Mutational Analysis of the Catalytic Domain of O-Linked N-Acetylglucosaminyl Transferase

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O-Linked N-acetylglucosaminyltransferase (OGT) catalyzes the transfer of O-linked GlcNAc to serine/threonine residues of a variety of target proteins, many of which have been implicated in such diseases as diabetes and neurodegeneration. The addition of O-GlcNAc to proteins occurs in response to fluctuations in cellular concentrations of UDP-GlcNAc, which result from nutrients entering the hexosamine biosynthetic pathway. However, the molecular mechanisms involved in sugar nucleotide recognition and transfer to protein are poorly understood. We employed site-directed mutagenesis to target potentially important amino acid residues within the two conserved catalytic domains of OGT (CD I and CD II), followed by an in vitro glycosylation assay to evaluate N-acetylglucosaminyltransferase activity after bacterial expression. Although many of the amino acid substitutions caused inactivation of the enzyme, we identified three amino acid residues (two in CD I and one in CD II) that produced viable enzymes when mutated. Structure-based homology modeling revealed that these permissive mutants may be either in or near the sugar nucleotide-binding site. Our findings suggest a model in which the two conserved regions of the catalytic domain, CD I and CD II, contribute to the formation of a UDP-GlcNAc-binding pocket that catalyzes the transfer of O-GlcNAc to substrate proteins. Identification of viable OGT mutants may facilitate examination of its role in nutrient sensing and signal transduction cascades.

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UDP-GlcNAc synthetized by the hexosamine biosynthetic pathway (HBP) (26, 27). Increased intracellular glucose concentrations result in increased flow through the HBP, leading to an elevation of UDP-GlcNAc and the O-GlcNAcylation of many proteins (28). Furthermore, elevated O-GlcNAcylation of proteins is linked to insulin resistance and the onset of diabetes (29–31). The determination of the function of OGT as a nutrient sensor and the mechanism underlying sugar nucleotide recognition and transfer are therefore of interest.

The OGT enzyme is comprised of two distinct regions, an N-terminal domain containing a varying number of tetratricopeptide repeat (TPR) motifs and a C-terminal catalytic domain (1, 2) (Fig. 1). The TPR motif mediates protein-protein interactions (32, 33), and a minimum of three TPRs are required for substrate binding (34). We have recently solved the three-dimensional structure of the TPR domain of OGT (35), which was shown to have a similar binding motif to importin-α; importin-α uses a string of conserved asparagines residues to bind a number of different, structurally unrelated substrates. The crystal structure also showed that the nucleoeytoplasmic form of OGT exists as a dimer and that residues involved in formation of the dimer interface are present in the TPR domain. The three reported isoforms of OGT, nucleoplasmic OGT (ncOGT), mitochondrial OGT (mOGT), and short OGT (sOGT), have 12.5, 9.5, and 2.5 TPRs, respectively (36). The C-terminal catalytic region of OGT contains two conserved domains known as CD I and CD II (37), originally identified by their organizational similarity with the catalytic domain and lectin domain of the GalNAc transferases (38, 39). The two conserved domains of OGT also show similarities to glycosyltransferases that have a single monosaccharide-binding site.

Our aim was to perform a mutational analysis of residues in the C-terminal catalytic domain that may be important for UDP-GlcNAc sensing, thus providing information regarding the catalytic roles of CD I and CD II. To identify potentially important amino acid residues that could be targeted for mutagenesis, sequence comparisons were made with OGT homologues, as well as with other glycosyltransferase families for which a mechanism has been proposed for catalytic function (38, 40–44). The approach taken was to make single amino acid substitutions at conserved residues in both the CD I and CD II regions of human mOGT (1), followed by recombinant expression in Escherichia coli. Because E. coli does not possess a gene for OGT, any OGT activity recovered from these extracts is due to expression of the recombinant enzyme. E. coli extracts were used in an in vitro assay system, where wild type mOGT and mutant OGT enzymes were tested for their ability to glycosylate Nup62, a nuclear pore protein known to be an excellent OGT substrate.

Here we show that many of the amino acid substitutions made in both the CD I and CD II domains of OGT are critical for function and may be involved in either maintaining structure or catalysis. Most of the residues mutated in OGT were inactive; however, two amino acids in CD I (Asp⁴²⁵ and Tyr⁴³⁸) and one amino acid in CD II (Phe⁷⁵²) retained similar levels of enzyme activity as wild type. Homology modeling of these residues to other glycosyltransferases in the glycogen phosphorylase...
superfamily suggests that they may be either in or close to the active site of the enzyme. These results provide important new insights into the structure/function of this enzyme and may help understand the mechanism by which OGT recognizes UDP-GlcNAc and catalyzes transfer to substrate proteins.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Thirty mOGT single point mutants were generated using the QuikChange site-directed mutagenesis kit from Stratagene (see TABLE ONE), with the pET-32 human mOGT expression vector (1) as a template. The sequence was confirmed in each case and was shown to produce an open reading frame with the appropriate amino acid change.

Production of Human mOGT and mOGT Mutants from an E. coli Protein Expression System—The pET-32 human mOGT expression vector and the pET-32 human mOGT mutant expression vectors were transformed into competent BL21(DE3) cells (Novagen). As previously described (45), the cells were grown overnight at room temperature and 220 rpm in LB medium (K.D. Medical) supplemented with 50 μg/ml Ampicillin (Sigma). The cells were centrifuged at 3000 rpm for 10 min in a Beckman GS-6r centrifuge, and the pellet was resuspended in 1/50 of the original volume of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mg/ml lysozyme, 0.1% Triton X-100, and complete mini EDTA-free protease inhibitor mixture tablet (Roche Applied Science). Lysozyme digestion was performed at room temperature for 5 min. The lysate was subjected to freeze/thaw cycles by sonication on ice (3 × 10 s) until DNA was sheared. The supernatant obtained after centrifugation at 14,000 × g for 10 min was frozen in aliquots and stored at −80 °C. The total protein concentrations were determined using the BCA protein assay protocol (Fierce). OGT levels were determined by immunoblotting using an anti-His tag antibody (AbCam).

O-GlcNAc Transferase Assay—As described previously (45), bacterial extracts containing human recombinant mOGT and mOGT mutant proteins were added to a 40-μl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 12.5 mM MgCl₂, 0.2 nCi of UDP-[14C]GlcNAc (American Radiolabeled Chemicals), and 1 μg of recombinant, purified Nup62. The reactions were incubated for 60 min at 37 °C with shaking and were stopped by adding 4 × SDS-PAGE sample buffer (Invitrogen) and boiling for 3 min. SDS-PAGE was performed using precast 4–12% NuPage gels (Invitrogen), followed by staining with Simply Blue Safestain (Invitrogen) for 60 min and destaining with distilled water for 60 min. Glycosylation of Nup62 with [14C]-GlcNAc was visualized after treatment with En3Hance (PerkinElmer Life Sciences) for 60 min by fluorography using BIOMAX-MR film (Kodak) or the FujiFilm BAS-1500 phosphorus imager. Densitometry of x-ray film was performed with Image J software, whereas densitometry of phosphorimaging data was performed with Image Gauge 3.0 software. OGT enzyme activity was also measured using ScintItstip wells (Wallac) pre-coated with Nup62 (1, 46). For kinetic assays, OGT concentrations were initially determined by Ponceau S staining of Western blots. Image J software was used to determine the level of OGT protein compared with total protein levels determined by BCA. More recent experiments used fast green staining of nitrocellulose, followed by quantitation by infrared imaging using an Odyssey Western blot scanner.

Native Gel Electrophoresis—E. coli lysates containing the same amounts of recombinant mOGT and mOGT mutant proteins were prepared in 2× native sample buffer (Invitrogen) and loaded onto a precast 4–12% Tris-glycine Novex gel (Invitrogen). Following electrophoresis, the gels were immunoblotted with an anti-His tag antibody (AbCam) to detect recombinant protein. Aldolase (160 kDa) and catalase (240 kDa) were used as molecular mass standards (Serva).

RESULTS

Site-directed Mutagenesis of CD I Amino Acid Residues—The catalytic region of OGT consists of two highly conserved domains, CD I and CD II. Because the CD I domain of OGT may encode part of the sugar nucleotide-binding domain (36), a number of acidic residues in this domain were mutated. Acidic residues in the galactosyltransferase family are known to be important for recognition and stabilization of the sugar nucleotide substrate (44, 47). Seven Asp residues and three Glu residues were mutated to Ala. Gly358 was substituted for Ser to evaluate a similar mutation in Arabidopsis OGT that causes disruption to gibberellin signaling (Spindly phenotype) (48). Three other Gly to Ser substitutions were used to determine whether other Gly residues within CD I would have an effect on the enzyme activity of mOGT. Three Tyr residues, two Phe residues, and a Trp residue were mutated to Ala to evaluate the importance of aromatic residues in CD I. A complete list of CD I mutations is presented in TABLE ONE.

Enzyme activity for wild type mOGT and the CD I mutant constructs was determined by the O-GlcNAc transferase assay (see “Experimental Procedures”), and incorporation of [C14]GlcNAc into Nup62 was visualized by fluorography (Fig. 2B). The bands were then quantified, and the resulting values were normalized as percentages of mOGT activity (Fig. 2A). All of the data were normalized to the concentration of OGT in each sample (Fig. 2C). The following mutations were not included for

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**TABLE ONE**

| OGT mutant constructs for CD I and CD II |  |
|---|---|
| CD I | CD II |
| Y387A | F721A |
| G402S | W735A |
| D407A | W748A |
| D422A | F752A |
| Y434A | F776A |
| D438A | L796A |
| F439A | W812A |
| G453S | C836S |
| F460A | C839S |
| G472A | W878A |
| E482A |  |
| D488A |  |
| D505A |  |
| W536A |  |
| G538S |  |
| Y539A |  |
| D549A |  |
| D554A |  |
| E56A |  |
| E568A |  |
enzymatic analysis, because we could not produce sufficient amounts of protein: Y387A, F439A, D505A, W536A, Y539A, D549A, D554A, and E556A. With the exception of Y434A and D422A, all of the CD I mutants analyzed had a significant inhibitory effect on OGT enzyme activity. D422A produced a 50–100% increase in activity, whereas all of the other acidic amino acid substitutions (D407A, D438A, E482A, and D488A) caused an 95–99% reduction in enzyme activity.

The Spindly mutation of Arabidopsis is in a conserved region of OGT corresponding to Gly538. Mutation of Gly538 to Ser produced a dramatic reduction in enzyme activity: 1% compared with wild type. This is the first demonstration that the Spindly mutation directly lowers OGT activity and points to the relevance of this residue in the mammalian OGT enzyme. Mutation to Ala of a neighboring residue, Tyr539, decreased OGT activity by 99%. Because glycine is not usually associated with catalytic activity in glycosyltransferases, three other Gly residues were substituted to Ser to determine whether this phenomenon was specific to Gly538 or whether other Gly residues had an effect on enzyme activity. Surprisingly, all three of these Gly residues reduced the enzyme activity by more than 98%. Finally, the remaining mutation, F460A, caused inactivation of the enzyme.

All of the above results were confirmed using an alternative, scintillation-based assay. The OGT glycosylation assay was performed in ScintiStrip wells coated with Nup62, and incorporation of [C14]GlcNAc into Nup62 was determined by scintillation counting (TABLE TWO).

| Construct | Activity (mOGT) | Construct | Activity (CD II) |
|-----------|----------------|-----------|-----------------|
| mOGT      | 100            | F721A     | 10              |
| G402S     | <1             | W735A     | <1              |
| D407A     | <1             | W748A     | <1              |
| D422A     | 80             | F752A     | 110             |
| Y434A     | 65             | F776A     | 10              |
| D438A     | <1             | L796A     | <1              |
| G453S     | 5              | W812A     | <1              |
| F460A     | <1             | C836S     | 10              |
| G472A     | <1             | C839S     | <1              |
| E482A     | 5              | W878A     | <1              |
| D488A     | <1             |           |                 |
| G538S     | <1             |           |                 |
| E568A     | <1             |           |                 |

FIGURE 2. Activity of CD I mutants compared with wild type mOGT. The ability of CD I mutants to glycosylate nuclear pore protein Nup62 was compared with wild type mOGT. A, the degree of Nup62 glycosylation was measured for mOGT (100%) and for each mutant by quantifying the level of [C14]GlcNAc incorporation. Enzyme activity was normalized to the level of protein expression, as shown in C. B, visualization of [C14]GlcNAc incorporation into Nup62. The OGT assay reaction mixture was run on a 4–12% acrylamide gel, followed by staining with Simply SafeBlue. The gel was then soaked in En3Hance scintillation fluid, and the scintillant was precipitated with 10% polyethylene glycol. The gel was then dried and exposed to a BAS-IIIImaging system (Fuji), followed by development with a BAS-1500 phosphorus imager system (Fuji). C, protein expression levels of wild type mOGT and CD I mutants. The results presented are representative of at least three separate experiments.

Site-directed Mutagenesis of CD II Amino Acid Residues—The CD II domain of OGT is predicted to be a lectin domain, to be an allosteric regulatory site, or to be directly involved in binding of the donor sugar. Because aromatic residues have been previously implicated in binding reactions of lectins and glycosyltransferases with lectin-like domains (49), all of the aromatic amino acid residues in CD II were mutated. Three Phe residues and four Trp residues were mutated to Ala. Two Cys residues (Cys836 and Cys839) were also mutated. A complete list of CD II mutations can be found in TABLE ONE. As with CD I mutant constructs, enzyme activity of CD II mutant constructs was determined by the O-GlcNAc transferase assay, and the results were visualized by flu-
Mutagenesis of O-GlcNAc Transferase

![FIGURE 3. Activity of CD II mutants compared with wild type mOGT. The ability of CD II mutants to glycolylate nuclear pore protein Nup62 was compared with wild type mOGT. A, the degree of Nup62 glycolylation was measured for mOGT (100%) and for each mutant by quantifying the level of [C-14]GlcNAC incorporation. Enzyme activity was normalized to the level of protein expression as shown in B. B, visualization of [C-14]UDP-GlcNAC incorporation into Nup62. C, protein expression levels of wild type mOGT and CD II mutants. The results presented are representative of at least three separate experiments. * C365S showed great variability, as also seen by scintillation counting (TABLE TWO).](http://www.jbc.org/)

TABLE THREE

| Construct | $K_m$ (mM) | $V_{max}$ (nmol/mg/min) |
|-----------|------------|-------------------------|
| mOGT      | 0.8        | 125                     |
| D422A     | 0.29       | 1580                    |
| Y434A     | 1.9        | 717.4                   |
| F752A     | 0.15       | 660                     |
| F776A     | 0.44       | 53                      |

The kinetic parameters of activating mutations D422A, Y434A, and F752A were analyzed and compared with mOGT. The $K_m$ and $V_{max}$ values were determined with respect to UDP-GlcNAC, using the concentration range 1.5–12 μM.

The results suggest that at least some of the amino acid residues found within the GTGPF motif are important for enzyme activity. However, examination of this region shows that many of these residues are highly conserved across many of the different OGT species and therefore may be important for maintaining the structure of the enzyme. These residues do not, however, show a great degree of primary sequence homology with glycogen phosphorylase, the enzyme with which the GTGPF superfamily classification was determined. Once again, the above results were confirmed using an alternative, scintillation-based assay (TABLE TWO).

Kinetic Analysis of mOGT and Mutant Constructs D422A, Y434A, and F752A—Having shown that three of the mutations made to OGT could transfer a similar or increased amount of sugar nucleotide to Nup62, we next wanted to compare the kinetic properties of each with the native enzyme. Enzyme activity with respect to increasing concentrations of radiolabeled [C-14]UDP-GlcNAC was measured by the O-GlcNac transferase assay, and the results are presented in TABLE THREE. Wild type OGT exhibited a $K_m$ value of $0.8 \mu$M and a $V_{max}$ value of $125$ nmol/mg/min (TABLE THREE and Ref. 45). OGT mutant D422A appeared to have an overall increase in activity (Fig. 2B), which was found to be due to a lower $K_m$ value than wild type (0.29 μM) and an almost 14-fold higher $V_{max}$ value (1580 nmol/mg/min). OGT mutant Y434A had approximately double the $K_m$ value of wild type (1.9 μM) and a 5-fold increase in the $V_{max}$ value (717.4 nmol/mg/min; TABLE THREE). Finally, the F752A OGT mutant had a lower $K_m$ value (0.15 μM) and a higher $V_{max}$ value (660 nmol/mg/min; TABLE THREE). For comparison, the kinetics of F776A were determined. This mutation was not inactive but showed a ~76% reduction in enzyme activity. F776A had a $K_m$ value of 0.44 μM and a $V_{max}$ value of 53 nmol/mg/min (TABLE THREE).

To make certain that the mutant proteins used for kinetic analysis were not aggregated and maintained their multimeric status, native gel electrophoresis was performed (Fig. 4). mOGT, D422A, Y434A, F752A, and F776A were compared with a monomeric form of OGT (35); however, all mutants behaved like wild type. This indicates that the single point mutations made within the catalytic domain did not affect the stability or the aggregation and multimerization of the protein.

Homology Modeling of OGT with Members of the GTGPF Superfamily—We have recently determined the crystal structure of the TPR domain of OGT (35); however, the structure of the catalytic region is yet to be resolved. Having generated and determined the activity of OGT was placed into the glycogen phosphorylase superfamily (glycogen phosphorylase/glycogen transferase family (GTGPF)) by Wrabl and Grishin (50), based on its predicted three-dimensional structure consisting of two Rossmann-type fold domains. Enzymes in this family share a common motif; however, the importance of amino acids in the GTGPF motif and the effect that these residues have on catalytic activity have not been studied. Two of the mutations produced are within this motif, L796A and W812A. As mentioned above, all Trp residues reduced OGT activity to background levels. The second mutation of Leu706 to Ala was not predicted to have a large impact on enzyme activity; however, this substitution caused a 99–100% reduction in the level of activity when compared with wild type. These results suggest that at least some of the amino acid residues found within the GTGPF motif are important for enzyme activity. However, examination of this region shows that many of these residues are highly conserved across many of the different OGT species and therefore may be important for maintaining the structure of the enzyme. These residues do not, however, show a great degree of primary sequence homology with glycogen phosphorylase, the enzyme with which the GTGPF superfamily classification was determined. Once again, the above results were confirmed using an alternative, scintillation-based assay (TABLE TWO).

orography of incorporated [C-14]GlcNAC into Nup62 (Fig. 3B). The bands were quantified, and the resulting values were normalized as percentages of mOGT activity (Fig. 3A). All of the data were normalized to the concentration of OGT in each sample (Fig. 3C).

All of the CD II mutant constructs that contained a Trp to Ala substitution (W735A, W748A, W812A, and W878A) showed a dramatic reduction in enzyme activity. OGT activity of these mutants was reduced by 96–100%. Of the three Phe residues to be mutated, one caused an ~98% reduction in enzyme activity. The other two Phe residues had much less effect on enzyme activity, with F752A having a similar level of activity as wild type and F776A being reduced to ~24% of normal activity.

N-Ethylmaleimide and Alloxan inhibit OGT presumably by interfering with critical cysteine residues. Furthermore, dithiothreitol (a disulfide bond reducing agent) is known to increase the activity of wild type OGT (45), the importance of disulfide bonds, and their effect on producing active enzyme. The mechanism underlying this increase in activation is unknown; however, dithiothreitol is known to affect disulfide bonds between adjacent Cys residues or Cys residues that are in close proximity to one another. Two candidate residues for this type of interaction were Cys836 and Cys839. Each of these Cys residues was substituted with a Ser, and their OGT activity was determined. Both mutant enzymes caused a reduction in the level of enzyme activity. The C836S mutant showed variable activity, ranging from an ~40–90% reduction in activity, whereas C839S showed an ~93% reduction in activity. These results suggest that both Cys residues may be important for function of the enzyme; however, the difference in the level of OGT activity indicates that these residues may not interact with each other.

The kinetic parameters of activating mutations D422A, Y434A, and F752A were analyzed and compared with mOGT. The $K_m$ and $V_{max}$ values were determined with respect to UDP-GlcNAC, using the concentration range 1.5–12 μM.

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the substitutions described above in the CD I and CD II domains of OGT, a paper was published by Wrabl and Grishin (50), suggesting that OGT was part of the GTGP superfamily. These authors performed an alignment of OGT with other GTs known to have a similar three-dimensional structure. Of the 78 proteins presented in this alignment, five had known Protein Data Bank structures. We used four of these proteins to model the OGT mutations, two of which are presented in Fig. 5 (B and C). The fifth protein structure from the Wrabl alignment was not included because its function is too far removed from OGT to be considered useful. The four proteins used for modeling were yeast glycogen phosphorylase (Protein Data Bank code 1YGP), bacteriophage T4 8-glucosyltransferase (Protein Data Bank code 1I39), E. coli UDP-GlcNAc 2-epimerase (Protein Data Bank code 1F6D; Fig. 5B), and E. coli MurG glycosyltransferase (Protein Data Bank code 1NLM; Fig. 5C). We focused primarily on the two E. coli proteins (Fig. 5, B and C), because both utilize UDP-GlcNAc for their respective functions, analogous to OGT. Fig. 5A shows the Wrabl sequence alignment used to model each mutation.

The positions of substitutions made in OGT were mapped to E. coli UDP-GlcNAc 2-epimerase (Fig. 5B), which catalyzes the interconversion of UDP-GlcNAc to UDP-N-acetylmannosamine (UDP-ManNac) (51). This protein has a single catalytic active site that is formed by residues found in the cleft between the two Rossman fold domains. Three OGT CD I mutants were found in close proximity to this active site (Y434A, D554A, and E556A), as were four CD II mutants (F721A, F752A, F776A, and W812A). Other mutants were found far from the active site and are unlikely to be involved in the catalytic mechanism. Similar results were found with the E. coli MurG glycosyltransferase (Fig. 5C). This protein catalyzes the transfer of GlcNAc, from UDP-GlcNAc to the N-acetylmuramyl pentapeptide during biosynthesis of the bacterial cell wall (52) and also has a single active site formed by residues found in the cleft between the two Rossman fold domains. Two CD I mutants were found close to the active site (D554A and E556A), as were four CD II mutants (F752A, F776A, F776A, and W812A). Again, other mutants were far removed from the presumptive active site.

Two other GTs were used to model the substitutions made in OGT: the glycogen phosphorylase, the GT on which the GTGP superfamily was based, and the bacteriophage T4 8-glucosyltransferase (data not shown). Glycogen phosphorylase is different from the other three GTs, because it has two distinct active sites. Two mutants from CD I (F439A and D549A) were close to one active site, whereas all eight mutations from CD II were found near the other. The bacteriophage T4 8-glucosyltransferase has one active site created from residues found in the cleft between the two Rossman folds and catalyzes the transfer of glucose from UDP-glucose to DNA (42). Three mutations from CD I (Y434A, D554A, and E556A) and six mutations from CD II (F721A, W735A, F752A, F776A, L796A, and W812A) were found either in or adjacent to the active site. In all four molecules, the two Cys mutations (C8365 and C8395) were at the extremities of the protein and are unlikely to directly affect catalytic activity.

If analogous, OGT probably has only one active site for UDP-GlcNAc that is formed between the cleft of the two Rossman fold domains, similar to three of the structures from the GPGBP superfamily (T4 8-glucosyltransferase, E. coli UDP-GlcNAc 2-epimerase, and E. coli MurG glycosyltransferase). If a close-up view of the active site is made for the two UDP-GlcNAc-binding proteins (Fig. 5, B and C), the results suggest that residues Phe721 and Phe752 are potentially involved in either catalysis or substrate binding, because both are found in close proximity to the bound substrate in all three proteins. It also seems possible that Trp812 may play a role in the active site, because it is present in both E. coli glycosyltransferases.

**DISCUSSION**

The importance of O-GlcNAc addition as a post-translational modification is becoming apparent, with many studies implicating O-GlcNAc as a regulatory mechanism for target proteins (reviewed in Refs. 25 and 53). Although the roles and physiological importance of O-GlcNAc addition are becoming clearer, the catalytic mechanism required for sugar nucleotide recognition and transfer to target proteins remains to be determined. The substrate specificity of OGT for its target proteins is known to occur via an interaction with the TPR region of the molecule (35, 54). In this paper, we have investigated the two conserved regions within the catalytic domain and show that many conserved residues are important for activity. Molecular modeling of these substitutions to molecules of the GPGBP superfamily suggest that residues in both CD I and CD II are required to form a single sugar nucleotide-binding domain that may be positioned in the cleft between the two Rossman fold subunits of OGT.

*Mutagenesis*—To select potentially important residues for mutagenesis, comparisons were made with sequences and structures of other glycosyltransferase families that had known mechanisms for catalysis. These included the OGT homologue from *Arabidopsis thaliana*, SPY, as well as galactosyltransferases and members of the GPGBP superfamily.

Spindly (SPY) is an essential component of the gibberellin signaling pathway, which is involved in many important plant functions such as growth, germination, leaf greening, flowering time, and seed filling (55), some of which are analogous to human insulin signaling. Mutations at the *spy* locus produce plants with a “slender” phenotype (56), the only known, naturally occurring mutation of the OGT enzyme. Mammalian gene knock-out studies have shown that a lack of OGT is embryonic lethal (36, 57), thus preventing in depth in vivo functional studies. Many of the known *Arabidopsis* SPY mutations are exon deletion mutants or multiple amino acid deletion mutants (48). One of the more interesting SPY mutations relevant to this study (spy-3) is a single, glycine to serine conversion (48, 58) corresponding to position 538 in the CD I domain of OGT. This single point mutation was sufficient to disrupt many of the signaling pathways regulated by gibberellin (56). We have shown that mutation of this residue in mammals is sufficient to inactivate OGT. Other Gly residues within CD I were also inactivating, an unusual finding because Gly residues are not normally associated with catalytically important regions of these glycosyltransferases.

Catalytically important residues of other GTs have been previously studied; however, each family of GTs appear to have a specific

**FIGURE 4. Native gel electrophoresis.** Mutant proteins used for kinetic analysis were assayed by nondenaturing gel electrophoresis to determine their monomerization and aggregation status. Wild type mOGT and mutant proteins were compared with a known OGT monomer (33). Lane 1, standards (catalase 140,000; aldolase 160,000); lane 2, monomeric OGT (containing mutations at residues Trp349 and Ile351); lane 3, mOGT; lane 4, D422A; lane 5, Y434A; lane 6, F752A; lane 7, F776A.
mechanism for transferring substrate to acceptor molecule, making it difficult to predict which amino acid(s) are involved in direct nucleophilic attack of the sugar nucleotide substrate. A study of glycogen synthase suggested that a C-terminal E\text{X}E motif is involved, with the two glutamic acids responsible for catalysis (40). A crystal structure of T4\text{glucosyltransferase} suggested that a Glu and an Asp were responsible for catalysis (41), whereas the crystal structure of \textit{Neisseria meningitidis} LgtC showed that two Asp residues and an Arg residue were critical (43). Clearly acidic residues such as Asp and Glu are commonly required for nucleophilic attack of the sugar nucleotide substrate and hence were targeted for mutagenesis here. Conversely, Trp residues have been implicated in enzymes that bind NAD and NAD+ (49), which are common allosteric regulators. Because it was postulated that the CD II region of OGT may contain an allosteric site, Trp and another aromatic amino acid, Phe, were also targeted for mutagenesis. In addition, the lectin-like domains of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family of enzymes, contain a (QXW)\textsubscript{3} repeat sequence at their C termini (44). This QXW repeat is not present in OGT, although two QXW sequences are found within the CD II region and close to the C-terminal end of the OGT sequence. These Trp residues were also targeted for mutagenesis.

**FIGURE 5. Homology modeling of OGT amino acid substitutions.** Using the sequence alignment from Wrabl et al. (50) [A], the amino acids substituted in the mOGT mutants were modeled onto the crystal structures of four members of the GPGTF superfamily: yeast glycogen phosphorylase, bacteriophage T4\text{glucosyltransferase}, \textit{E. coli} UDP-GlcNAc 2-epimerase, and \textit{E. coli} MurG glycosyltransferase. Results for \textit{E. coli} UDP-GlcNAc 2-epimerase (B) and \textit{E. coli} MurG glycosyltransferase (C) are shown here. Mutated residues are shown with an electron density map, with red residues representing inactivating substitutions and green residues representing activating residues (mutated residues are also highlighted with an asterisk in (A) using the same color scheme). Yellow residues indicate amino acids found within the active site and purple indicates bound sugar nucleotide. A magnified view of the active site is also shown for \textit{E. coli} UDP-GlcNAc 2-epimerase (B) and \textit{E. coli} MurG glycosyltransferase (C).
Homology Modeling of Permissive Mutations—Of the 20 mutations generated in the CD I domain of OGT, almost all of those that produced protein were inactivating, suggesting that the structure of OGT has limited flexibility, with little room for modification before the activity is affected. This is also supported by the fact that the OGT protein is highly conserved between species. Two residues in CD I, however, were permissive substitutions and increased the amount of GlcNAc transferred to Nup62. Y434A was modeled against the GPGTF superfamily members and was shown in E. coli UDP-GlcNAc 2-epimerase to be in close proximity to the active site. This may suggest that substituting Tyr for Ala in this position decreases the steric hindrance of UDP-GlcNAc within the active site, allowing it either better access or closer proximity to the attacking nucleophilic residues. The second permissive substitution, Asp\(^{422}\) to Ala, could not be modeled because we have no information as to its spatial localization. Because it is only 12 residues from Tyr\(^{434}\) it may be involved in the active site. Aspartic acid residues are often involved in the catalytic reactions of GTs; thus Asp\(^{422}\) may play a role in the catalytic mechanism of OGT.

Of the eight CD II mutants modeled against GPGTF superfamily members (Fig. 5), seven were found to be inactivating. The one permissive mutation from CD II (F752) was in the active site of all four models and seemed to be close enough to the bound substrate to possibly interact with the sugar nucleotide. Alternatively, mutation of Phe to Ala may cause reduction in steric hindrance within the binding pocket, thereby allowing better access for UDP-GlcNAc.

The Phe\(^{752}\) residue is conserved among higher eukaryotes but has diverged in some of the plant and viral GTs (Arabidopsis, Petunia, Hordeum, and Haemophilus influenza) (50). This is the case for seven of these eight residues, with Leu\(^{796}\) the only conserved residue in nearly all species (except H. influenza). Leu\(^{796}\) was the only residue found within the conserved GPGTF motif, as described by Wrabl and Grishin (50) and, because of its highly conserved nature, it was not surprising that this residue was inactivating. Again, however, Leu\(^{796}\) was not positioned close to the active site, indicating that its role is probably more structural than catalytic.

Implications for Nutrient Sensing—It has been postulated that OGT plays a key role in nutrient sensing within the cell through its interaction with UDP-GlcNAc, the terminal product of the HBP (25). Insulin resistance is associated with an increase in the O-GlcNAcylation of cellular enzymes, with an ultimate aim of determining the role of OGT in insulin resistance. Although a precise understanding of sugar nucleotide recognition must await a crystal structure, these mutations in OGT will provide useful tools for examining the biological and enzymatic functions of OGT.

REFERENCES

1. Lubas, W. A., Frank, D. W., Krause, M., and Hanover, J. A. (1997) J. Biol. Chem. 272, 9316–9324
2. Kreppel, L. K., Blomberg, M. A., and Hart, G. W. (1997) J. Biol. Chem. 272, 9308–9315
3. Haltiwanger, R. S., Busby, S., Grove, K., Li, S., Mason, D., Medina, L., Moloney, D., Philipsberg, G., and Scaccoi, R. (1997) Biochem. Biophys. Res. Commun. 231, 237–242
4. Miller, M. W., Caracciolo, M. R., Berlin, W. K., and Hanover, J. A. (1999) Arch. Biochem. Biophys. 367, 51–60
5. Comer, F. I., and Hart, G. W. (2001) Biochemistry 40, 7845–7852
6. Kamermura, K., Hayes, B. K., Comer, F. I., and Hart, G. W. (2002) J. Biol. Chem. 277, 19229–19235
7. Hanover, J. A., Cohen, C. K., Willingham, M. C., and Park, M. K. (1987) J. Biol. Chem. 262, 9887–9894
8. Holt, G. D., Snow, C. M., Senior, A., Haltiwanger, R. S., Gerace, L., and Hart, G. W. (1987) J. Cell Biol. 104, 1157–1164
9. Davis, I. L. and Blobel, G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7552–7556
10. Kelly, W. G., Dahmus, M. E., and Hart, G. W. (1993) J. Biol. Chem. 268, 10416–10424
11. Cervoni, L., Turano, C., Ferraro, A., Ciavatta, P., Marmoroni, F., and Eufemi, M. (1997) FEBS Lett. 417, 227–230
12. Jackson, S. P., and Tjian, R. (1988) Cell 55, 125–133
13. Yang, X., Su, K., Roos, M. D., Chang, Q., Paterson, A. J., and Kudlow, J. E. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6611–6616
14. Iyer, S. P., Akimoto, Y., and Hart, G. W. (2003) J. Biol. Chem. 278, 5399–5409
15. Takahashi, M., Tsujokia, Y., Yamada, T., Tsuob, Y., Okada, H., Yamamoto, T., and Lapositas, Z. (1999) Acta Neuropathol. 97, 635–641
16. Arnold, C. S., Johnson, G. V., Cole, R. N., Dong, D. L., Lee, M., and Hart, G. W. (1996) J. Biol. Chem. 271, 82741–82744
17. Ding, M., and Vandre, D. D. (1996) J. Biol. Chem. 271, 12555–12561
18. Zhang, F., Su, K., Yang, X., Rowe, D. B., Paterson, A. J., and Kudlow, J. E. (2003) Cell 115, 715–725
19. Han, I., and Kudlow, J. E. (1997) Mol. Cell. Biol. 17, 2550–2558
20. Cole, R. N., and Hart, G. W. (1999) J. Neurochem. 73, 418–428
21. Luthi, T., Haltiwanger, R. S., Greenberg, P., and Bahler, M. (1991) J. Neurochem. 56, 1493–1498
22. Chou, T. Y., Dang, C. V., and Hart, G. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4417–4421
23. Medina, L., Grove, K., and Haltiwanger, R. S. (1998) Glycobiology 8, 383–391
24. Shaw, P., Freeman, J., Bovey, R., and Iggo, R. (1996) Oncogene 12, 921–930
25. Hanover, J. A. (2001) FASEB J. 15, 1865–1876
26. McClain, D. A., Lubas, W. A., Cooksey, R. C., Hazel, M., Parker, G. J., Love, D. C., and Hanover, J. A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 10695–10699
27. Marshall, S., Bacote, V., and Traxinger, R. (1991) J. Biol. Chem. 266, 4706–4712
28. Buse, M. G., Robinson, K. A., Marshall, B. A., Hresko, R. C., and Mueckler, M. (2002) Am. J. Physiol. 283, E241–E250
29. Hazel, M., Cooksey, R. C., Jones, D., Parker, G., Neidigh, J. L., Witherbee, B., Gulve, E. A., and McClain, D. A. (2004) Endocrinology 145, 2118–2128
30. Hawkins, M., Barzilai, N., Chen, W., Angelov, I., Hu, M., Cohen, P., and Rossetti, L. (1996) Diabetes 45, 1734–1743
31. Wells, L., Vosseller, K., and Hart, G. W. (2003) Cell Mol. Life Sci. 60, 222–228
32. Goebel, M. and Yanagida, M. (1991) Trends Biochem. Sci. 16, 173–177
33. Liu, F. H., Wu, S. J., Hu, S. M., Hsiao, C. D., and Wang, C. (1999) J. Biol. Chem. 274, 34425–34432
34. Das, A. K., Cohen, P. W., and Barford, D. (1998) EMBO J. 17, 1192–1199
35. Inke, M., Rehwinkel, I., Lazarus, B. D., Izaurelde, E., Hanover, J. A., and Conti, E. (2004) Nat. Struct. Mol. Biol. 11, 1001–1007
36. Hanover, J. A., Yu, S., Lubas, W. B., Shin, S. H., Ragano-Caracciola, M., Kochran, J., and Love, D. C. (2003) Arch. Biochem. Biophys. 409, 287–297
37. Roos, M. D., and Hanover, J. A. (2000) Biochem. Biophys. Res. Commun. 271, 275–280
38. Hagen, F. K., Hanes, B., Rallo, F., deSa, D., and Tabak, L. A. (1999) J. Biol. Chem. 274, 6797–6803
39. Breton, C., and Imberty, A. (1999) Curr. Opin. Struct. Biol. 9, 563–571
40. Cid, E., Gornis, R. R., Geremia, R. A., Guinovart, J. J., and Ferrer, J. C. (2000) J. Biol. Chem. 275, 33614–33621
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41. Morera, S., Imberty, A., Aschke-Sonnenborn, U., Ruger, W., and Freemont, P. S. (1999) J. Mol. Biol. 292, 717–730
42. Vrielink, A., Ruger, W., Driessen, H. P., and Freemont, P. S. (1994) EMBO J. 13, 3413–3422
43. Persson, K., Ly, H. D., Dieckelmann, M., Wakarchuk, W. W., Withers, S. G., and Strynadka, N. C. (2001) Nat. Struct. Biol. 8, 166–175
44. Tenno, M., Saeki, A., Kezely, F. J., Elhammer, A. P., and Kurosaka, A. (2002) J. Biol. Chem. 277, 47088–47096
45. Lubas, W. A., and Hanover, J. A. (2000) J. Biol. Chem. 275, 10983–10988
46. Lubas, W. A., Smith, M., Starr, C. M., and Hanover, J. A. (1995) Biochemistry 34, 1686–1694
47. Zhang, Y., Malinovskii, V. A., Fiedler, T. J., and Brew, K. (1999) Glycobiology 9, 815–822
48. Jacobsen, S. E., Binkowski, K. A., and Olszewski, N. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9292–9296
49. Beattie, B. K., Prentice, G. A., and Merrill, A. R. (1996) Biochemistry 35, 15134–15142
50. Wrabl, J. O., and Grishin, N. V. (2001) J. Mol. Biol. 314, 365–374
51. Campbell, R. E., Mosimann, S. C., Tanner, M. E., and Strynadka, N. C. (2000) Biochemistry 39, 14993–15001
52. Hu, Y., Chen, L., Ha, S., Gross, B., Falcone, B., Walker, D., Mokhtarzadeh, M., and Walker, S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 845–849
53. Wells, L., and Hart, G. W. (2003) FEBS Lett. 546, 154–158
54. Iyer, S. P., and Hart, G. W. (2003) J. Biol. Chem. 278, 24608–24616
55. Hedden, P., and Phillips, A. L. (2000) Trends Plant Sci. 5, 523–530
56. Jacobson, S. E., and Olszewski, N. E. (1993) Plant Cell 5, 887–896
57. Shafi, R., Iyer, S. P., Ellies, L. G., O’Donnell, N., Marek, K. W., Chui, D., Hart, G. W., and Marth, J. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5735–5739
58. Robertson, M., Swain, S. M., Chandler, P. M., and Olszewski, N. E. (1998) Plant Cell 10, 995–1007
59. Hawkins, M., Barzilai, N., Liu, R., Hu, M., Chen, W., and Rossetti, L. (1997) J. Clin. Investig. 99, 2173–2182
60. Rossetti, L., Hawkins, M., Chen, W., Gindi, J., and Barzilai, N. (1995) J. Clin. Investig. 96, 132–140
61. Robinson, K. A., Weinstein, M. L., Lindenmayer, G. E., and Buse, M. G. (1995) Diabetes 44, 1438–1446
62. Kreppel, L. K., and Hart, G. W. (1999) J. Biol. Chem. 274, 32015–32022.
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