The Noncatalytic Portion of Human UDP-glucose:Glycoprotein Glucosyltransferase I Confers UDP-glucose Binding and Transferase Function to the Catalytic Domain*

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The eukaryotic cell monitors the fidelity of protein folding in the endoplasmic reticulum and only permits properly folded and/or assembled proteins to transit to the Golgi compartment in a process termed “quality control.” An endoplasmic reticulum (ER) luminal sensor for quality control is the UDP-glucose:glycoprotein glucosyltransferase that targets unfolded glycoproteins for transient, calcium-dependent glycosylation. This modification mediates glycoprotein interaction with the folding machinery comprised of calnexin or calreticulin in conjunction with ERp57. Two human UGT homologues, HUGT1 and HUGT2, exist that share 55% identity. The highest degree of identity resides in the COOH-terminal 20% of these proteins, the putative catalytic domain of HUGT1. However, only HUGT1 displays the expected functional activity. The contribution of the NH2-terminal remainder of HUGT1 to glucosyltransferase function is presently unknown. In this report we demonstrate that HUGT2 is localized to the ER in a manner that overlaps the distribution of HUGT1. Analysis of a series of HUGT1 and HUGT2 chimeric proteins demonstrated that the carboxyl-terminal region of HUGT2 contains a catalytic domain that is functional in place of the analogous portion of HUGT1. Whereas neither catalytic domain displayed detectable activity when expressed alone, co-expression of either catalytic domain with the noncatalytic amino-terminal portion of HUGT1 conferred UDP-Glc binding and transfer of glucose that was specific for unfolded glycoprotein substrates. The results indicate that the amino-terminal 80% of HUGT1 is required for activation of the catalytic domain, whereas the homologous portion of HUGT2 cannot provide this function.

Proteins targeted to the extracellular environment, the plasma membrane, intracellular organelles, and the Golgi compartment are translocated into the endoplasmic reticulum (ER) in an unfolded state. Protein folding occurs as the polypeptide enters the oxidizing environment of the ER lumen with the assistance of molecular chaperones, including BiP, calnexin (CNX), and calreticulin (CRT) (1). CNX and CRT are membrane-bound and soluble isoforms, respectively, of a lectin that promotes proper protein folding and disulfide bond formation through association with the protein-disulfide isomerase homologue ERp57 (2–4). The carbohydrate binding specificities of CNX and CRT are similar, if not identical, recognizing the glucose-α(1,3)-mannose glycosidic bond present on high man- nose-containing asparagine-linked oligosaccharides (5–7). Upon translocation of proteins into the ER lumen, a 14-oligosaccharide core containing glucose3-mannose9-N-acetylglu- osaminе2 (Glc3Man9GlcNAc2) is added en masse to consensus asparagine residues (Asn-X-Ser/Thr, where X is any amino acid except Pro). Subsequently, the terminal glucose is cleaved by glucosidase I, and the remaining glucose residues are cleaved by glucosidase II (8). A monoglucosylated oligosaccharide is an intermediate in the trimming process and is the ligand for CNX and CRT interaction. CNX/CRT association prevents trafficking of unfolded glycoproteins from the ER to the Golgi compartment as part of the cellular quality control mechanism.

Glycoprotein release from CNX/CRT is associated with cleavage of the remaining glucose residue by glucosidase II. However, the folding status of the glycoprotein is not recognized by glucosidase II. It is the specific recognition of unfolded substrates by UDP-Glcglycoprotein glucosyltransferase (UGT), an enzyme that catalyzes the re-addition of a single glucose residue to Man7–9GlcNAc6 oligosaccharide core structures, that mediates retention of glycoproteins in the ER for prolonged participation in the CNX/CRT cycle and completion of the folding process.

UGT is a large glycoprotein (175 kDa) for which only a small region, the COOH-terminal catalytic domain, has a known function. The catalytic domain contains the DXD motif that coordinates donor sugar binding (9), although the catalytic residues involved in sugar transfer are unknown. Although additional domains have yet to be defined, recent work has provided insight into the function of the remainder of the protein. Significantly, data support that UGT specifically recognizes proteins in an advanced, although incomplete, state of folding (10). The ability to assess not only hydrophobicity of a substrate, but also globular structure, indicates that a complex recognition mechanism exists, perhaps spanning a large region of UGT that is difficult to define. In addition to glycoprotein folding status, UGT also recognizes the oligosaccharide to which glucose is transferred. Specifically, UGT recognizes the line; PC, Protein C; UDP-Glc, uridine diphosphate-glucose; UGT, UDP-glucose:glycoprotein glucosyltransferase; 1Cat, first catalytic; 2Cat, second catalytic; 5-N3UDP-Glc, 5-azidouridine 5′-diphosphoglucose.

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‡ The abbreviations used are: ER, endoplasmic reticulum; CNX, calnexin; CRT, calreticulin; FITC, fluorescein isothiocyanate; Glc, glucose; GlcNAc, N-acetylglucosamine; HUGT, human UDP-glucose:glycoprotein glucosyltransferase; Man, mannose; PBS, phosphate-buffered salicylic acid; PO1, project, HL57346 and by NIGMS National Institutes of Health Grant HL57346.
innermost N-acetylglucosamine of the substrate (11), and prefers high-mannose structures containing 7–9 mannose residues (12). These findings suggest the participation of a binding pocket or cleft in the recognition of oligosaccharide structure for glucose transfer. Homologous bacterial glycosyltransferases comprised solely of the analogous catalytic domain share characteristics of substrate oligosaccharide recognition, but lack the requirement for detection of substrate folding status. These findings support the hypothesis that the primary function of the noncatalytic portion of UGT is to monitor protein folding status.

Previously, two human sequences with homology to known UGTs from Drosophila and Schizosaccharomyces pombe were isolated, HUGT1 and HUGT2 (13). Although indistinguishable in size and highly identical (83%) to one another in their proposed catalytic regions, the human homologues are significantly dissimilar (49%) in their noncatalytic portions. In addition, HUGT2 does not display the defined glucosyltransferase activity characteristic of UGT homologues, whereas HUGT1 is a functional UGT. As a consequence, we hypothesized that the noncatalytic portion of UGT might define the nature of the catalysis mediated by the COOH-terminal domain. To test this hypothesis, we studied genetically engineered chimeras of HUGT1 and HUGT2 to elucidate the requirements for the noncatalytic portion to regulate UGT activity. Surprisingly, the results demonstrate that the noncatalytic portion of HUGT1 does indeed activate a glucosyltransferase activity for both the HUGT1 and HUGT2 catalytic domains.

**EXPERIMENTAL PROCEDURES**

**Design of Constructs**—Previously described HUGT1 and HUGT2 constructs in pedAc (13) were modified by sequence replacement with fragments generated by polymerase chain reaction or by restriction endonuclease digestion. PCR fragments were designed to contain, at their ends, sequence-specific restriction endonuclease digestion sites for ligation. All DNA constructs incorporating sequences generated by PCR were verified by DNA sequence analysis. All constructs, except where noted, were tagged with the Protein C epitope to facilitate protein purification. The Protein C epitope mediates a calcium-dependent binding to an anti-Protein C antibody affinity matrix (Roche) that can be used for purification. The Protein C (PC) epitope, EDQVDPLRDLGK, was placed at the COOH terminus of each construct, just upstream of the terminal amino acids (KREEL for HUGT1 COOH termini or THDEL for HUGT2 COOH termini) to maintain the ER-retrieval signal function. This was also true of the 1N80-PC and 2N80-PC truncations, in that the PC epitope, linked to the appropriate retrieval signal, immediately followed these noncatalytic sequences. The non-PC-tagged versions of these two constructs were equipped with retrieval signals as well. The 1C80-PC, 1Cat-PC, and 2Cat-PC constructs were each designed to contain a signal sequence for translocation into the ER lumen. The signal peptides of HUGT1 and HUGT2 (13) were tethered directly to the sequences comprising the COOH-terminal 80% of HUGT1 or the catalytic domains of HUGT1 or HUGT2, and were predicted to be cleavable (SignalP server (14)).

**Production and Testing of HUGT2 Antibody**—The 16 COOH-terminal amino acids of HUGT2, HLENKKQDTLTHDEL, were chosen for synthesis of a multiantigenic peptide (Biomedical Research Core Facilities, University of Michigan). This peptide, in conjunction with Freund’s adjuvant, was used to produce a rabbit polyclonal antibody (Cocalico Biologicals, Inc., Reamstown, PA). The acidic multiantigenic peptide (9 mg) was conjugated to a 500-μl bed volume of Affi-Gel 15 (Bio-Rad) in 10 mM phosphate buffer, pH 7.4 (3 ml total volume), and unbound peptide was removed with acid elution (100 mM glycine, pH 2.5, 10% ethylene glycol, 0.02% NaN3). The antibody was precipitated from serum overnight with an equal volume of saturated ammonium sulfate, dialyzed against 1× phosphate-buffered saline (PBS), and affinity purified with acid elution (buffer same as above). Elution fractions were neutralized with 1.5 mM Tris, pH 8, and fractions with highest titer were dialyzed against 1× PBS.

COS-1 cells were transfected by the DEAE-dextran method with 6 μg of pEDAC (backbone vector), HUGT1, or HUGT2 per 10-cm dish. Each plate additionally contained 2 μg of the pEDpvr vector to permit selection of uninfected cells with G418. G418 was added 6–11 h post-transfection for a period of 24 h. Lysates were then collected in 1× Nonidet P-40 buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8, and 1× Complete EDTA-free protease inhibitor mixture (Roche). For Western blot analysis, 50 μg of total lysate was analyzed per sample (polyclonal rat antibody 6G733 for recognition of HUGT1 kindly provided by Armando Parodi) and the HUGT1 and HUGT2 antibodies were used at a dilution of 1:100. Secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (Invitrogen, Carlsbad, CA) was used at a dilution of 1:5000, followed by ECL detection (Amersham Biosciences).

For immunofluorescence, transfected cells were split onto coverslips in 6-well plates at 36 h post-transfection. HUGT2 was transfected alone or co-transfected with HUGT1-PC. Immunostaining and fluorescence microscopy was performed as above (15) with few exceptions. The HUGT2 antibody was used at a dilution of 1:100, as was the mouse monoclonal antibody to Protein C (catalog number 1814508; Roche). A third, monoclonal antibody capable of recognizing the KDEL retrieval signal (catalog item SPA827; Stressgen, Victoria, BC, Canada) was used at a dilution of 1:200 to display ER localization. Primary antibodies were left on cells overnight. Fluorescein isothiocyanate-conjugated α-mouse and Texas Red-conjugated α-rabbit secondary antibodies (Molecular Probes, Eugene, OR) were employed at 1:500 dilutions, and a 1:10,000 dilution of 4,6-diamidino-2-phenylindole was used to demonstrate nuclear staining. Cells were viewed with an Olympus BX60 fluorescence microscope at ×80 magnification.

**Immunoprecipitation of PC-tagged Constructs**—COS-1 cells co-transfected with the pEDpdr plasmid and pED or PC-tagged constructs (method same as described above) were washed twice with cold 1× PBS and lysed in 1% Nonidet P-40 buffer with protease inhibitors (same buffer as above). Each lysate was cleared of cellular debris and added to a 50-μl bed volume of α-Protein C affinity matrix (catalog number 1815024, Roche). For highest affinity interaction with the resin, the lysates were exposed with agitation at 4 °C overnight. They were then washed with four 1-ml aliquots of cold lysis buffer and eluted at 4 °C in 50–100 μl of a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 5 mM EDTA. EDTA dissociates the Protein C epitope from the affinity matrix.

**UGT Assay**—For each activity assay, a solution of 20 mg/ml thyroglobulin (7.5 mg/ml Tris-HCl, pH 7.4 (3 ml total volume), and unbound was placed at 4 °C, under a nitrogen atmosphere, on a Millipore 1225 sampling manifold (Millipore). Filters were washed with cold 10% trichloroacetic acid, cold 100% ethanol, and acetone. Filters were dried and subjected to liquid scintillation for determination of 14C incorporation. Four samples were processed per construct per experiment: duplicate samples for each of 2 volumes of eluate or lysate. Background radioactivity (measured in samples containing all components except enzyme) was subtracted from each reading, counts per microliter of eluate or per microgram of lysate were determined, and the four samples were averaged. For eluted samples, Western blots of the proteins used for activity assay were analyzed by NIH image software. This information was then used to determine the relative percentage of HUGT1 in the construct. Activities were determined after a 16-h incubation of substrate with enzyme to detect very low levels of activity. Therefore, the activity values do not represent a stringent kinetic analysis, but rather a value relative to wild-type HUGT1.

**Photaffinity Cross-linking Analysis**—Constructs purified on the basis of PC epitope (pEDAC, pH 7.4) were combined with an equal volume of CaCl2, such that the final mixture consisted of the following: 25 mM CaCl2, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 2.5 mM EDTA. Each standard reaction was initiated by adding the components of the mixture to a tube containing the cross-linker, [β-32P1]-azidoazidine 5-diphosphoglucone ([β-32P1]-azido-5-P[14C]G6P, 1.8–2.6 Ci/mmol; Affinity Labeling Technology, Lexington, KY) in a final volume of 4 μl and cross-linker concentration of 40 μM. After 1 min, samples were either maintained or exposed to UV with a handheld 254-nm lamp (UVG-11; UVP, Inc., Upland, CA) at a distance of 4 cm for a period of 90 s. Reactions were stopped with the addition of 2 volumes of 10% trichlo-
roacetate acid. For competition, cold UDP-Glc was added to the mixtures (final concentration, 0.2–1 mM) for 15 min prior to incubation with the cross-linker. The reactions then proceeded as above. Samples were processed for autoradiography by addition of SDS-PAGE loading buffer (responsible for smeary background of samples in Figs. 3C and 5D) or with a gel-loading buffer modified by the addition of 4 M urea as described previously (15) (Fig. 4C). The results shown in Figs. 3–5 are representative of two to three cross-linking analyses per construct in which consistent results were obtained.

\[ ^{[35S]} \text{Methionine/Cysteine Labeling of Transfected COS-1 Cells—} \]

COS-1 cells, in 10-cm plates, transfected with 3 μg of p1N80 or 2N80, 3 μg of 1Cat or 2Cat, and 2 mCi of pEDpur (transfection method described above), were washed twice with 1× PBS at the end of the transfection period, then incubated with 1 ml of Dulbecco’s modified Eagle’s medium without methionine or cysteine (Invitrogen) for 15 min. \[ ^{[35S]} \text{Methionine/cysteine (1000 Ci/mmol, Amersham Biosciences) in cysteine/methionine-free Dulbecco’s modified Eagle’s medium was then added for 1 h in a 37 °C incubator in a volume of 1 ml. Cells were washed twice with cold 1× PBS, lysates were collected in 1% Nonidet P-40 buffer (same as above), and PC purification was performed as described above.}

**RESULTS**

Expression of HUGT2—To analyze HUGT2 expression and function, an affinity purified rabbit polyclonal antibody was produced that reacts with the unique carboxyl-terminal 16 residues of HUGT2. The specificity of the antibody for HUGT2 was analyzed by Western blot reactivity using extracts from COS-1 cells transfected with vector alone or with vectors directing expression of HUGT1 and HUGT2 (Fig. 1A). Western blot analysis using an antibody specific for rat UGT1 detected increased levels of UGT1 specifically in HUGT1-transfected cells (lane 2, compare with lanes 1 and 3). In parallel Western blot analysis, HUGT2 antibody reacted uniquely with HUGT2 and not with HUGT1 (lane 6, compare with lane 5). In addition, the HUGT2 antibody detected the endogenous COS-1 cell UGT2 (lanes 4 and 5). The UGT2 signals in extracts from vector-transfected cells were likely specific, as stripping and reprobing with secondary antibody alone did not produce bands in this region (data not shown). In addition, unidentified bands in Fig. 1A, lanes 4–6, were attributable to the secondary antibody. These results demonstrate that the two polyclonal antibodies can distinguish between HUGT1 and HUGT2.

The intracellular localization of HUGT1 and HUGT2 was analyzed in transfected COS-1 cells. Although the HUGT2-specific antibody did not detect endogenous UGT2 by this method, it did recognize overexpressed HUGT2 (Fig. 1, panels B and E). The cellular distribution of HUGT2 reactivity was compared with reactivity with an α-KDEL antibody (Fig. 1, panel C) that recognizes the ER markers BiP and GRP94 as well as an unidentified 55-kDa protein. Upon merging these images, it became apparent that the majority of HUGT2 colocalized with the ER marker proteins, although there were some differences in their ER distributions (Fig. 1, panel D). Because a similar distributional disparity was previously described for HUGT1 (13), the expression pattern of HUGT2 was directly compared with that of a PC epitope-tagged HUGT1. Use of a PC-tagged form of HUGT1 was necessary because the rat UGT1 antibody, like α-HUGT2-peptide, was generated in rabbit and would not be distinguishable on the basis of reactivity to secondary fluorescence-labeled antibody. At the level of resolution achievable by light microscopy, the localization of PC-tagged HUGT1 (Fig. 1, panel F) was highly comparable with that of HUGT2 (Fig. 1, panel E) as evidenced by analysis of the merged images (Fig. 1, panel G).

The Catalytic Domain of HUGT2 Can Replace That of HUGT1—Previous studies demonstrated that recombinant HUGT2 expressed in transfected COS-1 cells was not active under conditions where recombinant HUGT1 was active (13). To analyze the contribution of distinct domains of the protein to catalysis, chimeras between HUGT1 and HUGT2 were constructed. Using sequence homology as a basis for comparison, both HUGT1 and HUGT2 were subdivided into 5 “domains” of approximately equal size (Figs. 2 and 3A). The initial set of chimeras was designed to determine whether or not the region of HUGT2 sharing homology with the highly conserved COOH terminus of active glucosyltransferases was competent for catalysis in the context of HUGT1. The first chimera, 1(80)/2(20)
PC, constituted an exchange of domain 5 of HUGT1, the catalytic domain, for domain 5 of HUGT2 on the full-length HUGT1 sequence (Fig. 3A, line III). PC refers to the Protein C epitope that was chosen for its ability to be dissociated from antibody following immunoadsorption. The numbers in parentheses refer to the approximate percentage of each molecule represented in consecutive amino acid sequence. The catalytic domain 5 is defined as previously described (13) on the basis of homology to sequences and structural information for members of the most closely related glycosyltransferase family (Family 8 (16)). A second chimera, 1(60)/2(40)-PC (Fig. 3A, line IV), extended the analysis to include domain 4 of HUGT2 in the swap in the event that the proposed catalytic domain was not inclusive of all residues required for UGT function. To control for the possibility that the absence of the HUGT2 function was attributable to a catalytic mutation, the chimeras 2(80)/1(20)-PC and 2(60)/1(40)-PC were constructed to replace the COOH-terminal region of HUGT2 with the analogous portions of HUGT1 (Fig. 3A, lines V and VI, respectively).

Recombinant proteins were purified from transfected COS-1 cell extracts by PC affinity chromatography and yields were measured by Western blot analysis using antibody that reacts with the PC tag (Fig. 3B). The UGT activities relative to that of wild-type HUGT1 were determined and normalized to PC epitope reactivity. Of the four chimeras, only the chimera containing the NH2-terminal 80% of HUGT1 adjoined to the catalytic domain-like portion of HUGT2, 1(80)/2(20)-PC, displayed activity in the standard assay (Fig. 3A, line III). An HUGT1 construct truncated after domain 4 (1N80-PC), and therefore missing the proposed catalytic domain, was not active (Fig. 3A, line VII), suggesting that the activity obtained from 1(80)/2(20)-PC required the catalytic domain-like region of HUGT2. Finally, the construct 1(60)/2(20)/1(20)-PC was also inactive (Fig. 3A, line VIII), suggesting that the subcatalytic region, domain 4, of HUGT2 may be defective for UGT activity. Analysis of the activity of chimeric constructs in cell lysates not subjected to the immunoprecipitation process generated similar relative amounts of activity, demonstrating that there was no loss of activity during the purification procedure (not shown).
To determine whether HUGT2 was inactive because of a defect in UDP-Glc binding, binding to a photoaffinity cross-linker, \([/\text{H}9252]-32\text{P}]5-\text{N}3\text{UDP-Glc}\), was measured. With the notable exception of 1N80-PC (Fig. 3C, lane 23), only those constructs displaying glucosyltransferase activity bound the cross-linker at levels above background (Fig. 3C, compare lanes 5 and 11 to lanes 8, 14, 17, 20, and 26). Cross-linker binding required exposure to ultraviolet light (Fig. 3C, compare lanes 5, 11, and 23 to lanes 4, 10, and 22, respectively) and was partially competed by the presence of a 5-fold excess of unlabeled UDP-Glc (Fig. 3C, compare lanes 5, 11, and 23 to lanes 6, 12, and 24, respectively), demonstrating specificity. Comparison of the relative protein levels evidenced by Western blot analysis in Fig. 3B to the labeling intensities in Fig. 3C demonstrated that the absence of cross-linking was not because of low recovery of immunoaffinity purified protein. Unexpectedly, the cross-linker binding specificity of the noncatalytic region of HUGT1, 1N80-PC (lanes 22–24), was confirmed in numerous experiments where it was clearer that significant binding did not take place in the absence of UV irradiation (not shown). The significance of these findings is not presently understood.

**Two Regions within the Noncatalytic Portion of HUGT1 Are Required for Glucosyltransferase Function**—Analysis of the first set of chimeras indicated that domain 4 of HUGT1 was necessary for function (Fig. 3A, compare line III with line IV). However, it could not confer glucosyltransferase function to HUGT2 (Fig. 3A, line VI). To identify HUGT1 domains that may, in combination with domain 4, activate HUGT2 catalytic

![Fig. 3](image-url)
activity, chimeric proteins were further extended to include domain 3 and domains 2 and 3, 2(20)/1(80)-PC and 2(20)/1(80)-PC, respectively (Fig. 4A, lines I and II, respectively). Of the two chimeras, only 2(20)/1(80)-PC was active (Fig. 4A, line II).

A truncation construct comprising the COOH-terminal 80% of HUGT1, 1C80-PC, was inactive (Fig. 4A, line III), verifying that the activity of the 2(20)/1(80)-PC chimera required the N terminus of HUGT2. An additional control, 1(20)/2(20)/1(60)-PC, was inactive as well (Fig. 4A, line IV), indicating that domain 2 of HUGT1 is also required to confer glucosyltransferase activity to the catalytic domain. A final control, 1(40)/2(20)/1(40)-PC, consistently displayed a low transferase activity (Fig. 4A, line V), localizing the regions required to confer glucosyltransferase activity to domains 2 and 4.

Photoaffinity cross-linking was used to measure the ability of these constructs to bind UDP-Glc for donor sugar transfer. Only constructs that had glucosyltransferase activity bound cross-linker above background levels in a manner that was UV-dependent and competed with a 25-fold molar excess of unlabeled UDP-Glc (Fig. 4C, lanes 5 and 6 and lanes 14 and 15). The reduced binding of 1(40)/2(20)/1(40)-PC to UDP-Glc (Fig. 4C, lane 14) correlated with the low level of UGT activity detected for this chimera. Western blot analysis of the amount of protein present (Fig. 4B) demonstrated that absence of activity and cross-linking were not a consequence of low protein yield from purification.

Co-expression of the NH₂-terminal Regulatory Domain of HUGT1 Activates the Catalytic Domains of HUGT1 and HUGT2—While analyzing the ability of the rat UGT homologue to bind the photoaffinity cross-linker [β-³²P]5-N₃UDP-Glc, Tessier et al. (9) detected cross-linking with a 37-kDa proteolytic fragment of RUGT. NH₂-terminal sequence analysis identified this fragment as the putative catalytic domain. The corresponding sequence was cloned, expressed, and shown to have a low level of transferase activity toward unfolded substrate, although its ability to bind cross-linker was not confirmed (9). The catalytic domains of HUGT1 and HUGT2 were therefore independently expressed for verification of glucosyltransferase function as a means to test the inhibitory potential of the noncatalytic region of HUGT2 (2N80, Fig. 5, line I) by in vitro competition assay. Surprisingly, both 1Cat-PC and 2Cat-PC were defective in transfer of glucose to thyroglobulin (Fig. 5A, lines II and III, respectively) and in binding to the photoaffinity cross-linker (Fig. 5D, lanes 5 and 8, respectively). The active proteolytic fragment of RUGT (9) is slightly larger than the region defined as the catalytic domain for HUGT1 and HUGT2 (this NH₂-terminal extension is denoted by an asterisk in Fig. 2). To determine whether a 12-residue extension of 1Cat-PC, or the analogous 11-residue extension of 2Cat-PC, was necessary for catalytic function and/or UDP-Glc cross-linking, two additional Cat constructs were made that incorporated these sequence extensions. By our analysis, nei-
The noncatalytic portion of HUGT1 can activate UGT function of the HUGT1 and HUGT2 catalytic domains. Panel A, schematic representation of catalytic and noncatalytic portions of HUGT1 and HUGT2 expressed independently (lines I-III, VIII, and IX) or together (lines IV-VII). All constructs were PC-tagged with the exception of the 1N80 and 2N80 constructs used in cotransfections. Activities shown were normalized to the expression levels quantified by Western analysis in panel B. With the exception of constructs in lines III, VIII, and IX, for which single measurements were obtained, activities described in panel A were derived from one of three separate transfection experiments, but are representative of the results consistently obtained. Ranges described represent separate quantification for the higher and lower bands of the catalytic domain doublets, respectively. Panel B, α-PC Western analysis of purified constructs used for the activity and cross-linking analyses described elsewhere in the figure. Although faint bands corresponding to the molecular weight of 2N80 appear to be coprecipitating with the Cat domains in lanes 7 and 8, a subsequent repeat of the Western blot with the same samples did not reproduce this PC reactivity (not shown). Panel C, immunoprecipitation of radiolabeled, untagged 1N80 or 2N80 constructs with radiolabeled, tagged chimeric constructs. Panel D, autoradiograph of photoaffinity samples analyzed as in Fig. 3, panel C. A lighter exposure is also shown for the 2Cat-PC and 1N80 co-transfection (lanes 16–18) because of an ambiguous, high background in the no UV lane (lane 13).
ther modified catalytic domain was active or capable of binding the UDP-Glc photoaffinity cross-linker (data not shown). In addition, a 1(80)/2(20)-PC construct incorporating the 11-residue extension of 2Cat retained activity of a level comparable with that of the original 1(80)/2(20)-PC construct (data not shown), indicating that the catalytic domain of HUGT2, as defined by either boundary, could function in place of the HUGT1 catalytic domain.

Given the apparent inability of either isolated catalytic domain to function independently, the capacity for the HUGT1 NH2-terminal region (1N80) to activate the catalytic potential of either 1Cat-PC or 2Cat-PC was tested by co-expression of the isolated domains in COS-1 cells. Interestingly, expression of either catalytic domain in the presence of the 1N80 construct partially reconstituted glucosyltransferase activity (Fig. 5A, lines IV and V) and binding to the photoaffinity cross-linker (Fig. 5D, lanes 11 and 14/17). 5-fold excess of unlabeled UDP-Glc used to demonstrate specificity in lanes 12 and 15/18). The reconstituted activity was specific for denatured, and not native, thyroglobulin (not shown). In addition, to regenerate catalytic activity, it was necessary to express both domains in the same cell as no measurable activity (Fig. 5A, lines VIII and IX) or stimulation of cross-linking to UDP-Glc (not shown) was obtained upon co-incubation of isolated domains purified from independently transfected cells.

Western blot analysis demonstrated that the levels of catalytic domain expression were similar in the presence and absence of co-expressed, untagged 1N80 (Fig. 5B, compare lanes 3 and 4 with lanes 5 and 6, respectively). Association between the co-expressed, separated domains was confirmed by the analysis of PC-absorbed [35S]Met/Cys-labeled cell lysate (Fig. 5C). Isolation of each PC-tagged Cat domain co-purified untagged 1N80 (Fig. 5C, lanes 2 and 3). Although the untagged, noncatalytic portion of HUGT2 (2N80) could also be pulled down with either 1Cat-PC or 2Cat-PC (Fig. 5C, lanes 7 and 8, respectively), there was no stimulation of catalysis (Fig. 5A, lines VI and VII, respectively) or binding to the UDP-Glc cross-linker (Fig. 5D, lanes 20 and 23, respectively).

DISCUSSION

The murine and human genomes encode two homologues of UGT (13). Although HUGT2 shares high identity with HUGT1 in the proposed catalytic domain and is of a similar size with 55% overall identity, the two differ in striking ways. Notably, HUGT2 is not active toward several known UGT substrates, including denatured thyroglobulin and denatured RNase B. Although HUGT1 and HUGT2 are transcribed in all tissues studied, transcription of HUGT1 is induced upon ER stress, whereas these conditions do not induce expression of HUGT2 mRNA (13).

The purpose of this study was to identify the amino acid sequences responsible for the functional disparity between HUGT1 and HUGT2. We first characterized the endogenous expression and cellular localization of HUGT2 as a means of demonstrating its potential to play some role as an ER glucosyltransferase. A specific rabbit antibody was successfully raised against the COOH-terminal 16 amino acids of HUGT2, a region sharing no homology with HUGT1. Immunofluorescence analysis of transfected cells demonstrated colocalization of HUGT1 and HUGT2, whereas the expression pattern for HUGT2 differed mildly from that observed for proteins recognized by an α-KDEL antibody, consistent with previous observations for HUGT1 (13). Importantly, endogenous UGT2 protein was detected in COS-1 cells, indicating that the endogenous gene is functional (not a pseudogene).

Glycosyltransferases homologous to HUGT1 and HUGT2 transfer their donor sugars in the presence of Mn2+ or Mg2+ in place of Ca2+, but neither of these cofactors conferred glucosyltransferase activity to HUGT2 (data not shown). Additionally, increasing the concentration of UDP-Glc in the activity assay had no affect on the catalysis, indicating that HUGT2 activity was not simply undetectable because of a reduced affinity for the donor sugar (data not shown).

To evaluate the dependence of catalytic function on the remainder of the UGT protein, chimeric proteins were produced and tested for UGT activity. Surprisingly, when the HUGT2 catalytic domain replaced that of HUGT1, the resulting chimeric protein was functional and bound the photoaffinity cross-linker [α-32P]5-UDP-Glc, indicating that the amino acid sequence of the HUGT2 catalytic domain had transferase activity and could bind UDP-Glc when placed in the context of HUGT1. It is plausible that the noncatalytic portions of these proteins dictate donor sugar specificities for their catalytic domains and that HUGT2 could transfer a different donor sugar as specified by its noncatalytic component. Alternatively, the catalytic domain of HUGT2 might not exist in an active or open conformation in the context of the remainder of the protein, therefore precluding it from functioning as a glucosyltransferase. For example, some region of the noncatalytic portion of HUGT2 may inhibit its glucosyltransferase activity through destabilization of the catalytic domain in a manner that requires the binding of an additional component for functional activity. Conversely, a cofactor may interact with the catalytic domain of HUGT2 and block access to UDP-Glc. A final possibility is that HUGT2 plays a role in cells that is independent of transferase function. For example, the nonfunctional ER mannosidase 1 homologue EDEM, instead of exhibiting mannosidase activity, appears to identify targets for ER-associated degradation (17–19). Interestingly, EDEM immunoprecipitation data identified an association with a protein of ~175 kDa. It is intriguing to conjecture that EDEM might associate with HUGT2 to successfully recognize unfolded substrates. Because EDEM is ER stress-inducible (20), it would suggest that HUGT1 provides this type of role because it is stress inducible, whereas HUGT2 is not. However, if the endogenous level of HUGT2 expression is sufficiently high and supported by a long half-life, its induction for the purpose of participation in ER-associated degradation may be unnecessary. Alternatively, its substrate specificity might extend to a select subset of proteins that have a high ER turnover rate. If HUGT2 does select unfolded glycoproteins for ER-associated degradation, it is possible that it is an inactive homologue for which a novel function has evolved, similar to EDEM.

The inability of HUGT1 glucosyltransferase activity to tolerate substitutions with analogous regions of HUGT2 appeared to result from a loss of affinity for UDP-Glc. This is supported by the following: 1) in all cases, the function of a chimeric protein correlated with its ability to bind the UDP-Glc photoaffinity cross-linker, and 2) glycoprotein substrate was not required for, nor did it enhance, binding of UDP-Glc to the wild-type HUGT1 (data not shown). Therefore, we propose that the conformations of the inactive chimeric proteins prevented their association with UDP-Glc. It is possible that structural changes in inactive chimeras blocked access of their catalytic domains to UDP-Glc. Alternatively, sequences within the N-terminal region of HUGT2 might have inhibited catalytic activity, either directly or via a cofactor. The spatial separation of the two regions of HUGT2 that inhibit HUGT1 activity (i.e., sub-N-terminal and subcatalytic) would suggest this inhibition might require an association between these regions in the tertiary fold. Similarly, the juxtaposition of the homologous regions in wild-type HUGT1 may confer transferase activity to the catalytic domain. Importantly, the divergence in homology

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between analogous domains of HUGT1 and HUGT2 does not correlate with the loss of activity associated with inactive chimeric proteins. In other words, the sub-NH2-terminal (domain 2) and subcatalytic (domain 4) regions of HUGT2 have the same degree of homology with HUGT1 as do the NH2-terminal (domain 1) and middle (domain 3) portions of HUGT2. In fact, the largest gap between the two homologues, a stretch of 8 amino acids present in HUGT1 but not HUGT2, is localized to domain 3; a swap of domain 3 of HUGT2 onto HUGT1 results in a chimera that maintains, albeit limited, activity. Importantly, removal of these 8 amino acids from HUGT1 had no effect on activity (not shown).

The boundaries of the catalytic domains of HUGT1 and HUGT2 were defined by amino acid homology with bacterial glycosyltransferases belonging to Family 8. The delineation was confirmed by modeling of the HUGT1 and HUGT2 sequence onto that of a Family 8 galactosyltransferase, Lgtc from Neisseria meningitides (for which the crystal structure was recently solved (21)) via the 3DPSSM Protein Fold Recognition server.2 Interestingly, when the catalytic domains were independently expressed, they were not active nor did they bind UDP-Glc photoaffinity cross-linking to the catalytic portion of HUGT1 partially restored functional activity (not shown). However, co-expression with the non-catalytic portion of HUGT1 partially restored functional activity and UDP-Glc photoaffinity cross-linking to the catalytic domains of both HUGT1 and HUGT2. During the preparation of this manuscript, Guerin and Parodi (22) reported that wild-type S. pombe UGT1 is readily cleaved to an active heterodimer consisting of a 38-kDa fragment comprising the COOH-terminal catalytic domain and a 135-kDa NH2-terminal fragment representing the remainder of the protein. In addition, co-expression of the separated domains in a UGT-null S. pombe mutant restored glucosyltransferase activity, whereas expression of the catalytic domain alone did not. Our results extend these observations by showing that the catalytic domains of HUGT1 and HUGT2 require some information from the non-catalytic region of HUGT1 to permit binding of UDP-Glc. It is possible that the analogous region of HUGT2 aids in recognition of a donor sugar other than UDP-Glc. This is compatible with the finding that the catalytic domain of HUGT2 can substitute that of HUGT1, and implies that the catalytic domains are interchangeable because they do not determine donor sugar specificity. Support for this interpretation awaits the identification of a donor sugar capable of binding wild-type HUGT2.

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REFERENCES
1. Gething, M. J., and Sambrook, J. (1992) Nature 355, 33–45.
2. Wada, I., Imai, S., Kai, M., Sakane, F., and Kanoh, H. (1995) J. Biol. Chem. 270, 20298–20304.
3. Oliver, J. D., van der Wal, F. J., Bulleid, N. J., and High, S. (1997) Science 275, 86–88.
4. Zapan, A., Darby, N. J., Tessier, D. C., Michalak, M., Bergeron, J. J., and Thomas, D. Y. (1998) J. Biol. Chem. 273, 6009–6012.
5. Hammond, C., Braakman, I., and Helenius, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 913–917.
6. Ware, F. E., Vassilakos, A., Peterson, P. A., Jackson, M. R., Lehrman, M. A., and Williams, D. B. (1995) J. Biol. Chem. 270, 4697–4704.
7. Spiro, R. G., Zhu, Q., Bhoyroo, V., and Soling, H. D. (1998) J. Biol. Chem. 271, 11588–11594.
8. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664.
9. Tessier, D. C., Digard, D., Zapan, A., Radominska-Pandya, A., Parodi, A. J., Bergeron, J. J., and Thomas, D. Y. (2000) Glycobiology 10, 403–412.
10. Trombetta, E. S., and Helenius, A. (2000) J. Cell Biol. 148, 1123–1129.
11. Sousa, M., and Parodi, A. J. (1995) EMBO J. 14, 4196–4203.
12. Trombetta, S. E., and Parodi, A. J. (1992) J. Biol. Chem. 267, 9236–9240.
13. Arnold, S. M., Fessler, L. I., Fessler, J. H., and Kaufman, R. J. (2000) Biochemistry 39, 2149–2163.
14. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng. 10, 1–6.
15. Radominska, A., and Drake, R. R. (1994) Methods Enzymol. 230, 330–339.
16. Campbell, J. A., Davies, G. J., Bulone, V., and Henriksen, B. (1997) Biochem. J. 326, 929–939.
17. Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L. O., Herscovics, A., and Nagata, K. (2001) EMBO Rep. 2, 415–420.
18. Molinari, M., Calanca, V., Galli, C., Lucea, P., and Paganetti, P. (2003) Science 299, 1397–1400.
19. Oda, Y., Hosokawa, N., Wada, I., and Nagata, K. (2003) Science 299, 1384–1387.
20. Yoshida, H., Matsu, T., Hosokawa, N., Nomura, R. J., Nagata, K., and Mori, K. (2003) Dev. Cell 4, 265–271.
21. Persson, K., Ly, H. D., Dieckelmann, M., Wakahachik, W. W., Withers, S. G., and Styrnadka, N. C. (2001) Nat. Struct. Biol. 8, 166–175.
22. Guerin, M., and Parodi, A. J. (2003) J. Biol. Chem. 278, 20540–20546.

2 www.bmm.icnet.uk/servers/3dpssm.