Structure and Function of the DUF2233 Domain in Bacteria and in the Human Mannose 6-Phosphate Uncovering Enzyme*

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Background: DUF2233 domain is present in bacteria and human UCE, which is implicated in lysosomal storage disorders.

Results: Functional residues in DUF2233 and UCE identified in the structure of a bacterial DUF2233 domain were investigated.

Conclusion: A function for this domain in bacteria is proposed, and functional residues in human UCE were identified.

Significance: This is the first structure/function study of this protein domain.

DUF2233, a domain of unknown function (DFU), is present in many bacterial and several viral proteins and was also identified in the mammalian transmembrane glycoprotein N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (“uncovering enzyme” (UCE)). We report the crystal structure of BACOVA_00430, a 315-residue protein from the human gut bacterium Bacteroides ovatus that is the first structural representative of the DUF2233 protein family. A notable feature of this structure is the presence of a surface cavity that is populated by residues that are highly conserved across the entire family. The crystal structure was used to model the luminal portion of human UCE (hUCE), which is involved in targeting of lysosomal enzymes. Mutational analysis of several residues in a highly conserved surface cavity of hUCE revealed that they are essential for function. The bacterial enzyme (BACOVA_00430) has ~1% of the catalytic activity of hUCE toward the substrate GlcNac-P-mannose, the precursor of the Man-6-P lysosomal targeting signal. GlcNac-1-P is a poor substrate for both enzymes. We conclude that, for at least a subset of proteins in this family, DUF2233 functions as a phosphodiester glycosidase.

N-Acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (uncovering enzyme (UCE))4, EC 3.1.4.45 converts N-acetylglucosamine-P-mannose diester to mannose-6-P monooester on newly synthesized lysosomal acid hydrolases, a key step in the targeting of these hydrolases to lysosomes (1). Disruption of the N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (Nagpa) gene that encodes UCE leads to excessive cellular secretion of acid hydrolases (2), and mutations have recently been associated with persistent stuttering in humans (3). Despite the importance of UCE, very limited information is available concerning its structure.

UCE, a type 1 transmembrane glycoprotein localized in the trans-Golgi network (4, 5) is synthesized as a 515-residue preproenzyme and subsequently processed by furin, which removes the 49-residue propiece (6). Analysis of the domain architecture of mature UCE revealed that ~50% of the luminal region of the protein (resides 130–325) is related to the domain of unknown function, DUF2233 (Pfam (7) protein family PF09992). This domain has been identified in proteins ranging in size from ~300 to 2,000 residues, is present in 48 unique domain organizations and combinations, and has been identified in ~1,200 bacterial proteins in addition to several viral and eukaryotic proteins.

We report the crystal structure of the first structural representative of the DUF2233 protein family, BACOVA_00430 from Bacteroides ovatus, at a resolution of 1.80 Å. Comparative

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The atomic coordinates and structure factors (code 3ohg) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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4 The abbreviations used are: UCE, uncovering enzyme; hUCE, human UCE; DUF, domain of unknown function; BACOVA, B. ovatus; Man-6-P, mannose-6-phosphate; GlcNac-1-P, N-acetylglucosamine 1-phosphate; MAD, multiwavelength anomalous diffraction; FATCAT, flexible structure alignment by chaining aligned fragment pairs allowing twists; SCOP, Structural Classification of Proteins; r.m.s.d., root mean square deviation; TM, transmembrane; ER, endoplasmic reticulum; HeLa, Henrietta Lacks; PNGase F, peptide-N-glycosidase F; PIPE, polymerase incomplete primer extension; TLS, translation/libration/screw; ManNAC, N-acetylmannosamine; TCEP, tris(2-carboxyethyl)phosphine HCl.
**Structure and Function of the DUF2233 Domain**

sequence analysis of the bacterial members of this family covering a sequence identity range of 30–95% revealed several conserved residues located in a cleft on the surface of the BACOVA_00430 structure, indicating some involvement in its function. The BACOVA_00430 structure was used as a template for modeling the luminal region of human UCE (hUCE). Site-directed mutagenesis of hUCE based on this model confirmed the predicted functional importance of some of these conserved residues. Similar mutational analyses were performed on BACOVA_00430. These studies provide the first structure-function analysis of DUF2233 proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-6-[3H]GlcNAc (37Ci/mmol) was obtained from PerkinElmer Life Sciences. GalNAc, GlcNAc, GlcNAc-1-phosphate, ManNAc, UDP-GlcNAc, and p-nitrophenyl N-acetyl-α- and β-D-glucosaminide and methyl α-D-mannopyranoside were purchased from Sigma. Monoclonal anti-HA antibody was obtained from Covance. Recombinant purified protein was expressed in E. coli (Stratagene) and was obtained from Millipore (Billerica, MA).

**Protein Production and Crystallization**—Clones were generated using the polymerase incomplete primer extension (PIPE) cloning method (8). The gene encoding BACOVA_00430 was amplified by polymerase chain reaction (PCR) from ATCC 8483 genomic DNA using amplified by polymerase chain reaction (PCR) from _B. ovis_ ATCC 8483 genomic DNA using _Pfu_ Turbo DNA polymerase (Stratagene) and I-PIPE (insert) primers (forward primer, 5'-ctgccctcgcgttaCTTCTTTTCTATAATCAACATACTGTTG-3' where the target sequence is in uppercase) that include sequences for the predicted 5'- and 3'-ends of the gene encoding the full-length protein. The expression vector, pSpeedET, which encodes an N-terminal tobacco etch virus protease-cleavable expression and purification tag (MGSDKIIHHHHHHENLYFQ↓G), was PCR-amplified with V-PIPE (vector) primers (forward primer, 5’-taagccccagatgggatg-3’; reverse primer, 5’-aaacccagtctttacctatttattcag-3’). V-PIPE and I-PIPE PCR products were mixed to anneal the amplified DNA fragments together. *Escherichia coli* GeneHogs (Invitrogen) competent cells were transformed with the I-PIPE/V-PIPE mixture and dispensed on selective LB agar plates. The cloning junctions were confirmed by DNA sequencing. The oligonucleotide primer used to amplify the BACOVA_00430 gene segment encoding residues Met1–Ala31 was deleted. Expression was performed in a selenomethionine-containing medium at 37 °C. Selenomethionine was incorporated via injection of methionine biosynthesis (9), which does not require a methionine auxotrophic strain. At the end of fermentation, lysozyme was added to the culture to a final concentration of 250 μg/ml, and the cells were harvested and broken. After one freeze/thaw cycle, the cells were homogenized and sonicated in lysis buffer (50 mM HEPES, pH 8.0, 50 mM NaCl, 0.5 mM imidazole, and 1 mM tris(2-carboxyethyl)phosphine HCl (TCEP)). The lysate was clarified by centrifugation at 32,500 g for 30 min. The soluble fraction was passed over nickel-chelating resin (GE Healthcare) pre-equilibrated with lysis buffer, the resin was washed with wash buffer (50 mM HEPES, pH 8.0, 300 mM NaCl, 40 mM imidazole, 10% (v/v) glycerol, and 1 mM TCEP), and the protein was eluted with elution buffer (20 mM HEPES, pH 8.0, 200 mM NaCl, 40 mM imidazole, and 1 mM TCEP). The eluate was buffer-exchanged with tobacco etch virus buffer (20 mM HEPES, pH 8.0, 200 mM NaCl, 40 mM imidazole, and 1 mM TCEP) using a PD-10 column (GE Healthcare) and incubated with 1 mg of tobacco etch virus protease/15 mg of eluted protein for 2 h at ambient temperature followed by overnight incubation at 4 °C. The protease-treated eluate was passed over nickel-chelating resin (GE Healthcare) pre-equilibrated with HEPES crystallization buffer (20 mM HEPES, pH 8.0, 200 mM NaCl, 40 mM imidazole, and 1 mM TCEP), and the resin was washed with the same buffer. The flow-through and wash fractions were combined and concentrated to 21 mg/ml by centrifugal ultrafiltration (Millipore) for crystallization trials.

BACOVA_00430 was crystallized using the nanodroplet vapor diffusion method (10) with standard Joint Center for Structural Genomics crystallization protocols (11, 12). Sitting drops composed of 200 nl of protein solution mixed with 200 nl of crystallization solution in a sitting drop format were equilibrated against a 50-μl reservoir at 277 K for 15 days prior to harvest. The crystallization reagent consisted of 0.2 M Li$_2$SO$_4$, 30% PEG 4000, and 0.1 M Tris, pH 8.5. Ethylene glycol was added to a final concentration of 10% (v/v) as a cryoprotectant.

Initial screening for diffraction and data collection was carried out using the Stanford automated mounting system (SAM) (13) at the Stanford Synchrotron Radiation Lightsource (Menlo Park, CA). Diffraction data were collected from a rod-shaped crystal of dimensions 200 × 20 × 20 μm and indexed in space group P6$_1$. The oligomeric state of BACOVA_00430 in solution was determined using size exclusion chromatography with a 1 × 30-cm$^2$ Superdex 200 size exclusion column (GE Healthcare) coupled with miniDAWN (Wyatt Technology) static light scattering and Optilab differential refractive index detectors (Wyatt Technology). The mobile phase consisted of 20 mM Tris, pH 8.0, 150 mM NaCl, and 0.02% (w/v) sodium azide. The molecular weight was calculated using ASTRA 5.1.5 software (Wyatt Technology).

**X-ray Data Collection, Structure Determination, and Refinement**—Multiwavelength anomalous diffraction (MAD) data were collected at the Stanford Synchrotron Radiation Lightsource on beamline 9-2 at wavelengths corresponding to the high energy remote (λ$_r$), inflection point (λ$_i$), and peak (λ$_p$) of a selenium MAD experiment using the Beamlime User Integrated Control Environment (BLU-ICE) (14) data collection environment. The data sets were collected at 100 K using a MarMosaic 325 charge-coupled device detector (Rayonix). The MAD data were integrated and reduced using MOSFLM (15) and scaled with the program SCALA (16). The heavy atom substructure was determined with SHELXD (17). Phasing was performed with autoSHARP (18), SOLOMON (19) (implemented in autoSHARP) was used for density modification, and ARP/wARP (20) was used for automatic model building to 1.80 Å resolution. Model completion and crystallographic refinement were performed with the _L_ data set using Coot (21) and REFMAC5 (22). The refinement protocol included the experi-
amental phase restraints in the form of Hendrickson-Lattman coefficients from autoSHARP and TLS refinement with one TLS group for the whole molecule. Data and refinement statistics are summarized in Table 1 (23–26).

Validation and Deposition—The quality of the crystal structure was analyzed using the Joint Center for Structural Genomics Quality Control server. This server verifies the stereochemical quality of the model using AutoDepInputTool (27), MolProbity (28), and WHATIF 5.0 (29); agreement between the atomic model and the data using SFcheck 4.0 (30) and RESOLVE (31); the protein sequence using ClustalW (32); atom occupancies using MOLEMAN2 (33); and the consistency of non-crystallographic symmetry pairs and evaluates difference in $R_{cryst}/R_{free}$, expected $R_{free}/R_{cryst}$ and maximum/minimum B-factors by parsing the refinement log file and Protein Data Bank header. Protein quaternary structure analysis was performed using the PISA server (34). Fig. 1B was adapted from an analysis using PDBsum (35), and Figs. 1A, 2, and 3A were prepared with PyMOL (36). Atomic coordinates and experimental structure factors were deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ under accession code 3ohg.

Cell Lines and Human UCE and BACOVA_00430 Constructs—HeLa cells were obtained from the ATCC. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 μg/ml penicillin, and 100 units/ml streptomycin. For mutational analysis, hUCE cDNA (4) was modified by addition of a C-terminal HA tag (YPYDVPDYA) and subcloned into the pcDNA3.1(-) expression vector (Invitrogen) using EcoRI and HindIII restriction sites. BACOVA_00430 cDNA was obtained from the Protein Structure Initiative Biology Materials Repository/DNASU (37) (Clone ID BoCD00384454). All mutations in the hUCE construct and the G288A mutation of BACOVA_00430 construct were introduced using QuikChange (Stratagene) site-directed mutagenesis protocols. For the H225Q and R227T double mutation BACOVA_00430 construct, the fragment of BACOVA between the EcoO109I site (264th base) and SpeI restriction site (612th base) was substituted with a fragment bearing a mutation BACOVA_00430 construct, the fragment of BACOVA between the EcoO109I site (264th base) and SpeI restriction site (612th base) was substituted with a fragment containing 3ohg.

RESULTS

Structure of BACOVA_00430—The cloning, expression, purification, and crystallization of BACOVA_00430 was carried out using standard Joint Center for Structural Genomics protocols as detailed under “Experimental Procedures.” The crystal structure of BACOVA_00430 was determined by MAD phasing to 1.80-Å resolution. Data collection, model, and refinement statistics are summarized in Table 1 (23–26). BACOVA_00430 is present as a monomer in the crystallographic asymmetric unit. Crystal packing analysis and analytical size exclusion chromatography support a monomer as the predominant oligomerization state in solution. The final model (Fig. 1) includes Gly(30) (which remains after cleavage of the expression and purification tag), residues 32–315 of the full-length protein (the predicted lipoprotein signal peptide, residues 1–31, was excluded from the protein construct), one chloride ion from the purification buffer, two sulfate ions from the crystallization reagent, 17 1,2-ethanediol molecules from the cryoprotectant, and 470 water molecules. The Matthews’ coefficient (40) is −4.0 Å³/Da with an estimated solvent content of ~70%. The Ramachandran plot produced by MolProbity (28) shows that 96.8% of the residues are in favored regions with none in the disallowed regions.

BACOVA_00430 consist of four domains, each of which bears some resemblance to the cystatin fold (SCOP code 54402) (41), which consists of a curved antiparallel $\beta$-sheet wrapped around an $\alpha$-helix (Fig. 1). The first domain, constituted by H1, $\beta$1, $\beta$2, and $\beta$3, resembles more closely the prototypical cystatin-like fold and is not included in the DUF2233 definition, which covers only domains 2–4 (residues 123–312) of BACOVA_00430. Interestingly, the C terminus of the bacterial protein reaches over from domain 4 and inserts its tail into...
Table 1

Data collection and refinement statistics for BACOVA_00430 (Protein Data Bank code 3ohg)

| Data collection statistics | Space group | P 61 |
|---------------------------|-------------|------|
| Unit cell parameters     | a = b = 96.9 Å, c = 91.1 Å |
| Datasets                  | 3, MAD-Sc; 3, MAD-Sc; 3, MAD-Sc |
| Wavelength (Å)            | 0.9184 |
| Resolution range (Å)     | 29.97±1.80 |
| Observations              | 172,338 |
| Unique reflections        | 44,948 |
| Completeness (%)          | 99.7 (99.4) |
| Mean I/σ(I)               | 7.1 (2.4) |
| Rmerge on 1 (Gσ2)         | 16.0 (62.3) |
| Rmerge on 1 (%)           | 8.1 (31.6) |
| Rmerge on 1 (%)           | 13.7 (53.6) |
| Highest resolution shell (Å) | 1.85-1.80 |

Model and refinement statistics:

| Dataset used in refinement | λ, MAD-Sc |
|----------------------------|-----------|
| Resolution range (Å)       | 29.97±1.80 |
| No. reflections (total)    | 44,927 |
| No. reflections (test)     | 2,262 |
| Completeness (%)           | 99.7 |
| Cutoff criteria            | F0 |
| Rmerge                      | 0.12 |
| Rmani                       | 0.15 |

Stereochemical parameters:

| Restrains (r.m.s.d. observed) | Bond angle (°) | 1.46 |
|-------------------------------|----------------|-----|
| Bond length (Å)               | 0.015 |
| Average protein isotropic B-value (Å²) | 20.3 |
| Wilson plot B-value (Å)       | 14.1 |
| ESU based on Rmerge (Å)       | 0.071 |
| Protein residues / atoms      | 265 / 2190 |
| Water molecules / chloride / sulfate / 1,2-ethanediol | 470 / 1 / 2 / 17 |

*Highest resolution shell.

Rmerge (redundancy-independent) = Σ||Fobs| - |Fcalc||/Σ|Fobs| (24), where N is the number of observations of a given reflection.

Rmerge (precision-indicating) = Σ(|Fobs| - |Fcalc|)/(Σ|Fobs|) (25, 26).

Typically, the number of unique reflections used in refinement is slightly less than the total number that were integrated and scaled. Reflections are excluded due to negative intensities and rounding errors in the resolution limits and cell parameters.

Rcryst = Σ|Fcalc| - |Fobs|/Σ|Fcalc|, where Fcalc and Fobs are the calculated and observed structure factor amplitudes, respectively.

Rmerge as for Rcryst, but for 5.0% of the total reflections chosen at random and omitted from refinement.

This value represents the total B that includes overall TLS refinement and residual B factors.

ESU, estimated overall coordinate error (23).

Table 1

| Sequence and Function of the DUF2233 Domain |
|--------------------------------------------|

**Structure and Function of the DUF2233 Domain**

- **Modeling Human UCE**—Mammalian UCEs are highly conserved type 1 transmembrane glycoproteins of ~515 residues with ~85% sequence identity. hUCE consists of a signal peptide (residues 1–25), a propeptide (residues 26–49), a luminal region (residues 50–448), a single TM region (residues 449–469), and a cytoplasmic tail (residues 470–515) (4). A sequence profile-based search for homologs of the entire luminal portion of hUCE (residues 50–448) using FFAS (45), which is very useful for finding remote homologs, identified a single significant hit to our BACOVA_00430 structure with a score of ~39.3 (scores ~9.5 indicate less than 3% false positives) and ~14% sequence identity between residues 50–298 of hUCE (comprising ~62% of the luminal region) and residues 36–282 of BACOVA_00430. When only those residues of hUCE that are included in the DUF2233-like domain definition (luminal residues 130–325) are queried using FFAS, only one significant hit (score ~58.7) is again found with ~20% sequence identity to residues 123–311 of BACOVA_00430. These results indicated that we could confidently use BACOVA_00430 as a template for modeling the corresponding region of hUCE. A model for hUCE residues 50–335, which accounts for ~70% of the Golgi luminal region, was built using I-TASSER (46) using explicit disulfide restraints between Cys51–Cys221, Cys115–Cys148, Cys132–Cys323, and Cys307–Cys314 (numbering is based on the hUCE sequence), corresponding to potential disulfide bonds identified in an earlier mass spectrometry study of a monomeric soluble construct of hUCE (47). The C-score of this final domain 2 and probably serves to stabilize the DUF2233 portion of the protein. Of the four domains, only domains 3 and 4 (residues 131–194 and 195–268, respectively) can be superimposed onto each other (r.m.s.d. of 2.2 Å over 41 Ca atoms and 22% sequence identity), suggesting possible gene duplication in this portion of the protein.

**Structural Comparisons**—A search for other proteins of similar structure was carried out using FATCAT (42) (flexible alignment mode) against the SCOP database (43) and DALI (44). When queried using the entire BACOVA_00430 structure, FATCAT returned only two hits with significant p value scores (~0.05): human latexin (Protein Data Bank code 2bo9; p = 0.0286; Ca r.m.s.d., ~3 Å; sequence identity, ~3%) and a protein of unknown function, YpmB, from Bacillus subtilis (Protein Data Bank code 2gu3; p = 0.04; Ca r.m.s.d., ~2 Å; sequence identity, ~3%). However, in both cases, the coverage is restricted to domain 1 of BACOVA_00430 because latexin and YpmB are both α and β (α + β) proteins belonging to the cystatin-like fold (and cystatin/monellin superfamily). No significant hits were found by FATCAT when the search was restricted to the DUF2233 domain. Similar results were obtained with a DALI search for the full BACOVA_00430 structure; all structural similarities were again limited to the N-terminal domain, which most closely resembles the prototypical cystatin-like fold. Thus, BACOVA_00430 is the first structural representative of the novel DUF2233 domain architecture.
model (energy-minimized and optimized hydrogen-bonding contacts by I-TASSER) was $-0.13$ (C-scores usually range from $-5$ to 2, and a higher C-score indicates higher confidence), and the TM-score for estimated accuracy of the model (48) was $0.70 \pm 0.12$ (a TM-score higher than 0.5 indicates correct topology of the model) with an r.m.s.d. of $6.4 \pm 3.9$ Å compared with the template. I-TASSER using disulfide restraints produced the most complete model, although several other procedures were tested including Modeler, M4T, Swiss-Model, and HHPred (using the Protein Structure Initiative Protein Model Portal), which produced only partial models.

Despite attempts to use the disulfide restraints, the I-TASSER model does not contain all four expected disulfide bonds. The model (Fig. 3) contains a disulfide bond between Cys$^{132}$-Cys$^{323}$, and Cys$^{307}$/Cys$^{314}$ and Cys$^{115}$/Cys$^{148}$ are relatively close to each other ($\sim 15$ and $11$ Å between C$\alpha$ atoms, respectively). Arg$^{328}$ and His$^{104}$ when mutated to Cys and Gln, respectively, are associated with persistent stuttering, and their location is visualized in the model. The major consequence of these mutations is impaired folding in the endoplasmic reticulum (ER) followed by degradation by the ER-associated protein degradation system (49). Thus, based on our model, we speculate that the impaired folding induced by these mutations could be a result of destabilization of the $\beta$-sheet in which His$^{104}$ resides as well as the potential to affect proper disulfide formation. In addition, three of the four N-linked glycosylation sites found by mass spectrometry in hUCE (Asn$^{208}$, Asn$^{214}$, Asn$^{296}$, and Asn$^{366}$) are solvent-exposed in our model. The conservation of disulfide and potential glycosylation sites indicates that the registry of the alignment used for modeling is likely correct. However, because of remote homology between the template and hUCE, it is expected that the accuracy may be low in some regions of the model. Nevertheless, the model provides the first three-dimensional view of the putative functional site of UCE and helped guide the mutational analysis.

**Mutational Analysis of Human UCE**—Next, we tested the consequences of mutating a number of the residues located in or near the surface cavity identified in the hUCE model that appears to be a putative active site. Most of these residues are conserved across the DUF2233 family (Asn$^{137}$, Gln$^{225}$, Thr$^{227}$, Arg$^{247}$, Asn$^{264}$, Asp$^{286}$, Gly$^{287}$, Gly$^{288}$, Gly$^{289}$, Ser$^{290}$, Thr$^{320}$, and Val$^{322}$) (Fig. 3). It was essential to take into account that hUCE is synthesized as an inactive preproenzyme that forms a tetramer in the ER and then traffics to the trans-Golgi network where the propiece is cleaved by furin to generate the active enzyme (6). Consequently, it was necessary to determine...
whether or not the mutant proteins could exit the ER and be processed by furin to ensure the mutation could be correlated with UCE activity.

HeLa cells transfected with plasmids encoding full-length WT or mutant hUCE containing a C-terminal HA tag were harvested, solubilized with Triton X-100, and analyzed to evaluate the effects of the mutations on cellular localization and activity of the proteins. First, aliquots were incubated with or without PNGase F to excise the N-linked glycans (high mannos and complex oligosaccharides) and then subjected to SDS-PAGE and Western blotting with anti-HA antibody. With two exceptions, N281A and V318A mutants, the untreated samples gave rise to two bands; the faster migrating band represents the ER form with high mannose glycans, and the slower migrating band represents the Golgi species with complex glycans (49) (Fig. 4). Following PNGase F treatment, the Golgi species migrated faster than the ER form, reflecting cleavage of the 24-residue propiece by furin in the trans-Golgi network in addition to the removal of the glycans. Evidence that the designation of these bands is correct is shown by the N281A and V318A mutants, which only exhibit the ER forms, with a single faster migrating band in the untreated samples and a single slower migrating band following PNGase F treatment. Exiting the ER seems to have been partially impaired for the G287A and G289A mutants, whereas all the other mutants trafficked to the Golgi and underwent furin cleavage similarly to the WT hUCE.

The remaining extract was used for hUCE activity measurements as summarized in Table 2. Among the residues in the most highly conserved patch, mutation of Asp286, Gly288, Gly289, and Ser290 to Ala resulted in the complete loss of hUCE activity with either no or only partial impairment of trafficking to the Golgi and furin cleavage. The G287A mutant exhibited
about 16% of WT hUCE activity when taking into account its partial impairment in trafficking to the Golgi, whereas the N284A mutant had only 22% of WT activity. Among the other mutants, the N137A, R247A, T320A, and V322A mutants exhibited 11, 87, 43, and 67% of WT activity, respectively. The effect of the N281A and V318A mutations on hUCE activity could not be explored because these constructs were retained in the ER. Interestingly, when Gln\(^{225}\) and Thr\(^{227}\) were mutared to the residues at equivalent positions in BACOVA_00430 (His and Arg, respectively), the hUCE activity was greatly decreased to 5.8 and 0.1%, respectively, relative to WT.

Mutation of Cys\(^{51}\) to Met had only a small effect on hUCE trafficking and activity (65% of WT), indicating that the Cys\(^{51}\)-Cys\(^{221}\) disulfide bond is not absolutely essential for folding or
enzyme activity (Fig. 4 and Table 3). The double mutant C51M/C221L also folded adequately and trafficked to the Golgi where it was cleaved by furin, but it had only 9.7/100% of WT activity. This result indicates that the C221L mutation leads to loss of enzymatic activity. By contrast, mutation of Cys115 and Cys132 greatly impaired folding of the enzyme as reflected by retention in the ER.

**BACOVA_00430 Exhibits Low Activity toward GlcNAc-P-Man**—The BACOVA_00430 protein was able to cleave GlcNAc from GlcNAc-P-Man but did so at a much slower rate than hUCE. Kinetic studies showed that its $V_{\text{max}}$ toward this substrate was only 1% of the UCE value, whereas the apparent $K_m$ for GlcNAc-P-Man was 10.9 $\mu$M versus 0.64 $\mu$M for hUCE (Table 4). Both proteins had much lower activity toward GlcNAc-1-P, and neither exhibited any activity toward $p$-nitrophenyl $N$-acetyl-$\alpha$- and $\beta$-$D$-glucosaminide substrates. This result is consistent with our previous finding that hUCE has a strong preference for substrates with the underlying phosphate in a diester linkage (50). To establish that the activity of the BACOVA_00430 protein toward these substrates was not the consequence of a contaminant in the preparation, a G288A BACOVA_00430 mutant was prepared and found to be completely inactive in this assay. Because the hUCE Q225H and T227R mutants were inactive, we tested the possibility that these residues may be important for increased activity toward GlcNAc-P-Man. However, mutating His$^{225}$ and Arg$^{227}$ of BACOVA_00430 to the corresponding residues in hUCE (Gln

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**TABLE 2**

**Mutational analysis of UCE**

| Mutation | Relative UCE activity | Traffic to Golgi |
|----------|-----------------------|-----------------|
| WT       | 100                   | Yes             |
| N137A    | 11.1 ± 1.2           | Yes             |
| N281A    | ND                   | No              |
| N284A    | 22.0 ± 4.6           | Yes             |
| D286A    | 0.2 ± 0.4            | Yes             |
| G287A    | 9.8 ± 1.0            | ~60% WT         |
| G288A    | 0.2 ± 0.4            | Yes             |
| G289A    | 0.4 ± 0.3            | ~50% WT         |
| S290A    | 0.6 ± 0.7            | Yes             |
| Q225H    | 5.8 ± 0.3            | Yes             |
| T227R    | 0.1 ± 0.1            | Yes             |
| R247A    | 87.0 ± 1.4           | Yes             |
| V318A    | ND                   | No              |
| T320A    | 43.0 ± 7.8           | Yes             |
| V322A    | 67.0 ± 2.1           | Yes             |

*The values represent units of enzyme activity divided by the intensity of the band of mature UCE determined by Western blot analysis with WT set to 100 and uniform sample loading. The values are the average of two to four determinations, each done in duplicate. ND, not detected, indicating failure of protein to traffic to the Golgi and undergo furin cleavage.

**TABLE 3**

**Effect of disulfide bond disruption on UCE activity**

| Relative UCE activity | Traffic to Golgi |
|-----------------------|-----------------|
| WT                    | 100             | Yes             |
| C51M                  | 64.7 ± 5.2      | Yes             |
| C51M/C221L            | 9.7 ± 1.0       | Yes             |
| C115S                 | 15.4 ± 1.6      | Trace           |
| C115S/C148V           | ND              | No              |
| C132V                 | ND              | No              |
| C132V/C221L           | ND              | No              |

*The values represent units of enzyme activity divided by the density of the band of mature UCE determined by Western blot analysis with WT set to 100. The values are the average of three determinations, each done in duplicate. ND not detected, indicating failure of protein to traffic to the Golgi and undergo furin cleavage.

**TABLE 4**

**Activity of UCE and BACOVA_00430 toward GlcNAc-P-mannose and GlcNAc-1-P**

The kinetic analyses were carried out in 50 mM Tris maleate, pH 6.7, 0.5% Triton X-100 buffer containing various concentrations of substrates in a final volume of 30 $\mu$L. The reactions contained 3 $\mu$L of hUCE and 9 $\mu$L of BACOVA_00430 for the GlcNAc-P-mannose assays and 0.5 $\mu$L of UCE and 30 $\mu$L of BACOVA_00430 for the GlcNAc-1-P assays. The values for GlcNAc-P-Man are the average of two determinations, and the values for GlcNAc-1-P are the average of five determinations.

| Substrates     | $K_m$ (mM) | $V_{\text{max}}$ (mol/h/mg) |
|----------------|------------|-----------------------------|
| GlcNAc-P-Man  | 0.64       | 10.9                        |
| GlcNAc-1-P    | 2.5        | 5.7                         |

*The values represent the average of five determinations.*
tested. The values are the average of two to three determinations.

FIGURE 5. Effect of various compounds on BACOVA_00430 and hUCE catalytic activity. The assays contained 0.41 mM [3H]GlcNAc-P-Man and the indicated concentration of the various compounds. Results are expressed as the percentage of control reactions lacking the respective compounds. *, not tested. The values are the average of two to three determinations.

and Thr, respectively) also resulted in a complete loss of enzyme activity.

The effects of a number of sugars, sugar phosphates, nucleotide sugars, and inorganic P$_i$ as inhibitors of the activity of BACOVA_00430 toward GlcNAc-P-Man were also investigated. All of the sugar phosphates as well as UDP-GlcNAc and inorganic P$_i$ were effective inhibitors, whereas the other nucleotide sugars and the simple sugars were very weak inhibitors (Fig. 5). This pattern differs from that observed for hUCE where GlcNAc-1-P is a more potent inhibitor than the other sugar phosphates, GlcNAc is a relatively good inhibitor, and inorganic phosphate is a weaker inhibitor. This pattern of inhibition of hUCE agrees with that reported previously for a partially purified preparation of rat liver UCE (51).

DISCUSSION

Elucidation of the crystal structure of BACOVA_00430 provides the first structural insights into proteins that contain DUF2233 domains. By focusing on the pattern and location of amino acids that are conserved within the DUF2233 family, we identified a surface cavity that is likely functionally important. Remarkably, among all known mammalian proteins, only one possesses the DUF2233 domain, namely UCE, a phosphodiester $\alpha$-N-acetylg glucosaminidase that catalyzes a critical step in the generation of the Man-6-P recognition signal on lysosomal acid hydrolases. The structure of BACOVA_00430 was used to model the catalytic domain of hUCE. This in turn provided a template for a structure-based mutational analysis and established that the highly conserved residues in the UCE surface cavity are essential for the catalytic function of the enzyme toward GlcNAc-P-Man.

The BACOVA_00430 protein exhibits only weak activity toward the GlcNAc-P-Man substrate. Kinetic analyses revealed that the apparent $K_m$ for this substrate is about 17 times higher than the corresponding value for hUCE, and the $V_{max}$ is only 1% of the value for hUCE. The finding that GlcNAc-1-P inhibited the enzymatic activity of BACOVA_00430 much more strongly than did GlcNAc points to a preference for the phosphorylated form of this aminosugar. This conclusion was confirmed by the fact that neither BACOVA_00430 nor hUCE has any detectable activity toward $p$-nitrophenyl N-acetyl-$\beta$-d-glucosaminide. However, both proteins exhibited poor activity toward GlcNAc-1-P, indicating that they function as phosphodiester glycosidases. Whereas BACOVA_00430 is inhibited equally by a variety of sugar phosphates, GlcNAc-1-P inhibits UCE much more than other sugar phosphates, consistent with having evolved to specifically recognize and act on GlcNAc-P-mannose. At this point, the physiological substrate(s) of BACOVA_00430 is unknown. In this regard, a number of bacterial cell wall components with sugar-P-sugar repeating structures could potentially be substrates for BACOVA_00430 and related bacterial proteins (52, 53), and the sulfate from the crystallization reagents that is near Gly$^{272}$-Gly$^{273}$ and Arg$^{239}$/Arg$^{303}$ may mimic the phosphate from the physiologically relevant substrate. Our results strongly hint at this possibility.

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