Structural biology | secretory pathway | enzyme | antiviral

A sparsely glycosylated proteins are a major protein modification that occurs extensively in eukaryotes. Glycosidases in the secretory pathway that trim N-linked glycans play key roles in protein quality control and in the specific modifications leading to mature glycoproteins. Inhibition of glycosidases in the secretory pathway is a proven therapeutic strategy, that holds great promise in the treatment of viral disease. The enzyme endo-α-1,2-mannosidase (MANEA) provides an alternative processing pathway to evade glucosidase inhibitors. We report the three-dimensional structure of human MANEA and complexes with enzyme inhibitors that we show act as antivirals for bovine viral diarrhea and human dengue viruses. The structure of MANEA will support inhibitor optimization and the development of more potent antivirals.

Significance

The glycosylation of proteins is a major protein modification that occurs extensively in eukaryotes. Glycosidases in the secretory pathway that trim N-linked glycans play key roles in protein quality control and in the specific modifications leading to mature glycoproteins. Inhibition of glycosidases in the secretory pathway is a proven therapeutic strategy, that holds great promise in the treatment of viral disease. The enzyme endo-α-1,2-mannosidase (MANEA) provides an alternative processing pathway to evade glucosidase inhibitors. We report the three-dimensional structure of human MANEA and complexes with enzyme inhibitors that we show act as antivirals for bovine viral diarrhea and human dengue viruses. The structure of MANEA will support inhibitor optimization and the development of more potent antivirals.

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glycoproteins within their envelope (12). Glycosylation is achieved by coopting host cell glycosylation machinery during replication. Substantial efforts have been deployed in the use of inhibitors of α-glucosidases I and II as antivirals (e.g., \(N\)-butyldeoxynojirimycin, 6-O-butanoylcastanospermine) for the treatment of HIV/AIDS (13), dengue (14, 15), and HBV (16, 17) (reviewed in refs. 18–20).

Inhibition of host glycosylation pathways, in particular ER glucosidases I and II and Golgi mannosidase I, interferes with the viral lifecycle by impairing protein folding and quality control, inducing the unfolded protein response or mistrafficking of viral glycoproteins. These changes can lead to impairment of secretion (17, 21–23) or fusion (24–26) or evasion of host immunity (27). In the case of HBV, when glucosidases are inhibited, mature HBV viral glycoproteins are still produced through the alternative processing provided by the endomannosidase pathway (23). However, the contribution of the endomannosidase pathway to viral protein assembly under normal conditions remains poorly studied, and the antiviral activity of inhibitors targeting MANEA has not yet been evaluated.

The \textit{Homo sapiens} endo-α-1,2-mannosidase gene, MANEA, is located on chromosome 6. The MANEA gene gives rise to three transcripts, only one of which encodes the complete protein product, MANEA. MANEA is categorized as a member of the glycoside hydrolase (GH) family 99 in the Carbohydrate-Active enZyme (CAZy) database (28), which also contains bacterial endo-α-1,2-mannanases from \textit{Bacteroides xylanisolvens} (BxGH99) and \textit{Bacteroides thetaiotaomicron} (BtGH99) that act on related structures within yeast mannan and that share ~40% identity with MANEA (29–31). MANEA encodes a 462-aa protein that consists of a single-pass type II membrane protein with a transmembrane helix followed by a stem region, followed by the catalytic domain (32). Here we reveal the structure of the catalytic domain of human MANEA. Through complexes with substrate, we show the architecture of the binding groove and identify key catalytic residues. We report structures with MANEA inhibitors that were designed based on the structure of the glucosylated high-mannose N-glycan. Finally, we show that inhibitors of MANEA act as antiviral agents against bovine viral diarrheal virus (BVDV) and dengue virus (DENV), confirming that MANEA is an antiviral target.

Results

Expression and Activity of Human MANEA GH99. To obtain soluble MANEA protein for structure and function studies to inform inhibitor design, attempts were made to express the gene. A truncated gene for MANEA, consisting of the catalytic domain beyond the stem domain (i.e., residues 98 to 462, hereinafter MANEA-Δ97), was synthesized in codon-optimized form. Expression trials in \textit{Escherichia coli} BL21(DE3) cells yielded only insoluble protein, but we were inspired by a report of soluble expression using cold-shock promoters (pCold-I vector) and coexpression of GroEL chaperones (33). Optimal yields of
soluble MANEA-Δ97 were obtained using Terrific Broth supplemented with glycerol and 20 mM MgCl₂, affording ~2 to 3 ng L⁻¹ (SI Appendix, Fig. S1A and Experimental Procedures). The recombinant protein was purified using the encoded N-terminal His₆-tag and was stable, with a Tₘ of 50 °C (SI Appendix, Fig. S1B).

Treatment of GlcMan₉GlcNAc₂ with recombinant MANEA-Δ97 released α-Glc-1,3-Man and Man₆GlcNAc₂ (SI Appendix, Fig. S1C). Enzyme kinetics were measured using α-Glc-1,3-α-Man-1,2,α-Man-OMe (GlcManOMe) as substrate with a coupled assay in which the product hydrolyzed by MANEA was detected using a D-mannose/D-fructose/D-glucose detection kit (Megazyme) (30). MANEA-Δ97 hydrolyzed GlcManOMe with a k₉₅ of 27.7 ± 1.0 min⁻¹ and a K₉₅ of 426 ± 33 μM (k₉₅/K₉₅ of 65 min⁻¹ μM⁻¹) (SI Appendix, Fig. S1D). The catalytic efficiency is similar to that displayed by BrGH99 on the epimeric Man₆OMe substrate (K₉₅ = 2.6 mM; k₉₅ = 180 min⁻¹; kₐₙ/k₉₅ = 69 min⁻¹ μM⁻¹) (30). The E404Q variant of MANEA-Δ97 was inactive on this substrate, consistent with the proposed mechanism (34).

Development of MANEA Inhibitors. GlcDMJ, a cell-permeable inhibitor of MANEA (35, 36), is composed of the well-known mannosidase iminosugar inhibitor deoxymannojirimycin (DMJ) modified with a glucosyl residue at the 3-position to enhance specificity and binding to MANEA by mimicking the substrate and benefitting from substrate-enzyme contacts in the −2 subsite (Fig. 2). The related compound GlcIFG (31), which is also cell-permeable (37), was developed through a similar approach applied to the azasugar isofagomine (IFG). Similarly, ManIFG was synthesized to match the stereochemistry of the substrate and the resultant mannose quantified using a D-mannose/D-fructose/D-glucose detection kit (Megazyme) (30). Because glucosylated mannans are substrates for bacterial endo-α-1,2-mannanases (i.e., for BrGH99: GlcIFG K₉₅ = 625 nM; ManIFG K₉₅ = 140 nm) (30), GlcIFG was preferred to DMJ in the −1 subsite.

The 3D Structure of Human MANEA Sheds Light on Eukaryotic Enzyme Specificity. Crystals of human MANEA-Δ97 (Fig. 3A) were obtained in several crystal forms (SI Appendix, Experimental Procedures). The initial crystal form, in space group P2₁2₁2₁, was diffraction to ~2.25 Å resolution, and structure solution by molecular replacement (using BrGH99 as a search model) was successful, leading to structures with R/ Rfree 18%/22% (SI Appendix, Table S1). Unfortunately, this crystal form suffered from occlusion of the active center by the His₆-tag and a metal ion assumed to be Ni²⁺. A second crystal form, P4₁2₁2, provided data of lower resolution (3 Å), but in this case we could build a loop (131 to 141) absent in the first crystal form; however, this crystal form could not be reliably reproduced. A third crystal form in space group P6₁ was obtained with Anderson–Evans polyoxotungstate [TeW₆O₂₄]₆⁻ (TEW), which has been used on...
The overall 3D structure of human MANEA is a single domain (βα8) barrel with a (partially; see below) open active center in which Glu404 and Glu407 (human numbering, as part of a conserved EWHE motif) are the catalytic residues in a neighboring group participation mechanism that proceeds through an epoxide intermediate (34) (Fig. 3 B and C). The structure is similar (Co rmsd >0.9 Å over 333 matched residues) to structures reported for BcGH99 and BsGH99 (31), with which MANEA shares 40% sequence identity. The positions of the −2 to +2 ligands in the bacterial and human GH99 proteins are equivalent and the sugar interactions in the −2 to +2 subsites, all of which bind mannosides with identical linkages in both high-mannose N-glycans (MANEA) and yeast mannan (bacterial endowed-α,1,2-mannanases) (29) are invariant.

A key difference between the substrates for the human and bacterial GH99 enzymes are the −2 sugar residues, which is glucose in high-mannose N-glycans, and mannos en yeast mannan. These differences are achieved by differences in recognition between the human and bacterial GH99 enzymes in the −2 subsite and its environs. In bacterial endo-α,1,2-mannanases, Trp126 (numbering for BsGH99) (31) forms a hydrophobic interaction with the C2 that was believed to be responsible for the selectivity of bacterial enzymes for ManMan vs. GlcMan substrates. In MANEA, the equivalent residue is Tyr189, which makes a water-mediated interaction with O2 of Glc in the −2 subsite (Fig. 3 D and E). Notably, we observed a loop (residues 191 to 201, hereinafter the “−2 loop”), absent in the bacterial structures, that was flexible and observed in different positions in the different MANEA crystal forms (Fig. 3F).

Two residues within the −2 loop are invariant across animal MANEAs: Asp195 and Gly198. Our structures reveal that Asp195 forms a hydrogen bond with the 3-OH of the −2 sugar (glucose) residue, and along with Asn197 H-bonding to O4, is a key determinant of binding of GlcMan structures. The second invariant residue, Gly198, enables the formation of a 195 to 198 invariant residue, Gly198, enables the formation of a 195 to 198 hydrogen-bond network with the −2 sugar (Fig. 3 D and F). MANEA isolated from human liver carcinoma cells processes triglucosylated N-glycans at a lower rate than monoglucosylated N-glycans (9). In the GlcIFG complex of MANEA, the −2 loop is closed over the active site, and in this conformation, triglucosylated N-glycans are unable to bind to human MANEA. However, the other observed conformations show flexibility in the loop that could allow these extended structures to bind. Of note, bovine MANEA does not process triglucosylated N-glycans (9). While both bovine and human MANEA have the −2 loop, a Ser227 (human)-to-Lys change in the bovine enzyme was proposed to have contributed to the difference in specificity. Ser227 lies adjacent to and points toward the −2 loop, while the side chain of Lys226 (numbering as in bovine) may interact with the loop and reduce its mobility (Fig. 3F). Interactions with the loop may explain why CyMe-GlcIFG binds nearly 50-fold more weakly than GlcIFG. Notably, bacterial GH99 endomannanases, which act on complex extended yeast mannan substrates have open active sites and do not contain this loop (SI Appendix, Fig. S5). To test this hypothesis, we determined the structure of BcGH99 with CyMe-GlcIFG (K<sub>i</sub> of 339 nM; tighter binding than that of GlcIFG alone, 625 nM). The cyclohexyl ring is visible (but mobile) in the density, and indeed the loop in MANEA will occlude binding of the cyclohexyl group (SI Appendix, Fig. S4).

Human MANEA as a Host Cell Antiviral Target. To explore the potential of the endomannosidase pathway as an antiviral target, we studied the effect of MANEA inhibitors on replication of BVDV, a pestivirus of the Flaviviridae family. Reinfection assays showed that increasing concentrations of GlcIFG, the tightest-binding ligand of MANEA, resulted in a decrease in the number of infected cells (measured as focus-forming units [FFUs]).

occasion in protein crystallography to act as a linking agent between molecules in the crystal lattice; examples include Protein Data Bank ID codes 4OUA, 4PHI, 4Z13, 6GS3, 6QSE, and 6N9O (reviewed in ref. 40). The new crystal form diffracted up to 1.8 Å resolution, generating largely anisotropic datasets. The overall 3D structure of human MANEA is a single domain (βα8) barrel with a (partially; see below) open active center in which Glu404 and Glu407 (human numbering, as part of a conserved EWHE motif) are the catalytic residues in a neighboring group participation mechanism that proceeds through an epoxide intermediate (34) (Fig. 3 B and C). The structure is similar (Co rmsd >0.9 Å over 333 matched residues) to structures reported for BcGH99 and BsGH99 (31), with which MANEA shares 40% sequence identity. The positions of the −2 to +2 ligands in the bacterial and human GH99 proteins are equivalent and the sugar interactions in the −2 to +2 subsites, all of which bind mannosides with identical linkages in both high-mannose N-glycans (MANEA) and yeast mannan (bacterial endowed-α,1,2-mannanases) (29) are invariant.

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Previously, changes in the N-glycan structure of vesicular stomatitis virus (VSV) G protein induced by the MANEA inhibitor GlcDMJ were identified by demonstrating a change in susceptibility to hydrolysis by endo-β-N-acetylglucosaminidase (endo H) (41). Therefore, we digested BVDV envelope glycoproteins E1/E2 with endo H.

Increased sensitivity of E1/E2 proteins to endo H cleavage was observed in both GlcIFG and the glucosidase I/II inhibitor N-[6-\{4″-azido-2″-nitrophenylamino\}hexyl]-1-deoxynojirimycin (NAP-DNJ), indicating an increased prevalence of high-mannose N-glycans. The increased sensitivity for GlcIFG was greater than that for NAP-DNJ, most likely reflecting the higher concentration of the former. Treatment with a combination of GlcIFG and NAP-DNJ provided an even greater sensitivity to endo H treatment, consistent with more effective cessation of N-glycan processing by blocking the glucosidase and endomannosidase pathways.

We next examined BVDV replication in the presence of a combination of GlcIFG and NAP-DNJ (Fig. 4C). These data showed that the combination of GlcIFG and NAP-DNJ gave a greater reduction than either agent alone, suggesting an additive antiviral effect from inhibiting both the α-glucosidase I/II and endomannosidase pathways. The addition of NAP-DNJ not only inhibits calnexin-mediated folding and quality control, but also requires viral glycans to undergo processing by MANEA, hence potentiating the antiviral effect.

To further define the antiviral potential of MANEA inhibitors, we extended these studies to DENV. The MANEA inhibitors GlcIFG, GlcDMJ, CyMe-GlcIFG, and ManIFG were used to study levels of viral particle formation and infectivity. Viral particle formation, as assessed by secreted viral RNA, was unaffected by treatment with MANEA inhibitors (Fig. 4D). However, treatment with GlcIFG, CyMe-GlcIFG, and GlcDMJ caused a reduction in plaque number, demonstrating that differences in glycosylation resulting from MANEA inhibition impair DENV infectivity. The greatest effect was seen for GlcDMJ, which reduced the number of plaque forming units by sixfold; ManIFG had no effect. The antiviral effect observed was at a relatively high concentration, but iminosugars are known to have difficulty in gaining access to the secretory pathway (21).

This proof-of-principle study demonstrates that GlcDMJ is a potential broad-spectrum antiviral, with changes in glycosylation reducing infectivity of the progeny. As it is the weakest-binding inhibitor, factors other than affinity, such as cell permeability, may be responsible for the potency of antiviral effects.

Summary

We report the 3D structure of Golgi MANEA, a eukaryotic N-glycosylation pathway glycosidase. The data provide a structural rationale for understanding the change in specificity of this enzyme for monoglucosylated, diglucosylated, and triglucosylated high-mannose N-glycans. We also show the potential for MANEA inhibitors to alter N-glycan structures of viral envelope glycoproteins and reduce viral infectivity. MANEA processes the Glc1–3Man9GlcNAc2 structure and provides a pathway for glycoprotein maturation independent of the classical α-glucosidase I/II-dependent pathways. Treatment of HBV with miglustat (N-butyldeoxynojirimycin, an inhibitor of α-glucosidase II) impaired viral DNA secretion and led to aberrant N-glycans on M proteins.

Fig. 4. Antiviral action of MANEA inhibitors. Results of BVDV reinfection assays in MDBK cells (A, B, and C) and DENV reinfection assays in Huh7.5 cells (D and E). (A) Percentage of FFU/mL relative to untreated cells at different concentrations of GlcIFG, at an MOI of 1. (B) Effect of MANEA inhibition (GlcIFG) and ER glucosidase II inhibition (NAP-DNJ) on the susceptibility of glycans on the BVDV E1/E2 protein to cleavage by endoH. (C) The combined effects of NAP-DNJ and GlcIFG on BVDV infectivity, as measured by FFU/mL. Experiments were performed in triplicate. (D) Secreted RNA levels in DENV-infected Huh7.5 cells. (E) Reinfectivity plaque assay from DENV-infected Huh7.5 cells. The horizontal bar in D and E indicates the mean.
glycoprotein, but other viral glycoproteins displayed mature glycans that arose through the endomannosidase pathway (23). Conversely, GlcDMJ treatment of VSV led to changes in G protein glycosylation (41).

These previous studies and the present work demonstrate that different viral glycoproteins have varying degrees of dependence on the α-glucosidase I/II and endomannosidase pathways for maturation, and that inhibition of MANEA alone can alter the infectivity of two encapsulated viruses. The human MANEA 3D structure along with the demonstrated antiviral activity of disaccharide imino and asa sugar inhibitors provide a foundation for future inhibitor and drug development work. Given the devastating consequences of global outbreaks of viral disease, the present work highlights the potential for MANEA as a new target for host-directed antiviral agents exploiting viral glycoprotein biosynthesis.

Methods
The methodology of this study is described in more detail in SI Appendix.

Expression, Characterization, and Structure Solution. In brief, recombinant MANEA-Δ97 featuring an N-terminal His tag was expressed in E. coli from a pColdI system coexpressing the groEL and groES genes encoding the GroEL/ES chaperone system. Recombinant MANEA-Δ97 was purified by metal-ion affinity and cation-exchange chromatography. Extensive crystal screening was performed using a coupled assay with GlcMan−GlcNaNac as the substrate, with subsequent permethylated and analyzed by matrix-assisted laser desorption/ionization mass spectroscopy. Michaelis–Menten kinetics was performed using a coupled assay with GlcMan−GlcNAc as the substrate and with standard curve generated from high-titer viral RNA isolated from C6/36-grown DENV2. The 95% confidence intervals were determined based on biological and technical variations and graphed using GraphPad Prism 6. The infectious DENV titers in supernatants collected were evaluated by a plaque assay.

Data Availability. All study data are included in the main text and SI Appendix.

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