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Non-muscle myosin IIA transports a Golgi glycosyltransferase to the endoplasmic reticulum by binding to its cytoplasmic tail

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ABSTRACT

The mechanism of the Golgi-to-ER transport of Golgi glycosyltransferases is not clear. We utilize a cell line expressing the core 2 N-acetylglucosaminyltransferase-M (C2GnT-M) tagged with c-Myc to explore this mechanism. By immunoprecipitation using anti-c-Myc antibodies coupled with proteomics analysis, we have identified several proteins including non-muscle myosin IIA (NMIIA), heat shock protein (HSP)-70 and ubiquitin activating enzyme E1 in the immunoprecipitate. Employing yeast-two-hybrid analysis and pulldown experiments, we show that the C-terminal region of the NMIIA heavy chain binds to the 1–6 amino acids in the cytoplasmic tail of C2GnT-M. We have found that NMIIA co-localizes with C2GnT-M at the periphery of the Golgi. In addition, inhibition or knockdown of NMIIA prevents the brefeldin A-induced collapse of the Golgi as shown by the inhibition of the migration of both Giantin, a Golgi matrix protein, and C2GnT-M, a Golgi non-matrix protein, to the ER. In contrast, knockdown of HSP70 retains Giantin in the Golgi but moves C2GnT-M to the ER, a process also blocked by inhibition or knockdown of NMIIA. Also, the intracellular distribution of C2GnT-M is not affected by knockdown of β -coatomer protein with or without inhibition of HSPs, suggesting that the Golgi-to-ER trafficking of C2GnT-M does not depend on coat protein complex-I. Further, inhibition of proteasome results in accumulation of ubiquitinated C2GnT-M, suggesting its degradation by proteasome. Therefore, NMIIA and not coat protein complex-I is responsible for transporting the Golgi glycosyltransferase to the ER for proteasomal degradation. The data suggest that NMIIA is involved in the Golgi remodeling.

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1. Introduction

Golgi structure–function relationships have been debated among cell biologists since the discovery of the Golgi in 1898 (Marsh and Howell, 2002; Nakamura, 2010), indicating that there is still a lot we do not know about this organelle. Golgi apparatus is a posttranslational modification and sorting station of the cargos in mammalian secretory pathway. It is a dynamic organelle constantly undergoing remodeling, and made of two groups of resident proteins, matrix and non-matrix (Munro, 2011; Puri et al., 2004). The Golgi matrix proteins, which include proteins such as GM130, Giantin, etc., form the Golgi scaffold and can serve as the

docking sites for transport vesicles (Appenzeller-Herzog and Hauri, 2006). The Golgi non-matrix proteins, which include enzymes such as glycosyltransferases, carry out posttranslational modification of the cargos as they pass through the Golgi. The Golgi glycosyltransferases are type II membrane proteins synthesized in the ER where they acquire proper conformation with the help of chaperones such as heat shock proteins (Walter and Buchner, 2002). Then, they are transported to various compartments of the Golgi largely according to the steps they participate in the assembly of conjugated glycans. The Golgi localization signals of glycosyltransferases reside in the N-terminal region, which includes a cytoplasmic tail, a transmembrane domain, and a stem (Aoki et al., 1992; Burke et al., 1994; Colley, 1997; El-Battari, 2006; Nilsson et al., 1996). The cytoplasmic tail contributes to the retention of a glycosyltransferase to the Golgi by binding to a protein in a glycosyltransferase-specific manner (Osman et al., 1996; Quintero et al., 2008; Wassler et al., 2001; Schmitz et al., 2008). To date, only four mammalian glycosyltransferases of which binding proteins have been identified (Grabenhorst and Conradt, 1999; Schaub et al., 2006; Tu et al., 2008; Tu and Banfield, 2010; Uliana et al., 2006). The ER-to-Golgi transport of the Golgi proteins is also determined by the cytoplasmic tail

Abbreviations: C2GnT-M/2, Core 2 β 1,6 N-acetylglucosaminyltransferase mucus-type or isozyme 2; NMIIA, non-muscle myosin IIA; HSP, heat shock protein; COP, coat protein complex.

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(Giraud and Maccioni, 2003; Guo and Linstedt, 2006; Watanabe and Riezman, 2004). Coat protein complex (COP) II is known to be involved in the ER-to-Golgi anterograde transport of Golgi proteins (King, 2000; Palmer et al., 2009; Presley et al., 1997; Storrie et al., 1998). But, whether this applies to Golgi glycosyltransferases is not known. Although the cytoplasmic tails of the Golgi enzymes have been shown to be essential for their Golgi-to-ER transport (Okamoto et al., 2008; Uemura et al., 2009), the exact role played by the cytoplasmic tails remains to be established.

Non-muscle myosin IIA (NMIIA) is a member of the actin-based molecular motors (Bray, 2001; Fath, 2005; Ludowyke et al., 2006; Richards and Cavalier-Smith, 2005), which is composed of two heavy chains and two pairs of light chains. The C-terminal region of NMIIA heavy chains contains the cargo-binding site while the N-terminal region contains ATPase and actin-binding sites, which enable NMIIA to walk on actin filaments to transport the cargos (Conti and Adelstein, 2008; Sellers, 2000). NMIIA is known to be involved in the Golgi-to-ER transport of Golgi proteins (Duran et al., 2003; Vazhappilly et al., 2010). However, its role in the Golgi-to-ER transport of glycosyltransferases is not known.

In this communication, we show that core 2 N-acetylglucosaminyltransferase-M/2 (C2GnT-M), the enzyme that synthesizes all three β 6GlcNAc branch structures found in secreted mucins (Cheng and Radhakrishnan, 2011; Ropp et al., 1991; Schwientek et al., 1999; Yeh et al., 1999), utilizes a COPI-independent mechanism for its Golgi-to-ER transport. We show that NMIIA is responsible for transporting C2GnT-M to the ER by binding to the cytoplasmic tail of this enzyme. After transported to the ER, the enzyme is degraded by proteasome. We conclude that NMIIA is involved in transporting this Golgi enzyme to the ER for recycling.

2. Methods

2.1. Materials

The reagents were obtained from the following suppliers: C-Myc (mouse monoclonal and rabbit polyclonal) and γ -tubulin (mouse monoclonal) Abs, Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal Abs against the peptide GKADGAEAKPAE located at the C-terminus of the NMIIA heavy chain, mouse monoclonal anti- β -actin Ab, trypsin (Type IX), and NMIIA inhibitor Blebbistatin, Sigma (St. Louis, MO); anti-human C2GnT-M goat polyclonal Abs, Everest Biotech (Oxfordshire, UK); rabbit polyclonal Abs (anti-Giantin, anti-protein disulfide isomerase (PDI), and anti-ubiquitin), and mouse anti- β -COP and anti-HSP70 Abs, Abcam (Cambridge, MA); horseradish peroxidase-conjugated secondary Abs (donkey anti-rabbit, donkey anti-mouse, and donkey anti-goat), Jackson ImmunoResearch (West Grove, PA); BFA, KNK437, and proteasome inhibitor, MG-132, EMD Chemicals (Brookfield, WI).

2.2. Cell culture and siRNA treatment

Panc1-bC2GnT-M (c-Myc) cells were prepared and cultured as previously described (Choi et al., 2005). Blebbistatin, BFA, KNK437, and MG-132 were dissolved in DMSO, and diluted in medium immediately before use. Cells treated with a corresponding amount of DMSO were used as the control. MYH9 (myosin, heavy polypeptide 9, non-muscle), HSPA8 (heat shock 70 kDa protein 8) and scrambled on-targetplus smartpool siRNA were purchased from Dharmacon (Chicago, IL). Pool of 3 siRNAs targeting COPB was obtained from Santa Cruz Biotechnology. Panc1-bC2GnT-M (c-Myc) cells were transfected with 150–300 nM siRNAs using Liofectamine RNAi MAX reagent (Invitrogen, Carlsbad, CA). After cultured for 72 h, cells were analyzed by Western blotting.

2.3. Co-immunoprecipitation (Co-IP) and transfection

For identification of proteins in the complexes pulled down by Co-IP, confluent Panc1-bC2GnT-M (c-Myc) cells cultured on a T-75 flask were harvested by trypsinization, neutralized with soybean trypsin inhibitor, and then lysed with 1.5 ml of a non-denaturing lysis buffer, which contained 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40 (w/w), and 1% (v/v) of mammalian protease inhibitor cocktail (Sigma). One milliliter of cell lysate was pre-cleared with 50 μ l of irrelevant antibody (1 mg/ml) of same species and isotype as that to be employed for IP for 1 h at 4 °C. It was followed by incubation with 100 μ l of a 50% slurry of protein G plus agarose (EMD) at 4 °C for 1 h with gentle rocking. An aliquot of the supernatant was incubated with anti-c-Myc C2GnT-M or NMIIA Abs (1.5 μ g Abs to 400 μ g protein in 1 ml cell lysate) overnight at 4 °C with gentle rocking. Then, 50 μ l of protein G agarose slurry was added and incubated for 1 h at 4 °C to capture the immunocomplexes, which were then analyzed by SDS-PAGE.

2.4. Gel electrophoresis and immunoblotting

Proteins were separated on SDS-PAGE on mini-gels and electro-transferred to PVDF membranes. After blocking in PBS solution containing 5% nonfat dried milk and 0.1% Tween-20 (PBST) at RT for 1 h, the membranes were incubated at 4 °C overnight in same blocking solution containing primary Abs. Following washing with PBS (pH 7.4) containing 0.1% Tween-20, the membranes were incubated with HRP-conjugated secondary Abs for 1 h. Immunoreactive bands were detected by Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate reagents and exposed to BioExpress Blue Basic Autorad chemiluminescence film (Kaysville, UT). The bands on the autoradiography films were scanned with ScanJet 6200C (Hewlett Packard) driven by Adobe Photoshop.

2.5. Immunofluorescence staining and confocal fluorescence microscopy

Panc1-bC2GnT-M (c-Myc) cells grown overnight on cover slips were fixed in 4% paraformaldehyde/PBS at RT for 30 min. After treated with mouse anti-c-Myc and rabbit anti-NMIIA Abs (1:100) at 37 °C for 1 h, the cells were stained with fluorochrome-conjugated DyLight 488 donkey anti-mouse Ab (green), and DyLight 594 donkey anti-rabbit Ab (red) (1:200) and mounted in ProLong Gold antifade reagent with and without DAPI (Invitrogen). After deparaffinization, human bronchial epithelial tissue sections were rinsed with water and then treated with Tris/EDTA (pH 9) buffer in a microwave for 20 min to retrieve the antigen. Following blocking with 1% donkey serum for 1 h at RT, the sections were incubated with anti-C2GnT-M goat polyclonal (1:50) and rabbit polyclonal anti-Giantin Abs (1:150) for 1 h at RT, followed by treatment for 1 h at RT with fluorochrome-conjugated DyLight 488 donkey anti-goat Ab (green), and DyLight 594 donkey anti-rabbit secondary Ab (red) (1:200). The treated tissue sections were mounted in ProLong Gold antifade reagent containing DAPI. Stained cells and tissue sections were viewed under a Zeiss 510 meta confocal laser scanning microscope. Images were analyzed using Zeiss 510 software. For some figures, image analysis was performed using the Adobe Photoshop and the ImageJ.

2.6. Proteomics analysis of electrophoretically separated proteins

The SDS-PAGE separated and Coomassie blue-stained bands were excised, trypsinized, and treated with 60% acetonitrile

containing 0.1% trifluoroacetic acid to extract the peptides. The peptides were analyzed by the Thermo Fisher LCQ Deca Plus system liquid chromatograph ion trap mass spectrometry with peaks subjected to MS/MS fragmentation by collision-induced dissociation. Full mass spectra of parent ions and MS/MS fragmentation data were processed using BioWorks 3.2 software based on SEQUEST algorithm. Proteomics analysis of these protein bands was carried out by the mass spectrometry and proteomics core facility at UNMC.

2.7. Cloning, expression and purification of the C-terminal region of human non-muscle myosin heavy chain 9 (MYH9) in *E. coli*

The RNA of A549 cells was used for PCR cloning of the cDNA of the C-terminal 1682–1960 aa of NMIIA heavy chain 9 (MYH9, accession no. NM.002473.3). The forward primer “MYH9-For” 5'-TAGGATCCATGATCCAGTTGCAGGAGGAAGT-3' and the reverse primer “MYH9-Rev” 5'-ATGAATTCATTTTCGGCAGGTTGGCCTCAGC-3' were used. The restriction sites BamHI and EcoRI are underlined. The purified PCR product (840 bp) was cloned into the 3' end of the GST fusion protein flanked by a Precision protease cleavage site (–) located in LeuGlcValLeuPheGlnGly-Pro sequence in the pGEX-6P-2 vector (GE health care, Piscataway, NJ). This plasmid DNA was amplified in high efficiency competent DH5 *Escherichia coli* (New England Biolab, Ipswich, MA) and then used to transform the BL21 (DE3) *E. coli*. A single clone was cultured in 100 ml LB medium containing ampicillin (100 µg/ml). The *E. coli* culture at the logarithmic phase (OD₆₀₀ ~0.6) was induced with 0.5 mM isopropylthiogalactoside (IPTG) at 37 °C for 3 h. The cell pellet was dissolved in 10 ml lysis buffer (10 ml PBS; 80 µl 100 mM PMSF; 10 µl 1 M DTT; 80 µl NP-40 and a tablet of Roche protease inhibitor cocktail mini (Indianapolis, IN) and lysed 3 times with French press (Thermo Scientific). The supernatant (13,000 × g, 40 min at 4 °C) was mixed with 1 ml of glutathione resin (Genscript, Piscataway, NJ) to immobilize the recombinant protein. The beads were suspended in 1.5 ml of PBS containing 40 µg of GST-tagged Precision protease (GE health care) to cleave the recombinant protein from the resin. After rocked at 4 °C for 16 h, the supernatant was mixed with 500 µl of fresh glutathione resin to remove the Precision GST-protease. The purity of the 32 kDa recombinant MYH9 protein in the supernatant was assessed with 10% SDS-PAGE.

2.8. Isolation of NMIIA in Panc1-bC2GnT-M (c-Myc) cell lysates or recombinant MYH9 with biotinylated N-terminal 21 and 7 amino acid (aa) peptides of hC2GnT-M

A 21 aa peptide, which includes the cytoplasmic tail (MVQWKRLCQLHYL) and a portion of the transmembrane domain (WALGCYM) located at the N-terminus of hC2GnT-M, was tagged with a biotin at the C-terminus (LifeTein LLC, South Plainfield, NJ). A 15 aa peptide (LCQLHYLWALGCYMK) tagged with biotin at the C-terminus served as the control. An N-terminal 7 aa peptide (MVQWKRL) was tagged with biotin at the Met (AAPPTec LLC, Louisville, KY). To isolate NMIIA, 20 µl of hC2GnT-M peptide in 25% acetic acid (0.1 mg/ml) was mixed with 20–40 µl of cell lysate (1.5–3.5 mg/ml of protein) containing 5% β-mercaptoethanol or 100 µl of purified C-terminal part of NMIIA heavy chain 9 (MYH9) (0.5 mg/ml). After incubation at 37 °C for 1 h, 100 µl of Dynabeads M-280 streptavidin (Dyna, Norway) was added. Following gentle rotation for additional 30 min, the beads with immobilized complexes were trapped with a magnet. The captured proteins were separated on 8% SDS-PAGE followed by Western blotting with rabbit anti-NMIIA Abs.

2.9. Trypsin digestion of the biotinylated peptide to remove the N-terminal 6 aa's

To identify the region of the biotinylated hC2GnT-M 21 aa peptide, which binds to NMIIA, this peptide was digested with trypsin at RT in a 5 mM potassium-phosphate buffer, pH 7.0 for 30 min with an enzyme-to-substrate ratio of 1:500 by weight. After addition of soybean trypsin inhibitor, the biotinylated peptide was isolated by Dynabeads and then exposed to the cell lysate or MYH9 solution to assess the ability of the biotinylated peptide to pull down NMIIA in the cell lysate or purified MYH9 peptide.

2.10. Yeast-two-hybrid analysis of the interactions between the 1507–1960 aa peptide of the NMIIA heavy chain and the cytoplasmic peptide of hC2GnT-M

The plasmids encoding GAL4(DBD)-C2GnT-M (1–10 aa) or GAL4(DBD)-C2GnT-M (1–10 aa, AAA⁶) were co-transformed with GAL4(AD)-NMIIA (444 aa peptide, 1507–1960, of NMIIA heavy chain) into yeast Y2HGold (Clontech). In the other series of experiments the plasmids encoding GAL4(DBD)-C2GnT-M^(1–14) (M¹VQWKRLCQLHYLW¹⁴), GAL4(DBD)-C2GnT-M^(Δ2,3), GAL4(DBD)-C2GnT-M^(Δ4–6), GAL4(DBD)-C2GnT-M^(Δ7–9), GAL4(DBD)-C2GnT-M^(Δ10–12) or GAL4(DBD)-C2GnT-M^(Δ13,14) were co-transformed with GAL4(AD)-NMIIA into yeast Y2HGold. Interaction of GAL4(DBD) and GAL4(AD) fusion proteins enables the cells to grow in the absence of His and in the presence of Aureobasidin A antibiotic. Cotransformants which survive Leu(–) and Try(–) conditions were spotted at 1.5 × 10⁷ cells/10 µl concentration onto plates lacking His and containing 100 ng/ml Aureobasidin A. The plates were incubated at 30 °C for 5–7 d before being photographed. The Gal4(DBD)-p53 and Gal4(AD)-SV40 large T antigen (Ali and DeCaprio, 2001) pair was used as a positive control while Gal4(DBD)-Lamin and Gal4(AD)-SV40 large T antigen pair was used as a negative control.

2.11. Miscellaneous

Protein concentrations were determined with the Coomassie Plus Protein Assay (Pierce Chemical Co., Rockford, IL) using BSA as the standard. Data are expressed as mean ± SEM. Analysis was performed using 2-sided *t*-test. A value of *P* < 0.05 was considered statistically significant.

3. Results

3.1. bC2GnT-M forms complexes with NMIIA in Panc1-bC2GnT-M (c-Myc) cells

Current study was initiated based on a preliminary observation that multiple c-Myc-carrying protein bands with sizes greater than that (55 kDa) of bC2GnT-M were detected in the Panc1-bC2GnT-M (c-Myc) cell lysates (data not shown). The results suggested that bC2GnT-M formed complexes with other cellular proteins. To identify these proteins, the complexes were isolated from the cell lysate using anti-c-Myc Abs plus protein G Agarose. The proteins in the complexes were separated on SDS-PAGE under non-reducing conditions and then stained with Coomassie blue (Fig. 1A). Proteomic analysis of the 4 high molecular weight bands showed: band 1 contained the heavy chains of NMIIA and NMIIIB, band 2 contained cytoskeletal keratin, type II and also heavy chains of NMIIA and IIB, band 3 contained cytoskeletal keratin, type II, and band 4 contained gelsolin isoform. Subsequent proteomics analysis of same samples run under reducing conditions identified additional proteins on Coomassie blue-stained bands. They include ubiquitin activating enzyme E1, HSP90 1β, and five different HSP70s (Fig. S1). Because

Fig. 1. The proteins pulled down from the lysates of Panc1-bC2GnT-M (c-Myc) cells with anti-c-Myc Abs. (A) Coomassie blue stained SDS-PAGE gel analyzed on the proteins in the complexes pulled down from the lysate of Panc1-bC2GnT-M (c-Myc) cells with mouse anti-c-Myc Abs. (B) Tryptic peptides derived from the 250 kDa protein band in (A) matched to the amino acid sequence of NMIIA heavy chain. SDS-PAGE was performed on 10% gel under non-reducing condition. Proteomics analysis of these four bands identified: non-muscle myosin IIA and IIB in 1st and 2nd bands, cytoskeletal keratin, type II in 2nd and 3rd band, and gelsolin in 4th band. The tryptic peptides are underlined. (C) Western blots of the cell lysates, the supernatants, and the complexes pulled down from the lysates of Panc1-bC2GnT-M (c-Myc) cells with control IgG, anti-c-Myc, or anti-NMIIA Abs. Top panel: NMIIA heavy chain Western blot. Bottom panel: C-Myc Western blot. The signal intensities of the bands were quantitated by Image J software. The efficiency of the pulldown was assessed by the ratio: amount of protein detected in each IP condition/input (in %). For this calculation, we considered the input (40 μ g of protein) and the amount of protein employed for the immunoprecipitation for each cell line (0.8 mg). About 90% of NMIIA and 57% of c-Myc were pulled down by anti-NMIIA Abs, 76% of NMIIA and 97% of c-Myc were pulled down by anti-c-Myc Abs. The results shown are representative of three independent experiments.

21 tryptic peptides derived from band 1 matched the amino acid sequence of the heavy chain of NMIIA (Fig. 1B), we chose to characterize NMIIA as a possible bC2GnT-M binding partner further.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.biocel.2012.04.004>.

We found that NMIIA was pulled down by anti-c-Myc Abs, and bC2GnT-M by anti-NMIIA Abs (Fig. 1C). The results confirmed that bC2GnT-M formed complexes with NMIIA. It was noted that about 57% of C2GnT-M was pulled down by anti-NMIIA Abs while about 76% of NMIIA was pulled down with anti-c-Myc Abs.

3.2. C2GnT-M interacts with the C-terminal region of NMIIA heavy chain via the cytoplasmic tail

Because the cytoplasmic tails of several Golgi glycosyltransferases were shown to form complexes with cytoplasmic proteins (Giraudo and Maccioni, 2003; Guo and Linstedt, 2006; Tu et al., 2008), we proceeded to determine if the cytoplasmic tail of C2GnT-M could pull down NMIIA. As shown in Fig. 2A, a C-terminally biotinylated 1–21 aa peptide of hC2GnT-M, which contained the

cytoplasmic tail (1–7 aa, MVQWKRL), pulled down NMIIA (lane 1). Following removal of the 6 amino acids (1–6 aa) with trypsin, the 7–21 aa peptide no longer could pull down NMIIA (lane 3). The results were confirmed by the pulldown of NMIIA using a biotinylated 1–7 aa peptide that contains the 6 aa's removed by trypsin (lane 2).

To identify the region of NMIIA heavy chain involved in its interaction with C2GnT-M, we performed a pull-down of the recombinant C-terminal region (1682–1960 aa) of the NMIIA heavy chain with the biotinylated 1–21 aa peptide of hC2GnT-M. As shown in Fig. 2B, the recombinant protein was pulled down by the 1–21 aa peptide of hC2GnT-M (lane 2) but not the 7–21 aa peptide (lane 1). These data indicate that the C-terminal portion of NMIIA heavy chain contains the sequence responsible for NMIIA binding to the C2GnT-M cytoplasmic tail.

The interaction of the cytoplasmic tail of hC2GnT-M with NMIIA was confirmed by yeast-two-hybrid analysis using a 454 aa peptide at the C-terminal region (1507–1960 aa) of the NMIIA heavy chain as the prey and 1–10 aa peptide of hC2GnT-M as the bait. The wild-type 1–10 aa peptide of hC2GnT-M showed a moderate

Fig. 2. Interactions of NMIIA heavy chain with hC2GnT-M cytoplasmic tail. (A) Western blot of NMIIA pulled down from Panc1-bC2GnT-M (c-Myc) cell lysates with biotinylated hC2GnT-M N-terminal peptide: 1–21 aa (lane 1), 1–7 aa (lane 2), and 7–21 aa (lane 3). The complexes pulled down with these peptides were run on 8% SDS-PAGE gel and blotted with rabbit anti-NMIIA Abs. Lane 4: Panc1-bC2GnT-M (c-Myc) cell lysate treated only with streptavidin dynabeads. (B) NMIIA western blot of the recombinant C-terminal region (1682–1960 aa) of NMIIA heavy chain pulled down with biotinylated hC2GnT-M (1–21 aa) peptide (lane 2) and control peptide (7–21 aa) (lane 1). (C) Yeast two-hybrid analysis of the interaction of hC2GnT-M peptide (1–10 aa) and the C-terminal 454 aa peptide (1507–1960 aa) of NMIIA heavy chain. Yeast cells at 1.5×10^7 cells/ $10 \mu\text{l}$ suspension were plated on growth medium lacking leucine, tryptophan and histidine in the presence of 100 ng/ml Aureobasidin A. Compared with the negative control (SV40-T antigen + Lamin), the 454 aa peptide (1507–1960 aa of the NMIIA heavy chain) showed a moderate interaction with hC2GnT-M (1–10 aa) peptide, which was reduced after WKR⁶ was replaced with AAA⁶. SV40-T + p53 is a positive control. (D) hC2GnT-M peptide (1–14 aa) deletion mutants – $\Delta 2,3$; $\Delta 4-6$; $\Delta 7-9$; $\Delta 10-12$; $\Delta 13,14$. (E) Yeast two-hybrid analysis of the interaction of the C-terminal 454 aa peptide (1507–1960 aa) of NMIIA heavy chain and hC2GnT-M peptides (C2GnT-M⁽¹⁻¹⁴⁾, C2GnT-M^(Δ2,3), C2GnT-M^(Δ4-6), C2GnT-M^(Δ7-9), C2GnT-M^(Δ10-12) and C2GnT-M^(Δ13,14)). All mutants except C2GnT-M^(Δ4-6) show the growth intensity comparable to that of the wild-type peptide in the absence of histidine and presence of Aureobasidin A.

interaction with the 454 aa peptide of the NMIIA heavy chain. The interaction was substantially reduced when WKR⁶ was replaced with AAA⁶, suggesting that these three amino acids play a critical role in the binding of C2GnT-M with NMIIA (Fig. 2C). Further experiment employing a series of deletion mutants has confirmed the importance of WKR for the binding of C2GnT-M with NMIIA. As shown in Fig. 2D and E, interaction of C2GnT-M cytoplasmic tail with NMIIA was substantially reduced after WKR⁶ deletion (C2GnT-M^(Δ4-6)). However, other mutants, C2GnT-M^(Δ2,3), C2GnT-M^(Δ7-9), C2GnT-M^(Δ10-12) or C2GnT-M^(Δ13,14), did not show an apparent reduction in their interaction with NMIIA heavy chain when compared with wild-type peptide.

3.3. Intracellular distribution of bC2GnT-M and NMIIA in Panc1-bC2GnT-M cells

To confirm that NMIIA formed complexes with bC2GnT-M in the cells, confocal immunofluorescence microscopy was employed. C2GnT-M was found to co-localize with Giantin, a Golgi matrix protein (Fig. 3A–C). Also, C2GnT-M co-localized with NMIIA at the cytoplasmic boundary of the Golgi stacks (Fig. 3D–G, arrows indicate peri-Golgi staining of NMIIA). Treatment with a NMIIA inhibitor Blebbistatin (100 μM , 1 h) (Chan et al., 2005; De Pina et al., 2007; Kovács et al., 2004; Straight et al., 2003) abolished co-localization of NMIIA with bC2GnT-M in the Golgi;

bC2GnT-M was retained in the Golgi while NMIIA was localized to the cytoplasm (Fig. 3H–K). The results indicate that bC2GnT-M and NMIIA no longer formed complexes in the Blebbistatin-treated cells, and another protein was predicted to be responsible for the Golgi retention of C2GnT-M. That result was confirmed by the failure of anti-c-Myc Abs to pull down NMIIA from the lysates of Panc1-bC2GnT-M cells treated with Blebbistatin (Fig. 3L).

We found that the Golgi localization of the recombinant C2GnT-M produced in Panc1-bC2GnT-M (c-Myc) cells was the same as that found in the goblet cells of human bronchial epithelium, which produces endogenous C2GnT-M (Fig. S2). The result indicates that the Panc1-bC2GnT-M (c-Myc) cell is a suitable model for studying the Golgi-ER trafficking of C2GnT-M.

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3.4. Inhibition or knockdown of NMIIA prevents BFA-induced collapse of the Golgi

The biological significance of the Golgi co-localization of NMIIA with C2GnT-M was examined by confocal immunofluorescence microscopy following inhibition or knockdown of NMIIA coupled with BFA treatment. We found that NMIIA siRNA or Blebbistatin treatment retained bC2GnT-M in the Golgi (Fig. 4B vs. A and F vs. G). As predicted, BFA treatment (36 μM for 30 min) resulted in

Fig. 3. Confocal immunofluorescence microscopic images of C2GnT-M, Giantin, and NMIIA in Panc1-bC2GnT-M (c-Myc) cells. (A–C) bC2GnT-M (green) co-localizes with Giantin (red), a Golgi matrix protein; (D–G) bC2GnT-M co-localizes with NMIIA at the Golgi periphery (arrows indicate peri-Golgi staining of NMIIA). (H–K) Treatment with a NMIIA inhibitor Blebbistatin abolishes co-localization of NMIIA with bC2GnT-M in the Golgi; bC2GnT-M is retained in the Golgi while NMIIA is localized in the cytoplasm. Bars, 10 μ m. G and K are enlarged images of F and J, respectively (arrows indicate peri-Golgi co-staining of NMIIA and C2GnT-M). (L) NMIIA Western blot of the complexes pulled down with anti-c-Myc Abs from lysates of Panc1-bC2GnT-M(c-Myc) cells treated with DMSO (–) or Blebbistatin (+). The amount of protein employed for the immunoprecipitation for both lanes is 0.7 mg. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the migration of C2GnT-M and Giantin from the Golgi to the ER (Fig. 4C, G, J and L vs. A, E, I and K, respectively). However, treatment with Blebbistatin or NMIIA siRNA followed by BFA resulted in the retention of C2GnT-M and Giantin in the Golgi (Fig. 4D and H). Quantitative measurement of the distribution of C2GnT-M in the cytoplasm relative to that in the Golgi for Fig. 4A–D and E–H is shown in Fig. 4M and N, respectively. The data indicate that NMIIA is involved in the Golgi-to-ER retrograde transport of Golgi proteins because inhibition or knockdown of NMIIA prevents bC2GnT-M as well as Giantin from moving out of the Golgi even in the presence of BFA.

3.5. Inhibition or knockdown of NMIIA inhibits the exit of C2GnT-M from the Golgi induced by inhibition of HSPs or knockdown of HSP70

BFA is thought to cause the collapse of the Golgi by suppressing the ER-to-Golgi anterograde transport and accelerating the Golgi-to-ER transport of Golgi proteins (Burgess et al., 1991; Donaldson et al., 1990; Helms and Rothman, 1992; Ivessa et al., 1995; Lippincott-Schwartz, 1993; Puri and Linstedt, 2003). The multiple effects of BFA make this agent an undesirable Golgi disruptive compound for studying Golgi remodeling. Therefore, another

agent with a simpler effect on Golgi structure is sought for studying this phenomenon.

Because several members of HSP70 were pulled down from the lysates of Panc1-bC2GnT-M cells with anti-c-Myc Abs (Fig. S1) and HSPs are chaperones known to facilitate folding of proteins in the ER and their Golgi targeting (Velez-Granell et al., 1994), we proceeded to examine the possible involvement of HSPs in the Golgi targeting of C2GnT-M. We found that HSP70 co-localized with C2GnT-M in the ER (Fig. S3) and inhibition of HSPs with KNK437 (50 μ M, 1 h) (Leu et al., 2009) resulted in the retention of C2GnT-M in the ER and Giantin in the Golgi (Fig. 5B and E vs. A and D, respectively; Fig. 5O vs. N). However, C2GnT-M was retained in the Golgi after the cells were treated with Blebbistatin before KNK437 (Fig. 5C, F and G). Similarly, cells treated with 150 nM HSP70 siRNAs showed the retention of C2GnT-M in the ER (Fig. 5J vs. H; and L). However, NMIIA knockdown followed by KNK437 treatment retained C2GnT-M in the Golgi (Fig. 5K vs. I; and L). The data are consistent with the involvement of NMIIA in the Golgi-to-ER retrograde transport of C2GnT-M. Interestingly, KNK437 treatment or HSP70 knockdown did not affect the Golgi architecture as shown by Giantin staining (Fig. 5E, I and J). This observation was different from the BFA-induced collapse of the Golgi which was shown by the migration of both C2GnT-M and Giantin from the Golgi to the ER (Fig. 4G and J). Formation of complexes between C2GnT-M and HSP70 was confirmed by the pull-down of HSP70 from the cell lysate by anti-c-Myc Abs (Fig. 5P).

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3.6. β -COP is not involved in the Golgi-to-ER transport of C2GnT-M

A recent report claiming the involvement of COPI in the Golgi-to-ER transport of peptidyl GalNAc transferases (Gill et al., 2010) prompted us to examine whether similar transport mechanism was also employed by C2GnT-M. We found that knockdown of β -COP (Fig. 6F), a COPI component (Waters et al., 1991; Lee and Goldberg, 2010), did not affect intracellular distribution of C2GnT-M (Fig. 6C vs. A; and E). Also, β -COP knockdown did not affect the KNK437-induced distribution of C2GnT-M to the ER (Fig. 6D vs. B; and E). Next, we examined β -COP distribution after inhibition of HSPs. In the control, β -COP evenly distributes throughout the cell, but after KNK437 treatment it is primarily located to the immediate vicinity of the Golgi (Fig. 6H vs. G). Under same conditions, C2GnT-M was found outside of the Golgi (Fig. 6J vs. I). In addition, NMIIA inhibition or knockdown did not alter β -COP distribution (Fig. S4). Collectively, the data suggest that COPI is not involved in the Golgi-to-ER transport of C2GnT-M.

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3.7. C2GnT-M degradation by proteasome

The identification of ubiquitin activating enzyme E1 in the complexes pulled down from the lysates of Panc1-bC2GnT-M (c-Myc) cells with anti-c-Myc Abs (Fig. S1) prompted us to examine if proteasome was involved in the degradation of C2GnT-M. We found that treatment of Panc1-bC2GnT-M cells with MG-132 (5 μ M, 24 h) resulted in an increase of not only total but also ubiquitinated C2GnT-M (Fig. 7A–C). The bulk of the cytoplasm was occupied by C2GnT-M in cells treated with MG-132 (Fig. 7E vs. D). In addition, Blebbistatin treatment resulted in an increase of NMIIA (Fig. 7F and G), but a decrease in ubiquitinated bC2GnT-M (Fig. 7H). The results suggest that upon inhibition of NMIIA, the Golgi-to-ER retrograde transport of bC2GnT-M is blocked, which leads to reduction of the amount of bC2GnT-M delivered by NMIIA to the ER for degradation

Fig. 4. NMIIA inhibition or knockdown followed by BFA treatment of Panc1-bc2GnT-M (c-Myc) cells retains C2GnT-M and Giantin in the Golgi. (A–D) Confocal immunofluorescence images of C2GnT-M (green) and NMIIA (red) in cells treated with DMSO (A), Blebbistatin (B), BFA (C), and Blebbistatin followed by BFA (D). (E–H) Confocal images of C2GnT-M (green) and Giantin (red) in cells treated with scrambled siRNA (E), NMIIA siRNA (F), scrambled siRNA followed by BFA (G), and NMIIA siRNA followed by BFA (H). The images were overlaid with DIC. Bars, 10 μ m. (I–L) Confocal images of C2GnT-M and Giantin in cells treated with DMSO (I) or BFA (J), and confocal images of C2GnT-M and an ER marker, protein disulfide isomerase (PDI) in cells treated with DMSO (K) or BFA (L). (M and N) Quantification of the intensity of bC2GnT-M fluorescence in the cytoplasm vs. that in the Golgi (=100%) as shown in A–D and E–H, respectively. Data were obtained from 50 cells in three independent experiments and expressed as mean percentage $\pm$ SEM; *, $P < 0.001$ for B, C, or D vs. A and F, G, or H vs. E. (O) NMIIA Western blot of the lysates of Panc1-bc2GnT-M cells (50 μ g) treated with scrambled or NMIIA-specific siRNAs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 5. NMIIA inhibition or knockdown prevents HSP inhibition or HSP70 knockdown-induced shifting of C2GnT-M cellular localization from Golgi to ER. (A–F) Confocal immunofluorescence images of bC2GnT-M (green) and Giantin (red) in Panc1-bC2GnT-M cells treated with DMSO (A and D), KNK437 (B and E), and Blebbistatin followed by KNK437 (C and F). D–F are enlarged images of A–C, respectively. Bars, 10 μm. (H–K) Confocal images of bC2GnT-M and Giantin in cells treated with scrambled siRNAs (H), scrambled siRNAs followed by KNK437 (I), HSP70 siRNAs (J), and NMIIA siRNAs followed by KNK437 (K). Bars, 10 μm. (G and L) Fluorescence intensity of C2GnT-M in the cytoplasm relative to that in the Golgi (=100%) in A–C and H–K was quantified and analyzed as described above. *, $P < 0.001$ for B or C vs. A, and I or J vs. H; and **, $P < 0.01$ for K vs. H. (M) HSP70 Western blot of the lysates of cells (50 μg) treated with scrambled or HSP70 specific siRNAs. KNK437 treatment or HSP70 knockdown retained Giantin in the Golgi and distributed bC2GnT-M to the ER. Golgi retention of both was observed when NMIIA was inhibited or knocked down coupled with inhibition of HSPs. (N and O) Confocal images of C2GnT-M and an ER marker, protein disulfide isomerase (PDI) in cells treated with DMSO (N) or KNK437 (O). (P) HSP70 Western blot of the complexes pulled down with non-specific IgG and anti-c-Myc Abs from cell lysates. The amount of protein employed for the immunoprecipitation was 0.8 mg. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 6. β -COP knockdown does not affect intracellular distribution of C2GnT-M in Panc1-bC2GnT-M (c-Myc) cells with or without treatment with KNK437. (A–D) Confocal images of bC2GnT-M and Giantin in cells treated with scrambled siRNAs (A), scrambled siRNAs followed by KNK437 (B), β -COP siRNAs (C), and β -COP siRNAs followed by KNK437 (D). Bars, 10 μ m. (E) Quantification of the C2GnT-M fluorescence signal in the cytosolic compartment relative to that of the Golgi (=100%). The analysis was performed on the complete confocal stacks of 50 cells from three independent experiments. Data are expressed as mean percentage $\pm$ SEM *, $P < 0.001$ for B vs. A, and D vs. C. The bC2GnT-M distribution was not affected after β -COP knockdown. KNK437 treatment has distributed bC2GnT-M to the ER even in the β -COP deficiency. (F) β -COP Western blot of the lysates of cells (50 μ g) treated with scrambled or β -COP-specific siRNAs. (G–J) Confocal images of β -COP and Giantin or C2GnT-M and Giantin in cells treated with DMSO or KNK437. Note that in control (G), β -COP distributes throughout the cell, but locates primarily in the Golgi area after KNK treatment (H). Unlike β -COP, C2GnT-M moves away from the Golgi following KNK437 treatment (I and J).

by proteasome. Interestingly, MG-132 treatment also increased NMIIA (Fig. 7F and G), suggesting that NMIIA was also degraded by proteasome.

4. Discussion

The cytoplasmic tails of Golgi glycosyltransferases have been shown to be involved in the ER-to-Golgi transport (Giraud et al., 2003; Watanabe and Riezman, 2004; Guo et al., 2008) and Golgi retention (Burke et al., 1994; Grabenhorst and Conradt, 1999; Uliana et al., 2006) of these enzymes. In this study, we have expanded the functional role of the cytoplasmic tails of

glycosyltransferases by showing that they also are involved in the Golgi-to-ER transport of these enzymes by binding to NMIIA. This NMIIA-mediated process is likely involved in Golgi remodeling.

We have found that NMIIA directly binds to C2GnT-M through the C-terminal 1682–1960 aa region of NMIIA heavy chain and the 4–6 aa of the cytoplasmic tail of C2GnT-M. The co-localization of C2GnT-M with NMIIA at the periphery of the Golgi coupled with the observation that a significant amount, i.e. 43%, of C2GnT-M is not pulled down with anti-NMIIA Abs (Fig. 1C) suggests that there is another pool of C2GnT-M that does not bind to NMIIA. The protein that binds to this pool of C2GnT-M is likely involved in the Golgi retention of this enzyme and it remains to be identified. In

Fig. 7. Proteasomal degradation of C2GnT-M in Panc1-bC2GnT-M (c-Myc) cells. (A) Western blot of bC2GnT-M in the lysates of cells (40 μ g) treated with DMSO or MG-132. Cell lysates were run on 8% SDS-PAGE with β -mercaptoethanol and then probed with anti-c-Myc Abs. (B) Densitometric analysis of the protein bands in (A). Data represent bC2GnT-M expressed relative to the density of γ -tubulin band and each bar represents mean $\pm$ SEM from three independent experiments. *, $P < 0.001$ for MG-132 vs. DMSO. (C) Ubiquitin Western blot of the complexes pulled down with anti-c-Myc Abs from the lysates of cells treated with DMSO or MG-132. Equal amounts of proteins in the lysates (0.8 mg) were used for anti-c-Myc Co-IP. (D and E) Confocal images of bC2GnT-M and NMIIA in cells treated with DMSO (D) or MG-132 (E). (F) NMIIA Western blot of the lysates of cells (40 μ g) treated with DMSO, Blebbistatin or MG-132. The 250 kDa band corresponded to NMIIA and γ -tubulin was used as a control for equal loading. (G) Densitometric analysis of the protein bands in (F). Data show the amounts of NMIIA relative to that of γ -tubulin and each bar represents mean $\pm$ SEM from three independent experiments. *, $P < 0.001$ for Blebbistatin or MG-132 vs. DMSO. (H) Ubiquitin Western blot of anti-c-Myc (bC2GnT-M) immunoprecipitate from the lysates of Panc1-bC2GnT-M (c-Myc) cells treated with DMSO or Blebbistatin. Co-IP samples were run on 12% SDS-PAGE with β -mercaptoethanol and then probed with anti-ubiquitin Abs. The amount of protein employed for the immunoprecipitation for both lane was 0.7 mg.

addition, our data suggest that NMIIA is involved in the Golgi-to-ER transport of not only C2GnT-M but also many other Golgi proteins because inhibition of NMIIA prevents the BFA-induced collapse of the Golgi, and the exit of many Golgi proteins including C2GnT-M but not Giantin when HSPs are inhibited or HSP70 is depleted. In addition, the NMIIA-mediated Golgi-to-ER transport of C2GnT-M is independent of COPI because knockdown of β -COP does not affect the distribution of C2GnT-M under basal or KNK437 treatment condition. Also, inhibition of NMIIA does not change β -COP distribution, and KNK437 treatment leaves β -COP concentrated around the Golgi. We conclude that C2GnT-M is transported from the Golgi

to the ER in a COPI-independent process which is in good agreement with several other reports showing an alternative route to COPI retrograde transport of glycosyltransferases (Girod et al., 1999; Jiang and Storrie, 2005; Lee et al., 2004; Storrie et al., 2000) but at variance with one recent report which claims the involvement of COPI in the Golgi-to-ER transport of peptidyl GalNAc transferases (Gill et al., 2010). However, we still do not know if COPI vesicles are involved in the intra-Golgi transportation of C2GnT-M (Cosson et al., 2002; Guo et al., 2008; Love et al., 1998; Lanoix et al., 1999).

One major function of HSPs is to help protein folding in the ER and Golgi targeting (Velez-Granell et al., 1994). The

Fig. 8. Proposed role of NMIIA in the Golgi-to-ER transport of C2GnT-M. Treatment of Panc1-bC2GnT-M cells with BFA, which inhibits the ER-to-Golgi transport, results in the collapse of the Golgi. Inhibition (with KNK437) or knockdown (with siRNA) of HSP70, which is required for the ER-to-Golgi transport of C2GnT-M, retains C2GnT-M in the ER but Giantin in the Golgi. Inhibition (with Blebbistatin) or knockdown (with siRNA) of NMIIA retains C2GnT-M and Giantin in the Golgi. Subsequent treatment with BFA or KNK437 still retains both C2GnT-M and Giantin in the Golgi. Therefore, NMIIA is required for the Golgi-to-ER transport of C2GnT-M. Inhibition or knockdown of NMIIA prevents the Golgi-to-ER movement of C2GnT-M even in the presence of reagents that are known to stimulate the exodus of this enzyme from the Golgi. Note that unlike BFA, KNK437 does not alter the Golgi morphology.

improperly folded proteins are degraded by the ER-assisted protein degradation mechanism (Pfeffer and Rothman, 1987; Helenius et al., 1992; Arias and Bendayan, 1993). We have found that treatment of the cells with KNK437 results in retention of Giantin in the Golgi but accumulation of C2GnT-M in the ER. This outcome is likely resulted from the inhibition of its anterograde transport by KNK437 coupled with unimpeded trafficking of C2GnT-M from Golgi to ER mediated by NMIIA (Fig. 8). The results support the role of HSP70s in assisting the folding of C2GnT-M and subsequent trafficking to the Golgi (Walter and Buchner, 2002; Triantafilou and Triantafilou, 2004; Hartl and Hayer-Hartl, 2009). These data also confirm the concept that Golgi is made of two different membrane proteins, one is represented by Giantin of which Golgi retention is resistant to KNK437, and the other one is represented by C2GnT-M of which Golgi retention is sensitive to KNK437. This feature makes KNK437 a useful inhibitor for studying the Golgi-to-ER retrograde transport of glycosyltransferases because, unlike BFA, KNK437 does not affect the Golgi architecture (Fig. 8).

We have found that C2GnT-M is degraded by proteasome because MG-132 treatment results in accumulation of total as well as ubiquitinated bc2GnT-M. NMIIA is involved in this process because Blebbistatin treatment leads to accumulation of NMIIA but a reduction in ubiquitinated bc2GnT-M. The results suggest that the final destination of NMIIA-dependent Golgi-to-ER transport of bc2GnT-M is proteasome. This conclusion is supported by the close association of bc2GnT-M and ubiquitin activating enzyme E1 as shown by the pulldown of ubiquitin activating enzyme E1 by anti-c-Myc Abs. The accumulation of NMIIA in Panc1-bC2GnT-M cells treated with MG-132 suggests that NMIIA is also degraded by proteasome. This observation confirms a recent report showing NMIIA involvement in proteasome-dependent degradation (Valiya Veettil et al., 2010; Vazhappilly et al., 2010).

We have shown that our approach of using a biotinylated cytoplasmic tail of a glycosyltransferase as a bait to isolate its cytoplasmic binding partners is quite useful because it may be used as a high throughput approach for identifying the cytoplasmic proteins that form complexes with a glycosyltransferase via its tail. This should be quite useful for glycosyltransferases of which immunoprecipitable antibodies are not yet available and thus immuno-precipitation approach is not feasible.

Fig. 9 summarizes the results of this study. Following the synthesis of C2GnT-M in the ER, C2GnT-M acquires a proper conformation as assisted by HSP70s. This enzyme is then transported, presumably on microtubules (McIlvain et al., 1993; Karecla and Kreis, 1992), to the Golgi, where it is retained by binding to a protein yet to

be identified. The Golgi-to-ER retrograde transport of this enzyme, presumably on actin filaments (De Pina et al., 2007), is determined by its direct interaction with NMIIA via its cytoplasmic tail and the C-terminal region of NMIIA. COPI is not involved in this process. The enzyme along with NMIIA is then degraded by proteasome. Our

Fig. 9. Intracellular trafficking and distribution of C2GnT-M. C2GnT-M is synthesized in the ER, where HSP70 helps fold C2GnT-M properly, and then transported to the Golgi, where the enzyme is retained by binding to a protein yet to be identified. C2GnT-M returns to the ER in a NMIIA-dependent but COPI-independent process. This process is mediated by the interaction of the cytoplasmic tail (1–6 aa) of C2GnT-M with the C-terminal portion (1682–1960 aa) of NMIIA heavy chain. C2GnT-M is degraded in the proteasome in a process mediated by ubiquitination.

work has contributed to a better understanding of the intracellular trafficking of glycosyltransferases, specifically the involvement of NMIIA in the Golgi-to-ER transport of C2GnT-M. The work also suggests that NMIIA is involved in the Golgi remodeling.

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