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UDP-*N*-acetylglucosamine transporter and UDP-galactose transporter form heterologous complexes in the Golgi membrane

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ABSTRACT

UDP-galactose transporter (UGT; SLC35A2) and UDP-*N*-acetylglucosamine transporter (NGT; SLC35A3) are evolutionarily related. We hypothesize that their role in glycosylation may be coupled through heterologous complex formation. Coimmunoprecipitation analysis and FLIM–FRET measurements performed on living cells showed that NGT and UGT form complexes when overexpressed in MDCK-RCA^r cells. We also postulate that the interaction of NGT and UGT may explain the dual localization of UGT2. For the first time we demonstrated in vivo homodimerization of the NGT nucleotide sugar transporter. In conclusion, we suggest that NGT and UGT function in glycosylation is combined via their mutual interaction.

Structured summary of protein interactions:
NGT physically interacts with **UGT2** by anti tag coimmunoprecipitation (View Interaction: 1, 2)
NGT physically interacts with **UGT1** by anti tag coimmunoprecipitation (View interaction)
UGT2 physically interacts with **NGT** by fluorescent resonance energy transfer (View interaction)
NGT physically interacts with **NGT** by fluorescent resonance energy transfer (View interaction)
UGT1 physically interacts with **UGT2** by anti tag coimmunoprecipitation (View interaction)
UGT1 physically interacts with **NGT** by fluorescent resonance energy transfer (View interaction)

1. Introduction

Cellular glycoconjugates play a variety of fundamental roles in the growth and development of eukaryotes, as well as in the host–pathogen interactions. The glycan moiety is synthesized and modified by glycosyltransferases acting in the lumen of the endoplasmic reticulum (ER) and Golgi apparatus. The substrates required by glycosyltransferases are sugars activated by the addition of a nucleoside mono- or diphosphate (UDP, GDP, or CMP). They are transported into the ER or Golgi apparatus by nucleotide sugar transporters (NSTs), which are hydrophobic proteins with a molecular weight of 30–45 kDa [1,2]. Most predictions determine in these multitransmembrane proteins an even number of spans, which results in the N and C termini facing the cytosol.

One of the best characterized NSTs is the UDP-galactose transporter (UGT; SLC35A2). Detailed characterization of UGT was possible after mutant cell lines, such as MDCK-RCA^r [3,4], CHO-Lec8 [5,6], and Had-1 [7], had been generated. Non-sense mutations identified in the mutant cells cause inhibition of UGT production, resulting in glycoconjugates significantly defective in galactosylation and sialylation [3,8]. Two splice variants of UDP-Gal transporter (UGT1 and UGT2) have been identified in human tissues, the CHO and MDCK cell lines [9–11].

Compared with mammalian UGT, knowledge regarding mammalian UDP-*N*-acetylglucosamine transporter (NGT) is limited. Among known UDP-GlcNAc transporters, SLC35A3 is assumed to play a main role in glycosylation [12,13], while the function of SLC35D2 [14] and SLC35B4 [15] multispecific transporters appears to be less important. Compared with SLC35A3, which is ubiquitously expressed, SLC35D2 and SLC35B4 are less common and tissue-specific. Immunofluorescent microscopic analysis of NGT (SLC35A3) overexpressed in CHO [13] and MDCK-RCA^r [16] cells demonstrated its localization in the Golgi apparatus.

NGT and UGT, two nucleotide sugar transporters involved in essential glycosylation processes, are evolutionarily related [2,17–19]. In addition, overexpression of NGT in UGT-defective cells partly restores galactosylation [17]. Therefore, we suspected that their role in glycosylation of macromolecules may be coupled and both transporters may partially replace function played by its partner. In an attempt to gain an insight into this hypothesis, we analyzed

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the ability of NGT and UGT to form heterologous complexes in the Golgi membrane.

2. Materials and methods

2.1. Construction of expression plasmids, cell maintenance and transfection

MDCK-RCA^r mutant cells were grown and transfected with expression plasmid(s) (Supplementary Table 1) as described previously [17,20]. Stable transfectants expressing NGT and UGT1/UGT2 combinations were selected in complete media containing 600 μg/ml G-418 sulfate (InvivoGen, Toulouse, France). For the control pull-down experiment, MDCK-RCA^r mutant cells and MDCK-RCA^r cells stably expressing either B4-FLAG (human UDP-xylose/*N*-acetylglucosamine transporter; SLC35B4) or UGT2-FLAG were transiently transfected with HA-HsNGT expression plasmid (Supplementary Table 1) and analyzed 4 days after transfection.

2.2. Cell lysis, coimmunoprecipitation and Western blotting

Cells (1×10^9) were washed in PBS and lysed using lysis buffer (50 mM Tris–HCl; pH 8.0 with 1% NP-40) supplemented with protease inhibitor cocktail and 1 mM EDTA. Lysates were centrifuged at 14000×*g* for 15 min. Supernatants were pre-incubated overnight at 4 °C with anti-HA rabbit polyclonal antibody (1.5 μg; Abcam, Cambridge, UK) and then incubated with Protein A-Sepharose® 4B (25 μl; Invitrogen, Camarillo, CA, USA) for 4 h at 4 °C. Samples were centrifuged at 2000×*g* for 5 min, pellets washed five times with lysis buffer, suspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, and incubated for 30 min at 65 °C. Proteins were separated in 10% SDS–PAGE gels and transferred onto nitrocellulose membranes (Whatman, Dassel, Germany). After blocking, incubation with anti-c-myc (0.1 μg/ml; Abcam) or anti-FLAG (1 ng/ml; Sigma–Aldrich) antibody, and subsequently with horseradish peroxidase-coupled goat anti-chicken IgY (0.2 μg/ml; Abcam) or anti-mouse IgG (1:10000; Promega, Madison, WI, USA) antibody was carried out. Immunoreactive proteins were visualized with an enhanced chemiluminescence system (PerkinElmer, Whaltam, MA, USA). Proteins subjected to coimmunoprecipitation were also separated by SDS–PAGE and stained with colloidal Coomassie Brilliant Blue (PAGEBLUE Protein Staining Solution; Fermentas, Vilnius, Lituania).

2.3. Confocal and fluorescence lifetime imaging microscopies of living cells

MDCK-RCA^r mutant cells were transiently transfected with expression construct(s). After 48 h cells were seeded onto the 40-mm cover glasses (Thermo Scientific, Rockford, IL, USA) placed in 60-mm culture dishes and allowed to grow for additional 24 h. The standard growth medium was replaced with the phenol-free minimal essential medium (Life Technologies, Paisley, UK) 16 h prior to imaging. Confocal microscopy images were acquired using the Zeiss LSM 510 META laser scanning confocal microscope (Zeiss, Jena, Germany). ImageJ software was used to analyze and process images (http://rsb.info.nih.gov/ij/). Time-resolved fluorescence imaging was performed with an LSM 510 microscope (Zeiss) equipped with a TCSPC FLIM module (PicoQuant, Berlin, Germany). The dishes with cells expressing fusion protein(s) were mounted in a dedicated observation stage chamber (PeCon GmbH, Erbach, Germany) and left for gas (5% CO_2) and temperature (37 °C) equilibration. Fluorescence of eGFP was excited at 470 nm with a pulsed laser (40 MHz) and collected through water immersion 63 × LCI Plan-Neofluar (NA 1.3) objective (Zeiss). The bandpass 500 ± 20 nm emission filter (Semrock, Lake Forest, IL, USA) was used and suitable controls to exclude the excitation cross-talk artifacts were performed. Laser power was adjusted to give an average photon rate of 10^4–10^5 photons to avoid the pile-up effect and significant photobleaching. About 500 photons per pixel were acquired and the photon statistics were further improved by 2 × 2 pixel binning. A robust fitting-free fast-FLIM approach was used to reconstruct FLIM images in which every pixel was colored according to the photon's average arrival time.

2.4. Analysis of FLIM–FRET experiments

Two florescence decay models for FLIM–FRET data analysis were applied. Validity of decay model was determined by weighted residuals, which for a good fit fluctuate randomly around zero with no obvious trends and by χ^2 value, which is close to 1 for a good fit. First, FLIM–FRET data were tail-fitted to single exponential decay. Such model described properly decays obtained from control cells, however, was insufficient when applied to decays obtained from cells expressing both eGFP-NGT and its mRFP-tagged putative interacting partners. In this instance data were tail-fitted to bi-exponential decay model: $I(t) = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2)$, where τ stands for fluorescence lifetime and A for amplitude of individual contribution. The applied model allows resolving both fluorescence lifetime and fractional contribution of interacting and non-interacting donor molecules. Fitting to a single-exponential decay model besides its invalidity may also lead to underestimation of FRET efficiency as in such case it is assumed that all donors interact with one or more acceptor(s). To calculate an average lifetime and fractional amplitudes, respective formulae were used: $\tau_{av} = (A_1\tau_1) + (A_2\tau_2)/A_1 + A_2$ and $A_i = A_i/A_i + A_{i+1}$.

3. Results and discussion

3.1. Coimmunoprecipitation demonstrates complex formation between NGT and UGT

Several reports demonstrated that NSTs function in the form of homodimers [21–23] or higher homooligomers [24], and UGT forms heterologous complexes with ceramide galactosyltransferase [25]. Recently we showed that UGT splice variants undergo partial relocalization when coexpressed, suggesting their mutual interaction [16]. Moreover, we demonstrated that overexpression of NGT may partially restore galactosylation of N-glycans in MDCK-RCA^r and CHO-Lec8 mutant cells defective in UGT activity [17]. Residual galactosylation detected in the mutant cells [11,26–28], at least in part, suggests that other NSTs may support this process in the absence of UGT. Taking into account similarity of NGT and UGT (Supplementary Fig. 1) [2,17–19] we assumed that these two NSTs may form heterologous complexes in the Golgi membrane.

To confirm our hypothesis, we first employed coimmunoprecipitation analysis. Putative complex formation was studied in UGT-deficient mutant MDCK-RCA^r cells, which theoretically should cause all NGT molecules to interact with recombinant UGTs exclusively. After overexpression of both UGT splice variants in MDCK-RCA^r cells a complex formation was observed (Fig. 1C), demonstrating optimal conditions of cell lysis and coimmunoprecipitation. Further we demonstrated that NGT and UGT2 as well as NGT and UGT1 formed complexes (Fig. 1A and B). These *in vitro* results for the first time revealed an interaction occurring between two different NSTs. However, coimmunoprecipitation performed on multitransmembrane proteins in the presence of detergents may result in non-specific, false positive results.