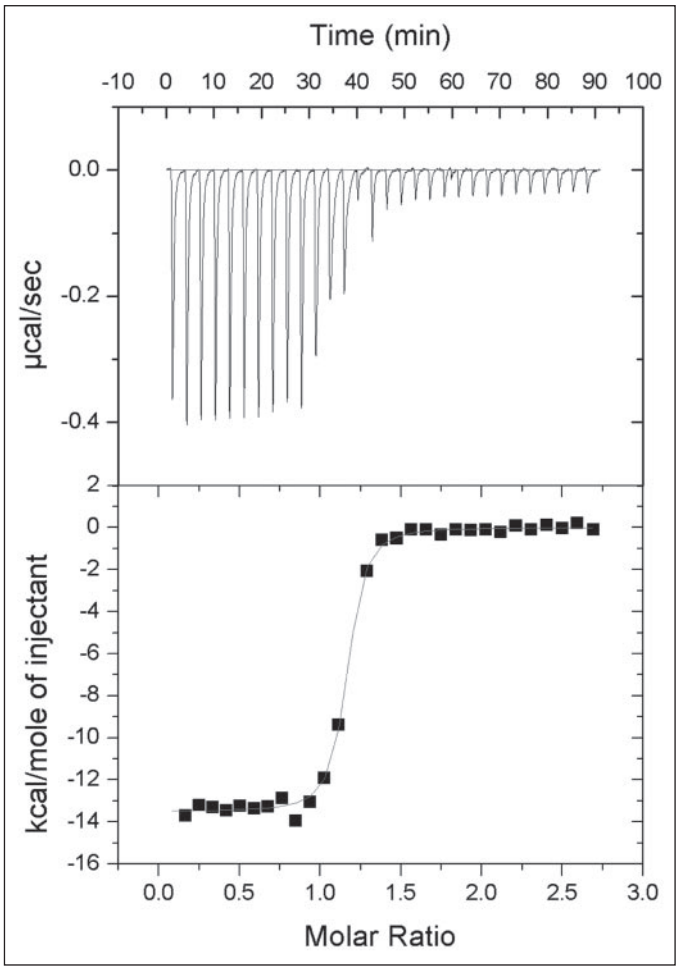

FIGURE 6. **Characterization of Sep15 binding to GT by isothermal titration calorimetry.** The *upper half* of this panel shows the binding isotherm. The *horizontal line* represents the base line. The *lower half* of this panel shows the amount of heat derived from each kcal/mol of injectant (Sep15). The *curved line* shows a fit of a single-site binding model to the data. Protein concentrations of *Drosophila* Sep15 and GT were 104 and 7 μM, respectively.

suggests a potential mechanism for the chemopreventive effects of dietary selenium (11).

In this study, we characterized interactions between members of Sep15 and GT families using a series of complementary genetic and biophysical techniques and found that this selenoprotein interacts with both human GT isozymes. The 1:1 stoichiometry of this complex has an apparent K_d value of 20 nM as determined by isothermal titration calorimetry using Sep15 and GT from *Drosophila*. In contrast, SelM (a sequence homologue of Sep15) does not interact with GT. Multiple sequence analyses identified a novel cysteine-rich domain that is conserved among the N termini of Sep15 proteins, which is not found in the sequences of SelM proteins. If the cysteine-rich domain of Sep15 is deleted, the truncated Sep15 protein no longer interacts with GT. However, when the cysteine-rich domain of Sep15 is fused to the N terminus of SelM, the resulting Sep15::SelM protein chimera forms a complex with GT.

The sequence of the cysteine-rich domain is highly conserved among Sep15 homologues from plants to humans (40) (Fig. 2*C*), suggesting that interactions between Sep15 and GT have been conserved throughout evolution. Mutating any one of these six conserved cysteine residues disrupts the interaction between Sep15 and GT. As four of these six cysteine residues form a pair of C*xx*C motifs, we speculated that these motifs might coordinate metal ions, such as iron or zinc. However, metal ion analysis of purified Sep15 protein samples did not identify specifically bound metal ions. It is more likely that these conserved cysteine residues form intramolecular disulfide bonds. Site-directed mutagenesis of cysteine residues may disrupt the proper arrangement of these disulfide bonds and compromise the structural integrity of the cysteine-rich domain, which in turn inhibits protein-protein interaction between Sep15 and GT. Taken together, these data unequivocally demonstrate that the cysteine-rich domain mediates the interaction between Sep15 and GT. However, the corresponding regions of GT that are involved in forming the binding surface for Sep15 remain to be established.

The fact that Sep15 does not possess the endoplasmic reticulum retention signal and its N-terminal part is responsible for maintaining Sep15 in the endoplasmic reticulum (41) suggests that its location in this cellular compartment is the result of the interaction with GT.

The biological significance of protein-protein interactions between Sep15 and GT within the endoplasmic reticulum is poorly understood. One possibility is that Sep15 modulates the enzymatic activity of GT by functioning as a protein co-factor that assists this folding sensor in assessing the structural fidelity of misfolded glycoproteins. However, our data show that GT-mediated labeling of thyroglobulin is not directly affected by Sep15. Sep15 appears to occur exclusively in the complex with GT, whereas GT is present in both selenoprotein-bound and selenoprotein-free form (11). Thus, it is possible that Sep15 is not required for the GT function *per se* but may be required for the recognition of glycoproteins with incorrectly formed disulfide bonds. It is an attractive possibility that the unusual C*x*U motif of Sep15 may participate in the reduction, isomerization, or oxidation of disulfide bonds of glycoprotein substrates of GT.

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Supplemental Data

EXPERIMENTAL PROCEDURES

Plasmids – The coding regions of SelM from *M. musculus* and Sep15 from *D. melanogaster* were PCR amplified from a cDNA clone (1) and a cDNA library (Clontech) with the following primers: 5'-TCCAGCCACCCATATGACCAACTACCGACCGGATTGGAACC-3' and 5'-GGTGGTGGTGCTCGAGGTCGTCGTGTTCTGAAGCTTCCTC-3' (residues 25-145 of SelM-U48C with a C-terminal hexahistidine-tag); and 5'-ATTCCATGGGACATCACCATCACCATCTCGAGCAAATTCAA-3' and 5'-ACCCCCATGGTTAGATCCTGTTGGT-3' (residues 16-178 of Sep15 with an N-terminal hexahistidine-tag). The resulting PCR fragments were digested with NdeI and XhoI or NcoI, and ligated into expression vectors pET21a or pET19b (Novagen). Sep15 without its N-terminal cysteine-rich domain (designated ΔCys-Sep15) was subcloned with the following primers: 5'-AAGGGCTAGCCACCATCACCATCACCATTTGGATCAGCAGCCG-3' and 5'-AAGGCTCGAGTTAGATCCTGTTGGT-3' (residues 62-178 of Sep15 with an N-terminal hexahistidine-tag). The resulting PCR product was digested with NheI and XhoI, and ligated into expression vector pET19b. A chimera *D. melanogaster* Sep15 and *M. musculus* SelM (designated Sep15::SelM) was generated by fusing the cysteine-rich domain of Sep15 to the N-terminus of SelM-U48C. The cysteine-rich domain of Sep15 (residues 16-61) was PCR amplified using the following primers: 5'-AAGGCATATGCTCGAGCAAATTCAAA-3' and 5'-AAGGCATATGAGTGCAGCATTGCTTACA-3'. This PCR product was digested with NdeI, and ligated into the pET21a vector containing SelM (residues 25-145 with a C-terminal hexahistidine-tag). Expected nucleotide sequences were verified by DNA sequencing.

NMR spectroscopy – Uniformly ^{15}N-labeled Sep15, Sep15 without its cysteine-rich domain (ΔCys-Sep15), SelM, and the Sep15::SelM chimera were produced by supplementing M9 media with 1 g/l ^{15}N-ammonium chloride and 3 g/l glucose supplemented with 0.5 ml/l of 0.5% thiamine, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.5 ml/l of 10 mg/ml biotin, and 100 μg/ml of carbencillin. These proteins were expressed in *Escherichia coli* BL21(DE3) cells (Novagen). Cell cultures were grown at 37°C until the OD_{600} reached 0.6. Protein expression was induced by adding 0.4 mM IPTG, and the cells were grown for 16 hours at 30°C. Following induction, bacterial cells were harvested by centrifugation at 4,700g for 15 minutes at 4°C, cell pellets were re-suspended in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% Triton-X-100, 10 mg/l lysozyme, 5 mg/l DNaseI, and EDTA-free Complete Protease Inhibitor Tablets (Roche)], and lysed by sonication. Cell lysates were centrifuged at 40,000g for 30 minutes at 4°C. Recombinant proteins were purified from cell lysates by immobilized metal-chelate affinity chromatography using Ni-NTA agarose (Qiagen). All proteins were further purified by ion exchange and gel filtration chromatography using MonoQ and Superdex 75 columns (Amersham Biosciences), respectively. All NMR experiments were acquired at 25°C with Varian Inova spectrometers operating at proton frequencies of 500 and 600 MHz using 1 mM protein samples dissolved in 25 mM Tris (D_{11}, 98%)(pH 7.0), 200 mM NaCl, 1 mM $CaCl_2$, and 5% D_2O. Protein-protein interactions were monitored by one-dimensional NMR using the strongest methyl resonance ^{15}N-edited technique as described previously (2).

Measurement of UDP-glucose:glycoprotein glucosyltransferase activity – The catalytic activity of GT was determined as described previously (3). Bovine thyroglobulin (20 mg/ml) was denatured for 16 h at room temperature in 5 mM Tris-HCl (pH 7.5) and 8 M urea. The protein solution was subsequently dialyzed against 25 mM Tris-HCl (pH 7.5) for four hours at 4ºC - the buffer was exchanged each hour. In a final volume of 50 μl, 0.5 μM GT contained in 10 mM $CaCl_2$ and 10 mM Tris-HCl, was incubated with 5 μM [^{14}C]-UDP glucose (303 mCi/mmol - MP Biomedicals), 20 μM of denatured thyroglobulin and 4:1, 2:1, 1:1, 1:2 and 1:10 molar equivalents of Sep15 to GT for 10 minutes at 37˚C. Samples were precipitated with 1 ml of cold 10% trichloroacetic acid for 30 minutes. Labeled thyroglobulin was collected on 24 mm GF/C glass microfiber filters, washed with 10 ml of cold 10% trichloroacetic acid, 1

ml of cold 100% ethanol, 1 ml of acetone, and dried. The amount of incorporated [^{14}C]-glucose was quantified using a liquid scintillation counter.

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Supplemental Fig. 1

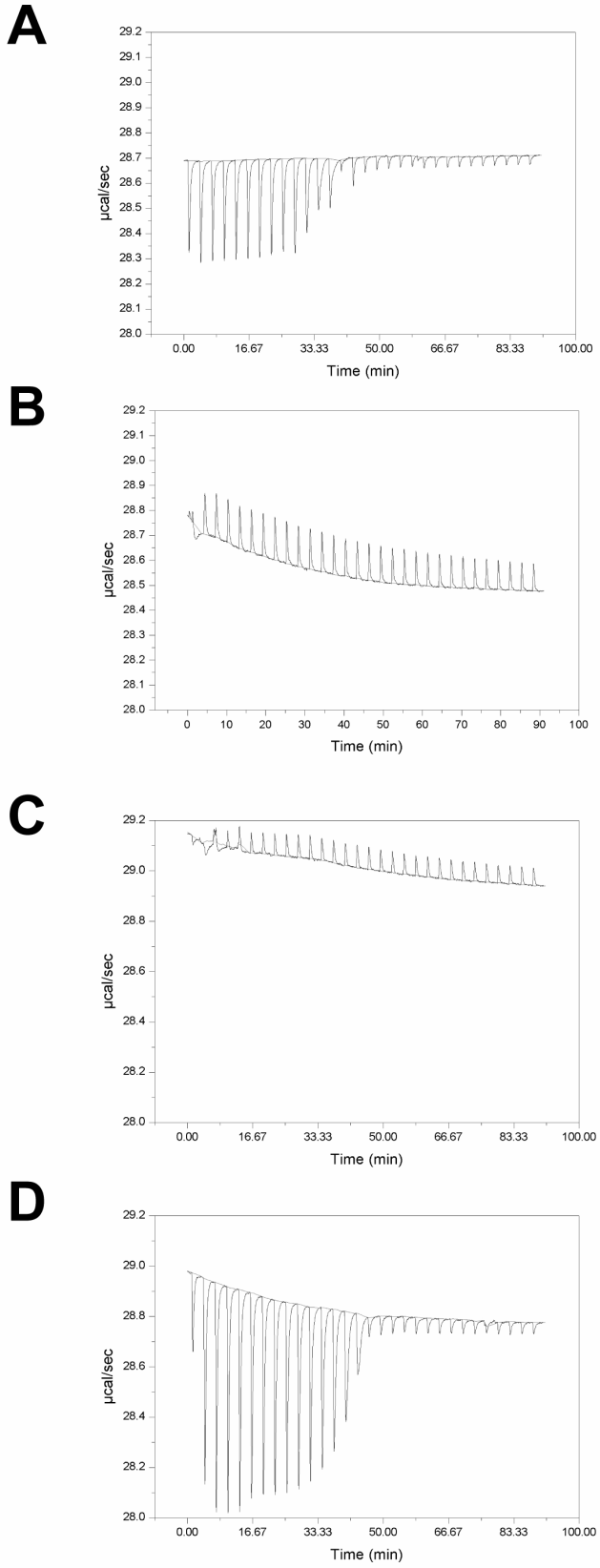

Fig. S1. Characterization of binding to GT by isothermal titration calorimetry. *A*, Sep15; *B*, ΔCys-Sep15; *C*, SelM; and *D*, Sep15::SelM chimera. These panels show the binding isotherms. The horizontal line represents the baseline.

Supplemental Fig. 2

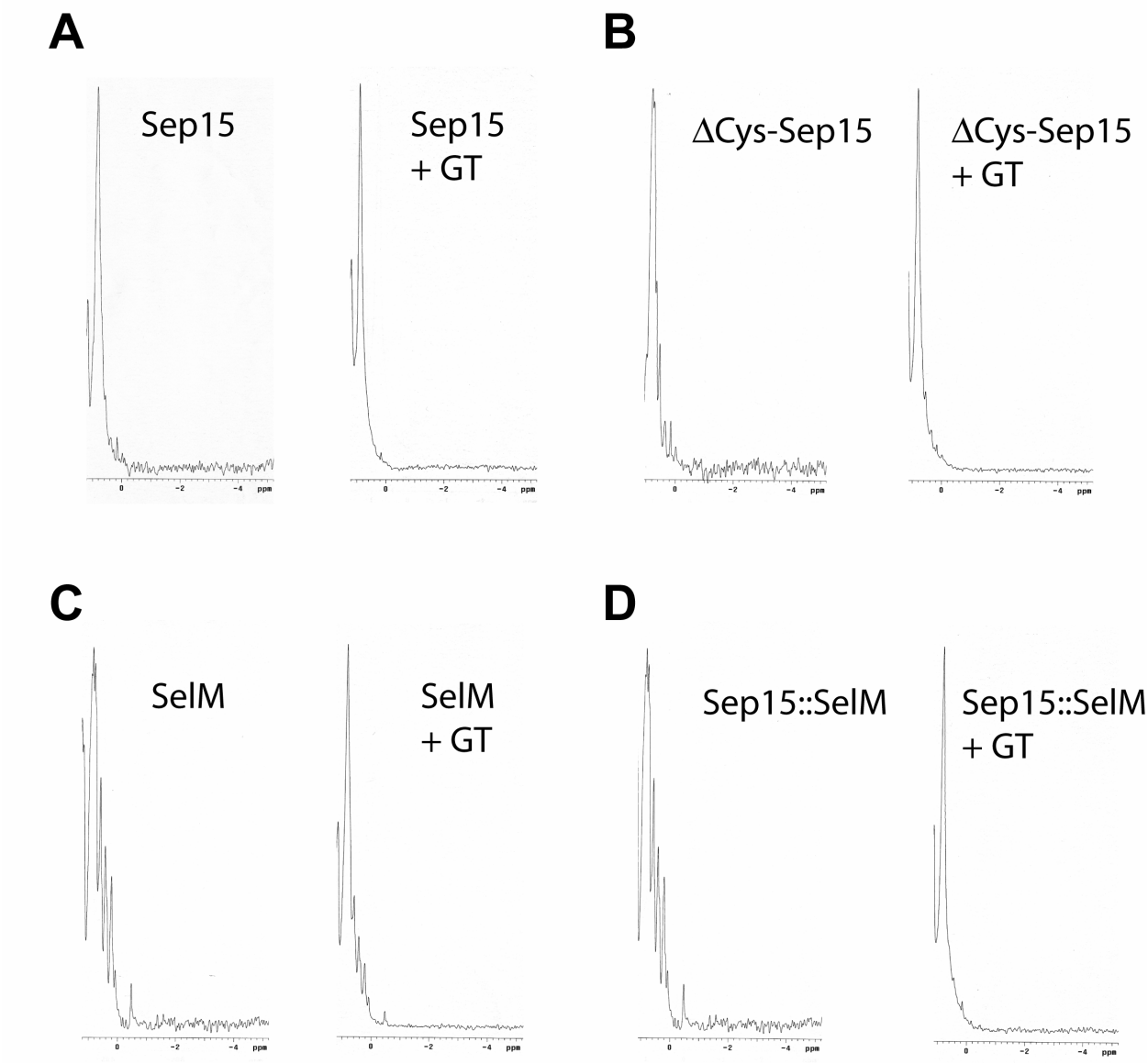

Fig. S2. Characterization of binding to GT by 1D NMR using the ^{15}N-edited strongest methyl resonance technique. *A*, 5.8 μM ^{15}N-labeled Sep15 (*left*) and following the addition of 13 μM GT (*right*). The resulting protein concentration was calculated to be 15.1 μM, indicating the formation of a complex. *B*, 3.7 μM ^{15}N-labeled ΔCys-Sep15 (*left*) and following the addition of 13 μM GT (*right*). The resulting protein concentration was calculated to be 16.7 μM, indicating that these two proteins do not interact. *C*, 5.0 μM ^{15}N-labeled SelM (*left*) and following the addition of 13 μM GT (*right*). The resulting protein concentration was calculated to be 18.0 μM, indicating that these two proteins do not interact. *D*, 3.3 μM ^{15}N-labeled Sep15::SelM (*left*) and following the addition of 13 μM GT (*right*). The resulting protein concentration was calculated to be 13.0 μM, indicating the formation of a complex.

Supplemental Fig. 3

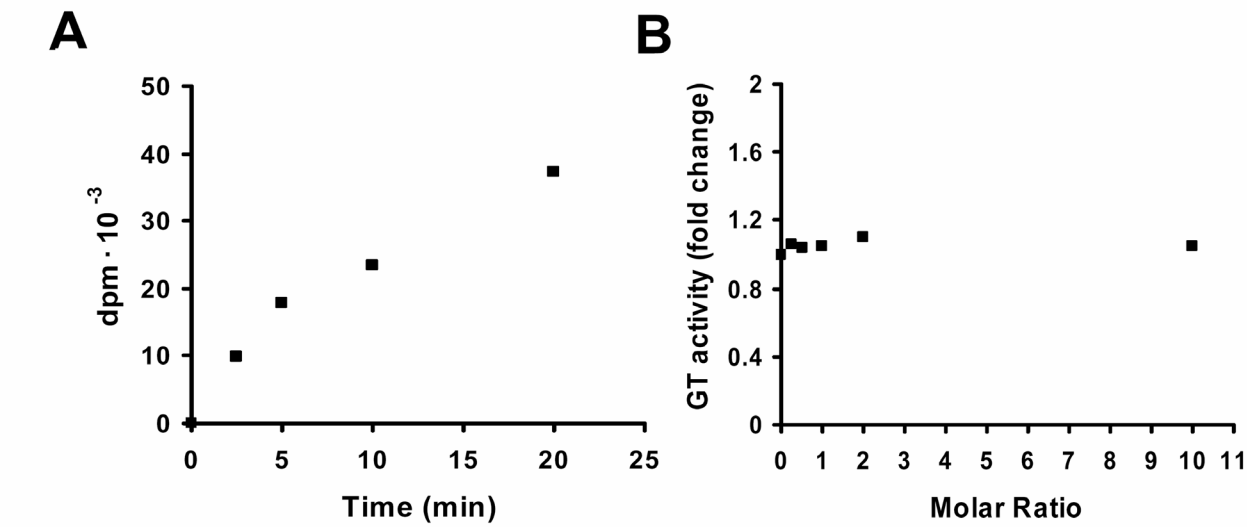

Fig. S3. Sep15 does not affect glucosylation of thyroglobulin. *A*, GT-catalyzed incorporation of [^{14}C]-glucose into denatured thyroglobulin as a function of time. *B*, Fold induction of GT activity as a function of the Sep15/GT molar ratio.