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Quantitative study of yeast Alg1 beta-1, 4 mannosyltransferase activity, a key enzyme involved in protein *N*-glycosylation

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ABSTRACT

Background: Asparagine (*N*)-linked glycosylation begins with a stepwise synthesis of the dolichol-linked oligosaccharide (DLO) precursor, Glc3Man9GlcNAc2-PP-Dol, which is catalyzed by a series of endoplasmic reticulum membrane-associated glycosyltransferases. Yeast *ALG1* (asparagine-linked glycosylation 1) encodes a β-1, 4 mannosyltransferase that adds the first mannose onto GlcNAc2-PP-Dol to produce a core trisaccharide Man1GlcNAc2-PP-Dol. *ALG1* is essential for yeast viability, and in humans mutations in the *ALG1* cause congenital disorders of glycosylation known as ALG1-CDG. Alg1 is difficult to purify because of its low expression level and as a consequence, has not been well studied biochemically. Here we report a new method to purify recombinant Alg1 in high yield, and a mass spectral approach for accurately measuring its β-1, 4 mannosyltransferase activity.
Methods: N-terminally truncated yeast His-tagged Alg1 protein was expressed in *Escherichia coli* and purified by HisTrap HP affinity chromatography. In combination with LC-MS technology, we established a novel assay to accurately measure Alg1 enzyme activity. In this assay, a chemically synthesized dolichol-linked oligosaccharide analogue, phytanyl-pyrophosphoryl-α-*N*, *N*′-diacetylchitobioside (PPGn2), was used as the acceptor for the β-1, 4 mannosyl transfer reaction.
Results: Using purified Alg1, its biochemical characteristics were investigated, including the apparent K_m and V_{max} values for acceptor, optimal conditions of activity, and the specificity of its nucleotide sugar donor. Furthermore, the effect of ALG1-CDG mutations on enzyme activity was also measured.
General significance: This work provides an efficient method for production of Alg1 and a new MS-based quantitative assay of its activity.

1. Introduction

In eukaryotes, asparagine (*N*)-linked glycosylation is one of the most important post-translational modifications that directly affects both the structural and biological function of glycoproteins [1,2]. *N*-glycan synthesis begins with a highly conserved multistep process, assembling a dolichyl-linked oligosaccharide (DLO) precursor, Glc3Man9GlcNAc2-PP-Dol on the endoplasmic reticulum (ER) membrane [3]. A series of ER glycosyltransferases, known as Alg (asparagine-linked glycosylation) proteins catalyze the synthesis of DLO [4]. Thirteen *ALG* genes have been cloned, which encode the twelve Alg glycosyltransferases that together lead to the production of Glc3Man9GlcNAc2-PP-Dol [5]. Many of these *ALG* genes, including *ALG1*, were first identified by Robbins and co-workers through a mutant screen for *S. cerevisiae* mutants blocked in the synthesis or extension of the DLO precursor [6,7]. Further genetic and biochemical experiments demonstrated that *ALG1* encodes an essential β-1, 4 mannosyltransferase, which adds the first mannose to GlcNAc2-PP-Dol (DPGn2) using GDP-mannose to produce Man1GlcNAc2-PP-Dol (DPGn2-man) [8]. In humans, mutations in *ALG1* lead to congenital disorders of glycosylation (CDG) termed as ALG1-CDG. ALG1-CDG patients suffer from broad multisystem defects with varying degrees of clinical severity. To date, fifty-two different human *ALG1* mutations have been reported, making ALG1-CDG the third most common CDG type [9–15]. The pathogenicity of human *alg1* mutations has been tested by a yeast complementation assay that measures the inability of human *alg1* mutant alleles to rescue the hypoglycosylation phenotype of a yeast temperature sensitive *alg1* mutant [10,12]. However, these yeast assays are limited in advancing our understanding of ALG1-CDG because the clinical severity of *alg1*

Abbreviations: Alg, asparagine-linked glycosylation; DLO, dolichol-linked oligosaccharide; DPGn2, dolichol pyrophosphate-GlcNAc2; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; ER, endoplasmic reticulum; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; PCR, polymerase chain reaction; PPGn2, phytanyl-pyrophosphate-GlcNAc2; TMD, transmembrane-spanning domain; UPLC, ultra-performance liquid chromatography.

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human phenotypes often fails to correlate with the severity of their phenotypes when expressed in yeast [12]. These observations underscore the need for new methods to assay Alg1 as a means of correlating its activity with the clinical severity of potential mutants.

GlcNAc2-PP-Dol (DPGn2) is the natural substrate of Alg1. Because dolichol is structurally complex (containing up to twenty hydrophobic isoprene units), it is difficult to synthesize. Alg1 *in vitro* assays using DPGn2 require its labor-intensive purification from microsomal membranes of cells or tissues [16,17]. Thus, a major advance in the analyses of DLO biosynthesis *in vitro* was the development of phytanyl-pyrophosphate-GlcNAc2 (PPGn2) as a DPGn2 mimic for Alg1 mannosyltransferase activity [18,19]. Phytanol is much shorter than dolichol (four versus twenty isoprene units) and can be conveniently obtained via chemical synthesis [20]. Importantly, Alg1 displays similar affinity for PPGn2 and DPGn2 *in vitro* [18]. Thus the use of PPGn2 as acceptor substrate has been widely used for Alg1 enzymatic studies *in vitro*. In this assay, the donor, GDP-[^{3}H]Man is used radiolabel the product, PPGn2-[^{3}H]Man, which is analyzed by HPLC. This method is constrained by the need to use radiolabeling. To bypass the need for radiolabeled donors, fluorescent reagents have been used to label oligosaccharide products [21]. The major drawback of fluorescent labeling methods is they require an additional step. Recently, mass spectrometry (MS) techniques have become the most powerful tools for glycan and glycoconjugate analyses [22] but as yet, these have not been applied to quantitate Alg glycosyltransferase enzymatic activity *in vitro*.

To comprehensively and quantitatively study enzymatic activity of Alg1, purified proteins are required, suggesting the importance of production and purification of recombinant Alg1 protein. Additionally, recombinant Alg1 has been exploited to synthesize Manβ1-4GlcNAcβ1-4GlcNAc (Gn2-Man) core trisaccharide to overcome difficulties in the chemical synthesis of the β-1, 4-mannoside linkage [23,24]. Using PPGn2 as the acceptor substrate, this chemoenzymatic approach can efficiently generate Gn2-Man core oligosaccharide, which can be elongated subsequently by downstream Alg glycosyltransferases to produce each of the different LLO intermediates of *N*-glycans. However, a sufficient amount of recombinant Alg1 protein is required in this strategy. Several approaches have been reported for purifying recombinant Alg1 protein. For instance, yeast Alg1 protein with deletion of the *N*-terminal transmembrane domain was successfully overexpressed in *E. coli*, and lysate from these transformed cells was confirmed to contain β-1, 4 mannosyltransferase activity, but the Alg1 protein yield was too low for purification. Instead of using purified proteins, the lysate was directly immobilized onto a nickel (II) chelation column and then used for the mannosylation of PPGn2 [23].

In this study, we describe a new method to overproduce and purify yeast Alg1 protein. The β-1, 4 mannosyltransferase activity of purified Alg1 protein was quantitatively characterized by a new *in vitro* assay, in which amide normal-phase liquid chromatography (LC) coupled with ESI-MS technologies were used to directly detect and analyze the oligosaccharides produced in a reaction containing purified Alg1 and GDP-mannose. Furthermore, this assay was applied to analyze patient-relevant missense mutations in Alg1 to judge their clinical severity.

2. Materials and methods

2.1. Plasmid constructions

The expression plasmid pET28-His6-Alg1ΔTM encodes His6-tagged *S. cerevisiae* Alg1 that lacks its predicted *N*-terminal 34 amino acid transmembrane spanning domain (TM) (hence forth referred to as His-Alg1ΔTM). It was constructed by PCR amplification of a 1245 bp *ALG1* fragment that was cloned in the *NheI* and *XhoI* sites of pET28 (Merck, Kenilworth, NJ, USA), in frame with the sequence encoding an *N*-terminal His6-tag. Mutations that altered various amino acids (see Table 1) were introduced using overlapping PCR with mutagenic primers (BGI, Shenzhen, China) and verified by DNA sequence analysis. Sequences of primers used in plasmid constructions are available upon request.

Table 1
Specific activities of Alg1 mutant proteins. One unit (U) is defined as the enzyme activity that transfers 1 μmol of mannose to PPGn2 per minute under standard assay conditions.

Enzyme	Specific activity (U/mg)
Wild type	6.25
S. cerevisiae mutants	
E278K	0
G310D	0
D363A	0.83
D370A	0.58
CDG related mutants	
K266W	5.56
Q331P	0
M366V	2.51
V385X	0

2.2. Expression and purification of recombinant His-Alg1ΔTM

pET28-His6-Alg1ΔTM was transformed into *E. coli* Rosetta cells (DE3, Merck, Kenilworth, NJ, USA) and transformants were selected on plates containing kanamycin (50 μg/mL) and chloramphenicol (34 μg/mL). Single colonies were cultured in Luria-Bertani (LB, 1% tryptone, 0.5% yeast extract and 1% NaCl) or Terrific-Broth (TB, 1.2% tryptone, 2.4% yeast extract and 0.5% glycerol) at 37 °C to an OD_{600} of 1.0. The cultures were cooled to 16 °C and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sangon Biotech, Shanghai, China), shaking overnight at 16 °C. Cells were harvested and resuspended in [150 mM NaCl; 50 mM Tris/HCl (pH 8.0)], then disrupted by sonication on ice. Membrane fractions were prepared by centrifugation of the cell lysate to remove cellular debris (4000 ×*g*, 20 min), followed by pelleting of the membranes (20,000 ×*g*, 90 min). The supernatant was removed and the pelleted membrane fraction was solubilized for 1 h in the above buffer (50 mM Tris/HCl and 150 mM NaCl, pH 8.0) containing 1% Triton X-100. Insoluble material was removed (12,000 ×*g*, 20 min) and His-Alg1ΔTM was purified from the resulting supernatant by using a HisTrap HP affinity column (GE Healthcare, Buckinghamshire, UK). The column was washed with buffer (50 mM Tris/HCl and 150 mM NaCl with 1% Triton X-100, pH 8.0) containing 60 mM imidazole and eluted with 500 mM imidazole. All fractions were analysed by SDS-PAGE. His6-Alg1ΔTM was dialyzed against [25 mM Tris/HCl (pH 8.0), 50 mM NaCl] overnight at 4 °C, using 10 kDa cutoff tubing (Sangon Biotech, Shanghai, China). After dialysis, the protein solution was centrifuged with Amicon Ultra 10 K NMWL (Millipore, MA, USA), and the concentration was measured using the BCA assay (Sangon Biotech, Shanghai, China).

Expression levels of His-Alg1ΔTM in *E. coli* were analyzed by western blot. Ten μg of *E. coli* lysates were separated by SDS-PAGE and transferred to a PVDF membrane. Immunoblotting was performed with an anti-His mouse antibody (TransGen Biotech, Beijing, China) as the primary antibody, the anti-mouse IgG (H + L) HRP Conjugate (TransGen Biotech, Beijing, China) as the secondary antibody, and detected by enhanced chemiluminescence (ECL) (Bio-Rad, CA, USA).

2.3. Quantitative assay of enzyme activity

The lipid-linked acceptor analogue PPGn2 (Fig. 1A) was chemically synthesized as reported [24]. Standard transferase assay conditions are as follows. PPGn2 (50 μM), GDP-Man (2 mM, Sigma-Aldrich, St. Louis, MO, USA) and His-Alg1ΔTM (80 ng/mL) were incubated in 20 mM Tris/HCl, 1 mM dithiothreitol (DTT), 0.15 mM EDTA, 0.13% NP-40 and 10 mM $MgCl_2$ (50 μL, pH 7.2) at 30 °C. After 30 min, the reaction was stopped by heating at 100 °C for 2 min. 0.2 mL of hydrogen chloride

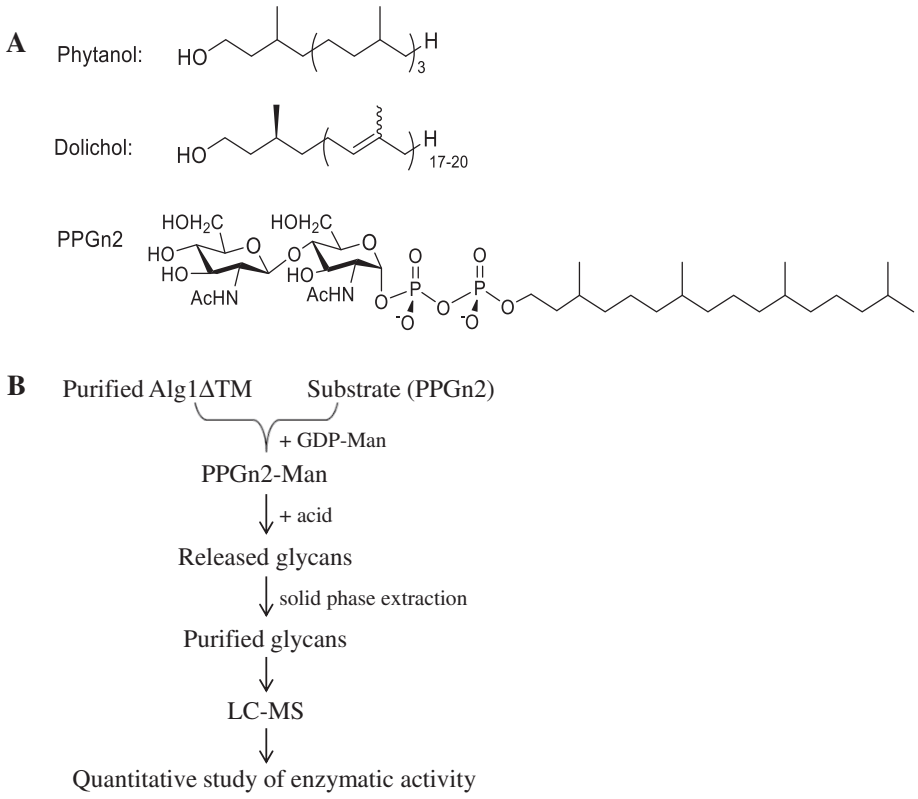

Fig. 1. The structure of PPGn2 (A) and the flowchart for assaying its Alg1-dependent conversion to PPGn2-Man (B). (A) Phytanol consists of 4 isopentane units and dolichol consists of up to 20 isoprene units. PPGn2 was synthesized from phytanol and chitobiose octaacetate as reported [24]. (B) The procedure of the assay system described in this study.

(20 mM) in a 4:1 ratio of H_2O: MeOH was added and the mixture was heated to 100 °C for 1 h to hydrolyze the glycans. The water-soluble fraction was desalted by solid-phase extraction using 1 mL Supelclean ENVI-Carb slurry (Sigma, MO, USA) equilibrated with 2% acetonitrile. The column was washed by 2% acetonitrile (10 mL), and oligosaccharide was eluted with 25% acetonitrile (3.0 mL) and lyophilized. Desalted oligosaccharides were injected into a Dionex Ultimate 3000 UPLC (Thermo Scientific, MA, USA, conditions: column, Waters Acquity UPLC BEH Amide Column 1.7 μm 2.1 × 100 mm; eluent A, CH_3CN; eluent B, H_2O; gradient: 0–2 min, 20% B; 2–15 min, 20–50% B; 15–18 min, 50% B; flow rate, 0.2 mL/min). The ESI-MS of eluate was measured on a TSQ Quantum Ultra (Thermo Scientific, MA, USA) in the mass range of 400–800 *m/z* (positive mode). Oligosaccharide transfer rate was quantified by calculating the peak intensity in LC-ESI-MS using Xcalibur (Version 2.0, Thermo Scientific, USA).

2.4. Characterization of recombinant His-Alg1ΔTM

Enzymatic assays were performed using the standard conditions mentioned above, and with variations of several parameters, including temperature, pH and divalent metal ions. The apparent K_m and V_{max} values for PPGn2 (10–200 μM) were calculated by nonlinear regression curve fitting (OriginPro 7.5, OriginLab Corp.), with His-Alg1ΔTM (8 ng/mL) and reaction time of 10 min. The substrate specificity of His-Alg1ΔTM was confirmed by screening UDP-Glc, UDP-GlcNAc and GDP-Man, all at the same concentration (2 mM). The optimized reaction condition, determined to be 30 °C, pH 9.0 with 10 mM Mg^{2+} was applied to obtain the maximum rate with which His-Alg1ΔTM catalyzed conversion of PPGn2 to PPGn2-Man.

The stereochemistry of the linkage between Man and GlcNAc in Gn2-Man was determined by mannosidase digestion. Gn2-Man (200 μM) was incubated with 0.1 U of β-mannosidase (*Helix pomatia*, Sigma-Aldrich, MO, USA) in 50 mM sodium citrate (50 μL, pH 4.4) at 25 °C for 16 h. The reaction mixture was analyzed by LC-MS as described above.

3. Results

3.1. Overproduction and purification of recombinant His-Alg1ΔTM

To facilitate purification, *His-ALG1ΔTM,* which encodes His-tagged Alg1 lacking the 34 amino acid hydrophobic *N*-terminal TMD was expressed in *E. coli*, and the level of His-Alg1ΔTM in bacterial lysates was analyzed by Western blot with anti-His antibody (Fig. 2A). Compared to lysates from uninduced control cells (Fig. 2A, lane 1), His-Alg1ΔTM expression in cells grown in LB and induced with IPTG could be detected, but the yield was quite low (Fig. 2A, lane 2). On the other hand, when cells were grown in TB and induced with IPTG, His-Alg1ΔTM expression increased dramatically; ~50 fold higher than in LB (Fig. 2A, lane 3). His-Alg1ΔTM was then purified by a HisTrap HP affinity column and applied to SDS-PAGE (Fig. 2B). In contrast to the uninduced fraction (Fig. 2B, lane 1), an additional band at 46 kDa, which corresponds to the molecular weight of Alg1ΔTM with 6His-tag, was observed after induction with IPTG (Fig. 2B, lane 2). This recombinant His-Alg1ΔTM protein was associated mainly in the cell membrane fraction (Fig. 2B, lane 3). A single band could be solubilized and purified from the membrane fraction following the procedure described in the Materials and methods section (Fig. 2B, lane 4). The yield of purified His-Alg1ΔTM was about 20 mg per liter culture. Purified His-Alg1ΔTM could be stored at 4 °C for weeks without significant loss of activity, suggesting its high stability. In addition, this recombinant Alg1 was detected as dimer by HPLC analysis (date not shown). This result consists with our previous report, which confirms the dimerization of Alg1 in yeast cells [25]. The specific activity of purified His-Alg1ΔTM

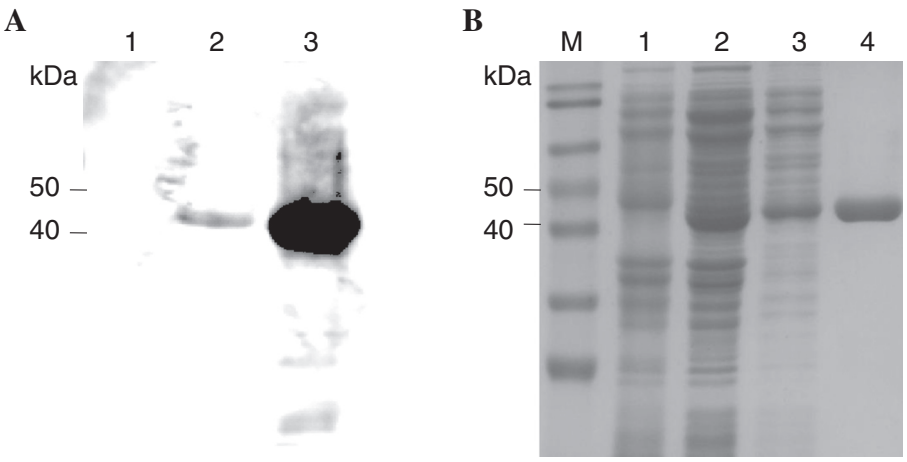

Fig. 2. Western blot analysis of recombinant His-Alg1ΔTM. (A) *E. coli* lysates were analyzed by SDS-PAGE and immunoblotted with anti-His antibody: uninduced *E. coli* (lane 1); *E. coli* grown in LB and induced with IPTG (lane 2); *E. coli* grown in TB and induced with IPTG (lane 3). (B) *E. coli* cells grown in TB. SDS-PAGE: uninduced *E. coli* lysate (lane 1); *E. coli* lysate induced with IPTG (lane 2); Triton X-100 extracted membrane fraction (lane 3); purified His-Alg1ΔTM (lane 4). "M" indicates molecular mass markers.

was calculated as 6.25 U/mg under standard assay conditions (Table 1), exhibiting a 20,000-fold increase compared with a crude yeast microsomal extract [26] and a 600-fold increase compared with *E. coli* cell lysates or nickel (II) beads trapped Alg1ΔTM [18,23,27].

3.2. Quantitative analysis of His-Alg1ΔTM activity using LC-MS

The mannose acceptor PPGn2 (Fig. 1A) was prepared as reported [24]. Mannosylation of PPGn2 was catalyzed by purified His-Alg1ΔTM and produced PPGn2-Man (Fig. 1B). After acid hydrolysis of the lipid chain, saccharide moieties were purified and applied to LC-MS for quantitative analysis. As shown in Fig. 3A, two groups of peaks could be detected after UPLC. These groups were predicted to correspond to Gn2 and Gn2-Man. The following ESI-MS analysis showed that the first two peaks eluted at 6.29 and 6.67 min originated from the substrate PPGn2, and possessed the mass peak *m/z*: 447 ($[Gn2 + Na]^+$, Fig. 3B). The latter eluted two peaks at 9.09 and 9.40 min originated from the product, PPGn2-Man, and possessed the mass peak m/z: 609 ($[Gn2\text{-}Man + Na]^+$, Fig. 3C). Further investigation showed that two peaks in each group had consistent fragmentations in MS/MS analyses (data not shown), thereby suggesting they are glycan anomeric isomers, which were designated alpha (α) and beta (β). This idea was confirmed since the two peaks in each group shifted to one peak (Fig. 3D) after the glycans were reduced in 1% sodium borohydride. Conversion rate was calculated using peak areas of substrate (6.29 and 6.67 min) and product (9.09 and 9.40 min).

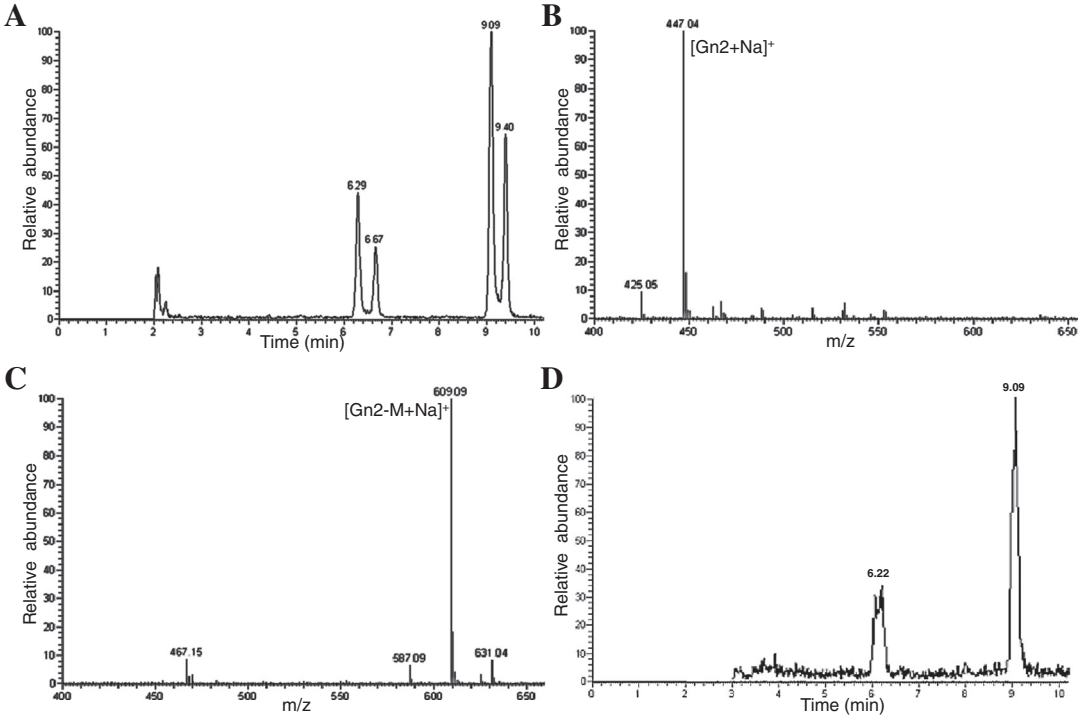

Fig. 3. LC-MS analyses to quantify the His-Alg1ΔTM activity. (A) The UPLC chromatogram of released glycans. Peaks eluted earlier represent Gn2, and peaks eluted later represent Gn2-Man. (B) Mass spectra of peaks eluted at 6.29 and 6.67 min in UPLC. (C) Mass spectra of peaks eluted at 9.09 and 9.40 min in UPLC. (D) The UPLC chromatogram of glycans reduced by sodium borohydride.

3.3. Characterization of His-Alg1ΔTM

The kinetic parameters of purified recombinant His-Alg1ΔTM were measured with a fixed concentration of 2 mM GDP-Man (Fig. 4A). The K_m value was calculated as 38.3 μM and the V_{max} value as 33.2 pmol·min^{-1}. The optimal conditions for enzyme activity was 30 °C (Fig. 4B), and pH 9.0 (Fig. 4C). Since most glycosyltransferases require additional divalent metal cations to remain active [17,26–29], we also examined the effect of various divalent metal ions. His-Alg1ΔTM was inactive in the presence of Ni^{2+}, Zn^{2+}, and EDTA, whereas it displayed optimal activity in the presence of Mg^{2+} (Fig. 4D). His-Alg1ΔTM also showed some activity in the presence Mn^{2+} and Co^{2+}. Three nucleotide sugar substrates are used in DLO synthesis as glycosylation donors, namely UDP-*N*-acetylglucosamine (UDP-GlcNAc), GDP-Man, and UDP-glucose (UDP-Glc). To determine Alg1 sugar donor specificity, each of these three nucleotide sugars was tested as substrates. This experiment demonstrated that recombinant His-Alg1ΔTM specifically exhibited transferase activity with GDP-Man, and not with UDP-GlcNAc or UDP-Glc (Fig. 4E).

We determined that optimal conditions were 30 °C, pH 9.0, with 10 mM Mg^{2+}. Under these conditions, we observed that a complete conversion of PPGn2 to PPGn2-Man was achieved by using 800 ng/mL of His-Alg1ΔTM. The UPLC chromatogram after 30 min of reaction is shown in Fig. 5A. To test the stereochemistry of newly formed Man-GlcNAc linkage, Gn2-Man was digested by β-mannosidase (see the Materials and methods section) followed by UPLC (Fig. 5B). After a 16 h digestion, the peaks correspond to Gn2-Man disappeared and two peaks correspond to Gn2 were observed, demonstrating that His-Alg1ΔTM catalyzed β-mannosylation.

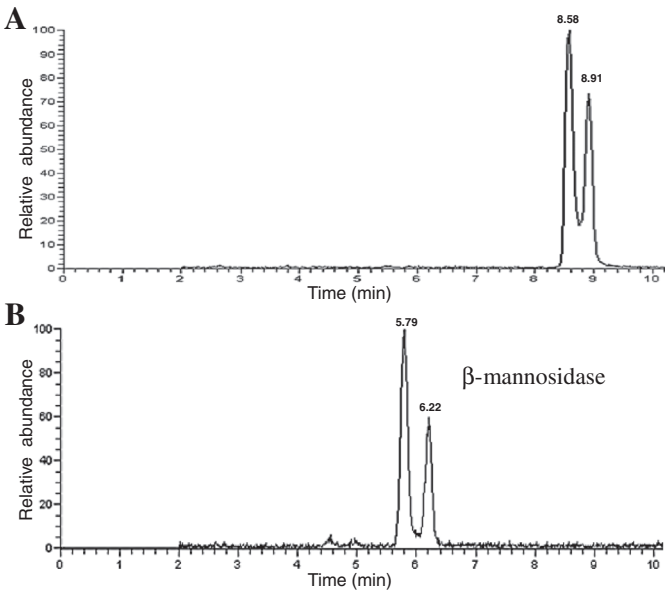

Fig. 5. Chromatogram of PPGn2-Man before and after β-mannosidase digestion. (A) The UPLC chromatogram of the reaction system using the optimized conditions. The two peaks eluted at about 9 min represent Gn2-Man. (B) The UPLC chromatogram of the β-mannosidase digestion reaction system using Gn2-Man as substrate. The two peaks eluted at about 6 min represent Gn2.

3.4. Enzyme activity of various His-Alg1ΔTM mutants

Previous analyses of Alg1 in *S. cerevisiae* reported that, *in vivo*, mutants E278K and G310D are completely inactive and result in lethality, whereas aspartic acids in the DX_6D domain (D363 or D370, Fig. 6A) are not essential for catalysis or viability [25]. In addition, partial loss of function mutations identified in three ALG1-CDG patients [9,11] implicated R276W, Q342P, M377V, and C396X (X indicates a nonsense mutation that leads to a premature stop) as being important for Alg1

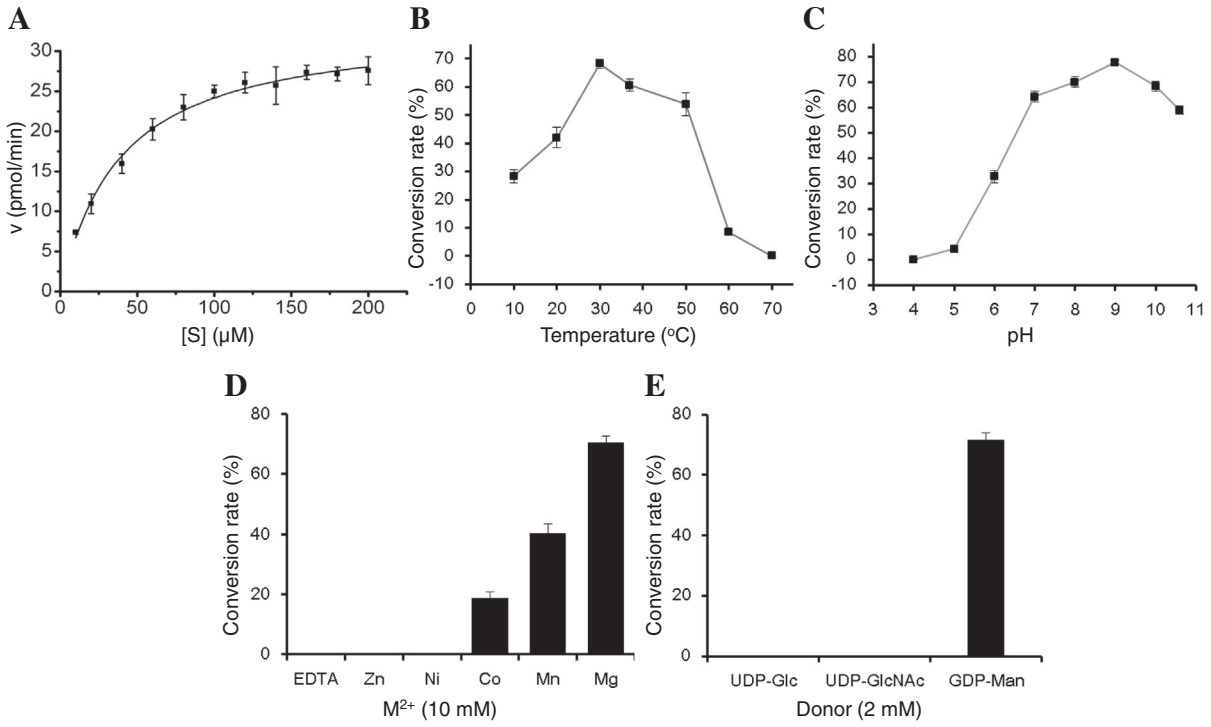

Fig. 4. Optimization of His-Alg1ΔTM enzymatic activity. (A) The K_m (38.3 μM) and V_{max} (33.2 pmol·min^{-1}) values for the substrate PPGn2, whose concentration ranged from 10 to 200 μM, were calculated by nonlinear regression with a constant concentration of 2 mM GDP-mannose. (B) Optimal temperature was evaluated, using seven temperatures (10 °C, 20 °C, 30 °C, 37 °C, 50 °C, 60 °C and 70 °C). (C) Optimal pH was examined by using different buffers, including sodium acetate-acetic acid buffer (pH 4, 5 and 6); Tris/HCl buffer (pH 7 and 8); glycine/NaOH buffer (pH 9, 10 and 10.6.) (D) Divalent cation dependency was examined using 10 mM ions (Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+} and Mg^{2+}, respectively) or 10 mM EDTA for depletion conditions. (E) The specificity of the nucleotide sugar donor was examined by performing the reaction with GDP-Man, UDP-GlcNAc, or UDP-Glc. Each data point represents the mean ± SD value calculated from three independent experiments.

function. The human mutations correspond to K266W, Q331P, M366V, and V385X in *S. cerevisiae* Alg1 (Fig. 6B). To measure the effect of these mutations on Alg1 enzyme activity, each of the mutations were introduced into *His-ALG1ΔTM*, expressed in *E. coli*, and purified as described above. Most of the mutant proteins could be purified at a similar yield as the wild-type His-Alg1ΔTM, except V385X (Fig. 6C). The specific activity of each of these mutant proteins was determined (Table 1). Consistent with the previously reported *in vivo* assay, E278K and G310D mutants proved to be inactive, and specific activities of D363A and D370A decreased (7.5 and 10.8 fold, respectively) compared with wild-type His-Alg1ΔTM. Among the CDG-related mutants, K266W displayed activity equal to the wild-type, whereas M366V decreased by 2.5-fold, and Q331P and V385X completely lost their activities.

4. Discussion

During DLO biosynthesis, Alg1 catalyzes the transfer of mannose from GDP-Man to DPGn2, resulting in the formation of DPGn2-Man. This is the first mannosylation step in the *N*-linked glycosylation pathway and is essential for proper glycoprotein function in all eukaryotes. Despite its importance, an understanding of this reaction has been stymied by the difficulties involved in the purification of Alg1 and of its acceptor substrate, as well as a simple means of assaying the conversion of acceptor to product. Here, we describe new and simple methods to purify and assay recombinant Alg1 and its product. We apply these methods to study a series of Alg1 mutant proteins related to human ALG1-CDGs as proof of principle for their utility in relating loss of Alg1 β-1, 4 mannosyltransferases activity to CDG pathology.

Alg1 purification is challenging because of its hydrophobicity. Like most other ER-associated Alg glycosyltransferases, Alg1 possesses multiple TMDs and exhibits low expression levels and stability. Truncated Alg1 that is deleted for the *N*-terminal 34 amino acid displays an activity similar to the full length wild type Alg1 protein, as well as an affinity to PPGn2 that is comparable to the natural dolichol-linked acceptor [18, 19]. To date, *in vitro* studies of Alg1 activity have used either cell lysates of *E. coli* expressing recombinant *ALG1* [18] or Alg1 immobilized by nickel (II) chelation resin [23]. In this study, we demonstrated that overproduction and purification of recombinant yeast His-tagged Alg1ΔTM, lacking only the first N-terminal TMD was successful in *E. coli*. High yield of recombinant Alg1 was dependent on the growth medium; expression was low in LB and required a richer TB medium, in which Alg1 levels increased by ~50 fold. This surprising result indicated that the richer medium and additional glycerol may promote Alg1 expression or stability. It should also be noted that other tags, including thioredoxin, *N*-utilization substance, and glutathione *S*-transferase (GST) were ineffective for Alg1 expression in LB (data not shown).

Despite the absence of the first predicted *N*-terminal transmembrane domain, recombinant His-Alg1ΔTM protein nevertheless remained associated in the membrane fraction of cells (Fig. 2B). This membrane association is consistent with the predicted structure of Alg 1 [25], which suggested that Alg1 may contain up to four membrane spanning domains. The observation that His-Alg1 ΔTM is membrane associated is consistent with those structural predictions. Current studies aimed at further elucidating the structural basis for Alg1 ΔTM membrane association are in progress. Nevertheless, this membrane association enabled an additional enrichment step, whereby His-Alg1ΔTM can

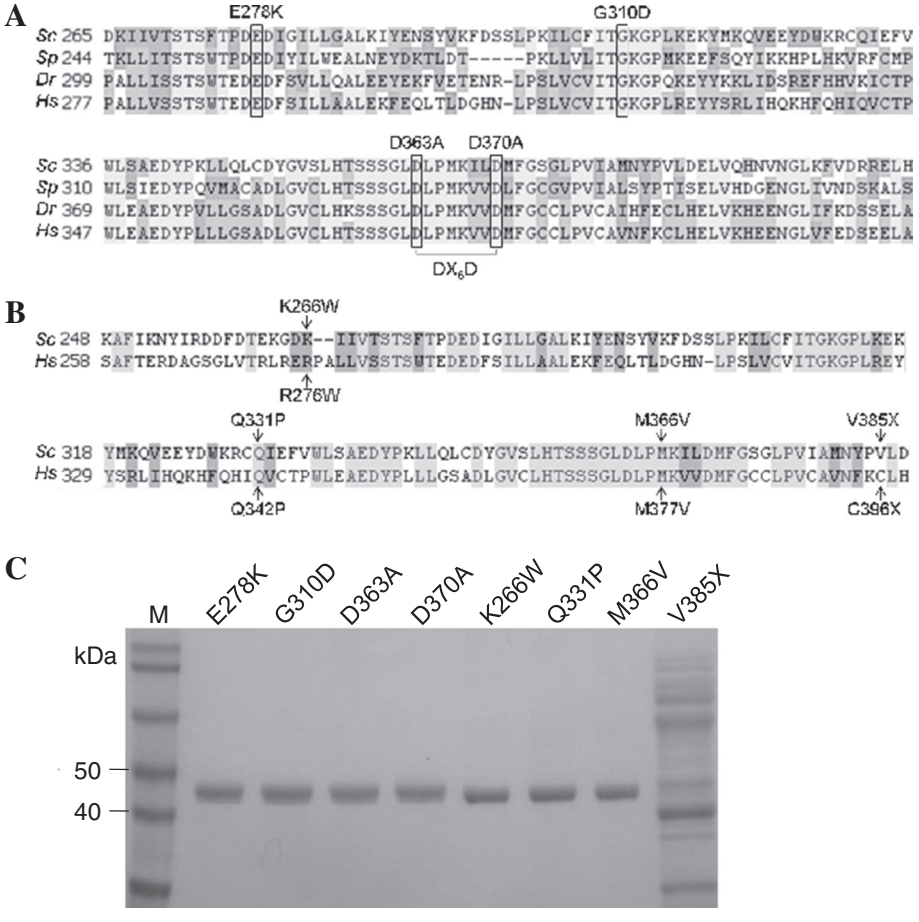

Fig. 6. Design and purification of His-Alg1ΔTM mutant proteins. (A) Alignment of *S. cerevisiae* (*Sc*), *Schizosaccharomyces pombe* (*Sp*), *Danio rerio* (*Dr*) and *Homo sapiens* (*Hs*) Alg1. Each of the mutations analyzed are boxed, with the alterations after mutagenisis indicated above. (B) Alignment of *S. cerevisiae* (*Sc*) and human (*Hs*) Alg1. Mutations are indicated by arrows, with the yeast mutants indicated to the top, and the corresponding human mutations indicated beneath the protein sequence. (C) SDS-PAGE of purified His-Alg1ΔTM mutants, including E278K, G310D, D363A, D370A, K266W, Q331P, M366V and V385X (X = stop codon). "M" indicates the molecular mass markers.

be solubilized with 1% of Triton-X, applied to a His-Trap affinity column for high yield and purity (Fig. 2B). The availability of a sufficient amount of His-Alg1ΔTM opens the way for performing the glycosylation reaction on a large scale and using PPGn2-Man as substrate for further downstream enzymatic reactions. In addition, purified His-Alg1ΔTM will facilitate high resolution structural studies that may reveal new information about the molecular mechanisms of glycosyltransferases in DLO biosynthesis pathway.

To study Alg1 activity, preparation of lipid-linked acceptor DPGn2 is essential. However dolichol, a long-chain lipid that is insoluble in water, is not commercially available and must be isolated from pig liver with low purity [30]. Pioneering work by Flitsch and her colleagues developed a phytanyl-linked substrate (PPGn2) to evaluate the mannosylation activity of Alg proteins instead of the dolichol-linked acceptor [19,24]. The practical use of the soluble PPGn2 opened the door to quantitative study on enzymatic activity of Alg glycosyltransferase. Taking advantage of this technique, we synthesized PPGn2 from phytol and chitobiose octaacetate [24], and then applied it to our new assay as the acceptor substrate for Alg1 instead of DPGn2. In addition to the high yield purification of Alg1 and PPGn2, another past remaining challenge in quantifying Alg1 activity was product analysis, which relied on removal of lipid from oligosaccharides, and oligosaccharide labeling prior to HPLC analysis [21,31,32]. To bypass the need for this two-step procedure, we applied LC-ESI-MS to measure product formation directly. To avoid the ionization suppression of oligosaccharides by the salt from HPLC eluent [33], we used only water and acetonitrile in the mobile phase to ensure the oligosaccharide signals in positive-mode ESI-MS were strong enough to be detected.

Accordingly, various biochemical parameters of purified His-Alg1ΔTM were examined. The calculated apparent *Km* value (38.3 μM, Fig. 4A) coincides well with the reported values of crude Alg1 from both yeast and recombinant *E. coli* cell lysates [18,23,26]. We also measured biochemical characteristics, including optimal temperature (Fig. 4B), pH value (Fig. 4C), and divalent metal ions requirements (Fig. 4D). Conversion of PPGn2 to its mannosylated product was almost 100%, under optimized conditions (Fig. 5A). Furthermore, Alg1 has a strict glycosylation donor selectivity, exclusively using GDP-Man. This donor specificity guarantees the structural homogeneity of oligosaccharide precursors whose correct structure is required for recognition by oligosaccharyltransferase [34].

Previous *in vivo* mutational analyses of yeast *ALG1* revealed that neither D363 nor D370 of the DX_6D domain are required for normal growth, whereas the E278K and G310D mutant alleles caused complete loss of Alg1 function [25]. In this present study, our *in vitro* assay provided a more quantitative analysis of how these four mutations (E278K, G310D, D363A, and D370A) influence Alg1 activity (Fig. 6A). Consistent with *in vivo* experiments, E278K and G310D mutants resulted in a complete loss of Alg1 activity (Table 1) [25]. However, in contrast to *in vivo* activity, the D363A and D370A mutations decreased Alg1 activity by 7.5- and 10.8-fold, respectively (compared to wild-type). Similar to the EX_7E domain found in most members of the PFAM glycosyltransferase 1 group (GT1-PFAM accession number PF00534), the DX_6D motif of Alg1 may be important for catalysis [35], although clearly not essential, at least in yeast.

We also analyzed *ALG1* mutations that correspond to those found in human ALG1-CDG patients. Mutation sites were chosen from three patients with heterozygous mutations of R276W (paternal allele) and C396X (maternal allele) [9]; heterozygous mutations of S258L (paternal allele) and Q342P (maternal allele) [11]; and homozygous mutations of M377V [9]. Sequence alignment of Alg1 from *S. cerevisiae* and *H. sapiens* demonstrated that K266W, Q331P, M366V and V385X are the corresponding Alg1 mutations in yeast (Fig. 6B). Purification of each of these proteins allowed us to measure each of their specific activities (Table 1). For the first patient, although V385X was completely inactive, K266W maintained almost 90% of wild-type activity, leading to mild CDG symptoms [9]. In the second patient, as human S258L has no conservation in yeast, only mutant Q331P was examined. To date, patients homozygous for the S258L mutation are terminally fatal. In our study, Q331P exhibited no transferase activity, which may explain the severe CDG symptoms combined with S258L [11,12]. The third patient also suffered mild CDG symptoms, which is predictable because homozygous mutation M366V retained only 40% of wild-type activity. Taken together these results demonstrate that the purification and assay system we describe could accurately measure Alg1 activity, and that the changes in activity due to specific mutations can be correlated with the severity of human ALG-CDG phenotypes. This may open a new avenue for estimating the severity of human CDG.

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