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Immunocytochemical analysis for intracellular dynamics of C1GalT associated with molecular chaperone, Cosmc

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Abstract

The core 1 structure Galβ1-3GalNAcα1-Ser/Thr (T antigen), the major constituent of *O*-glycan core structure, is synthesized by cooperation of core 1 synthase (C1GalT) and its specific molecular chaperone, Cosmc. The chaperone function of Cosmc has been well investigated biochemically. In this study, we established monoclonal antibodies specifically recognizing either C1GalT or Cosmc, respectively, and investigated the sub-cellular localization of each protein to elucidate how they cooperate to synthesize the core 1 structure.

A sequential immunocytochemical analysis of the human colon cancer cell line, LSB, demonstrated different localization of two proteins. C1GalT was localized in Golgi apparatus, while Cosmc was localized in endoplasmic reticulum. In contrast, the LSC cells, which do not have core 1 synthase activity due to a missense mutation in the Cosmc gene, did not express the C1GalT protein. Although the treatment with a proteasome inhibitor, lactacystin, of LSC cells resulted in the increased expression of C1GalT protein, the distribution of C1GalT was not in Golgi apparatus as seen in LSB cells. On the contrary, overexpression of Cosmc but not C1GalT lead to precise localization of C1GalT protein, which distributed in Golgi apparatus and recovered the core 1 synthase activity in LSC cells. These results suggest that the intracellular dynamics of C1GalT is controlled by its specific molecular chaperon, Cosmc, in association with core 1 synthase activity.

Keywords: Core 1 structure; Glycosyltransferase; C1GalT; Cosmc; Monoclonal antibody

The core 1 structure, the so-called Thomsen Friedenreich antigen (T antigen), is one of the initial compounds in *O*-glycan biosynthesis, and is further glycosylated to present a variety of extended *O*-glycan structures in the body. The structure is synthesized by transferring galactose from UDP-Gal to a GalNAcα1-Ser/Thr (Tn antigen) with β1,3 linkage. As for the synthetic enzymes for the core 1 structure, two responsible proteins, C1GalT and Cosmc, have been identified in human and mouse. C1GalT was isolated by protein purification methods by monitoring galactosyltransferase activity, while Cosmc was identified as an

Abbreviations: C1GalT, core 1 β1,3-galactosyltransferase; Cosmc, core 1 β1,3-galactosyltransferase-specific molecular chaperone; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; HEK, human embryonic kidney 293T; Ig, immunoglobulin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TBS, Tris-buffer saline; PNA, peanut agglutinin; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum.

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associated protein with C1GalT during its purification [1,2]. It was also cloned by us using a homology search in the database [3]. We at first mistook it for the second enzyme responsible for core 1 synthesis. Later, we noticed that it is not an enzyme as reported by the Ju and Cummings' group, and we reported an erratum. Though both proteins have a typical topology as a glycosyltransferase, Cosmc does not exhibit any apparent enzyme activity, but reportedly has a supportive function for C1GalT activity as a kind of chaperone [2].

The core 1 structure plays important roles in lots of biological phenomena. The knock-out mouse lacking the *C1GalT* gene died by the embryonic day 14th due to hemorrhage developing in the brain [4], which clearly demonstrated the indispensable roles of the core 1 structure for life. The incomplete synthesis of core 1 structure has been reported in association with two human diseases, IgA nephropathy (IgAN) and Tn-syndrome [5,6]. IgAN is a disease characterized by IgA1 deposits in the renal glomerular mesangium, resulting in chronic renal failure after more than a 20-year clinical history of disease. It has been proposed that a small percentage of IgA1 molecules in serum of IgAN patients have aberrant underglycosylated *O*-glycans in the hinge region with unknown mechanisms. In Tn-syndrome patients, their *Cosmc* genes have been demonstrated to be somatically mutated in blood cells, and they cannot synthesize the core 1 structure in such cells. The blood cells lacking Cosmc show the Tn antigen expression due to the lack of core 1 structure, and the patients show moderate pancytopenia and hemolytic anemia.

The regulated expression of core 1 structure appears to require collaborative action by these two proteins. Two studies have pointed out a possibility that somatic mutations or transcriptional repression in the *Cosmc* gene serve to decrease core 1 synthase activity, resulting in a decrease in the core 1 structure in IgAN patients [7,8]. Analogical disruptions are seen in cultured cells, such as the human T leukemic cell line, Jurkat, and human colon cancer cell line, LSC. These cells do not have core 1 synthase activity due to a mutation in Cosmc [3]. However, it still remains to be elucidated why C1GalT enzyme activity is lost due to the mutation of the *Cosmc* gene, and whether the expression of C1GalT enzyme is altered in these cell lines by the changes.

To date, some glycosyltransferases, such as β1,4-galactosyltransferase 1, have been identified as Golgi-resident enzymes [9], and others, such as protein *O*-fucosyltransferase 1, have been identified as enzymes retained in endoplasmic reticulum (ER) [10]. Human C1GalT has been described as a Golgi-retention glycosyltransferase according to its typical type II topology having the hydrophobic domains on its N-terminus. On the other hand, Cosmc is hypothesized to localize in ER due to its chaperone function. Taken together, these observations predict that the two proteins collaborate with each other to synthesize core 1 structure, but are present in different subcellular compartments in cells.

To shed more light on the molecular interaction between C1GalT and Cosmc, we generated specific monoclonal antibodies (mAbs) that react to C1GalT or Cosmc, respectively, and investigated the intracellular localization of both proteins endogenously expressed in LSB cells. We also compared the expression of C1GalT in the LSC cells that lack core 1 synthase activity. In this study, we immunocytochemically demonstrate the intracellular dynamics for C1GalT that is regulated by Cosmc.

Materials and methods

Cells and reagents. 293T cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). LSB and LSC cells from human colorectal cancer cell lines were kindly provided by Dr. Itzkowitz in our previous study [11]. The anti-Tn mAb, HB T1 (IgM), and anti-sTn mAb, HB STn1 (IgG1), were both purchased from Dako (A/S, Glostrup, Denmark), while fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA) lectin was purchased from Vector Laboratories Inc. (Burlingame, CA). For immunostaining, an EP892Y, anti-GM130 monoclonal antibody was purchased from Epitomics (Burlingame, CA) and anti-calnexin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Hoechst 33342 was purchased from Molecular Probes (Eugene, OR).

mAb preparation. To generate monoclonal antibodies (mAbs) against C1GalT or Cosmc, we used epitope-tagged recombinant forms of C1GalT and Cosmc as immunogens. C-Terminal of C1GalT was fused with GST tag and Cosmc was fused with His tag, which were expressed in *Escherichia coli* and purified. BALB/c mouse was administered immunogens as previously described [12], and the generated hybridoma colonies were screened by ELISA and Western blotting.

Expression of full-length C1GalT and Cosmc. For the expression of full-length C1GalT and Cosmc, the cDNA of C1GalT and Cosmc was inserted into pDEST12.2, using the GATEWAY system (Invitrogen) [3]. The putative catalytic domains of human C1GalT and Cosmc were inserted into pFLAG-CMV3 (Sigma, St. Louis, MO) and expressed as a secreted protein fused with FLAG-tag, respectively. Plasmids were then transiently transfected into 293T and LSC cells using Lipofectamine 2000. After 48 h, the cells ($\sim 10^6$) of each transfectant were subjected to several analyses.

Western blotting. Whole cell lysate of LSB, LSC and mock, C1GalT or Cosmc-transfected 293T cells was separated by 10% SDS–PAGE under reducing or non-reducing conditions. Each gel was transferred to a polyvinylidene difluoride (PVDF) membrane (GE healthcare, Amersham, UK), probed with 4A2 mAb or 1E4 mAb antibody as previously described [12].

Flow cytometric analysis. For flow cytometry, the cells were incubated with each mAb or lectin. After incubation with the first antibody, the cells were stained with FITC-conjugated goat anti-mouse IgG or IgM (ICN Pharmaceuticals, Inc.) and then subjected to flow cytometric analysis with a FACSCalibur (BD Biosciences).

Drug treatment of LSC cells. After 48 h incubation, LSC cells were incubated with lactacystin (10 μM) for 18 h. Drug-treated cells were attached to slide glasses by cytospin and immunocytochemistry was performed with 4A2 mAb.

Immunocytochemistry with fluorescent probes. For immunostaining with fluorescent probes, we performed as previously described [12]. In brief, antigens in cell lines and lactacystin (10 μM) treated LSC cells were detected by the indirect immunofluorescence method with Prolong Gold antifade reagent (Molecular Probes).

Results

Characterization of mAbs against C1GalT and Cosmc

The recombinant C1GalT and Cosmc proteins were expressed in human embryonic kidney 293T cells. As

shown in Fig. 1A, Western blot analyses using anti-C1GalT 4A2 mAb (4A2 mAb) or anti-Cosmc 1E5 mAb (1E5 mAb) specifically showed approximately a 48 kDa band of C1GalT or a 36 kDa band of Cosmc, respectively. 293T cells transfected with C1GalT showed a 75–80 kDa band which is probably a dimerized form of C1GalT because of its overexpression. Thus, each 4A2 or 1E5 mAb detected specifically either C1GalT (48 kDa) or Cosmc (36 kDa). Cosmc was not detected in 293T-mock cells because of its low endogenous amount.

Subcellular localization of C1GalT and Cosmc in LSB cells

Though both LSB and LSC cells were isolated from the LS174T human colon cancer cell line, LSB cells show the core 1 synthase activity and LSC cells do not [13,14]. In order to elucidate the subcellular localization of C1GalT and Cosmc in LSB cells, we performed an immunocytochemical study on LSB cells using 4A2 and 1E5 mAb with either anti-GM130 as a Golgi marker or anti-calnexin antibody as an ER marker.

In LSB cells, the bright signals of 4A2 mAb exclusively overlapped the signals of anti-GM130 antibody but not those of anti-calnexin antibody as shown in Fig. 1B. These results indicate that almost all C1GalT proteins localize in Golgi apparatus with GM130. On the contrary, signals of anti-calnexin antibody showed a typical pattern of ER staining in which diffuse positive signals surround a nucleus. The calnexin signals did not overlap those of C1GalT in LSB cells. In Fig. 1C, Cosmc signals were not overlapped with those of GM130, but well overlapped with calnexin signals. These results demonstrated that Cosmc distributes mainly in ER rather than in Golgi apparatus.

LSC did not express C1GalT

As demonstrated in the previous study, a single nucleotide insertion of thymidine between T53 and C54 in the *Cosmc* gene of LSC cells made Cosmc truncated and impaired, although the LSC *Cosmc* gene was fully transcribed [3]. To investigate the possible underlying mechanism in the absence of functional Cosmc, we examined the C1GalT expression in LSC cells with 4A2 mAb. Fig. 2A shows differential expression of C1GalT between LSB and LSC cells. Again, LSB cells showed clear expres-

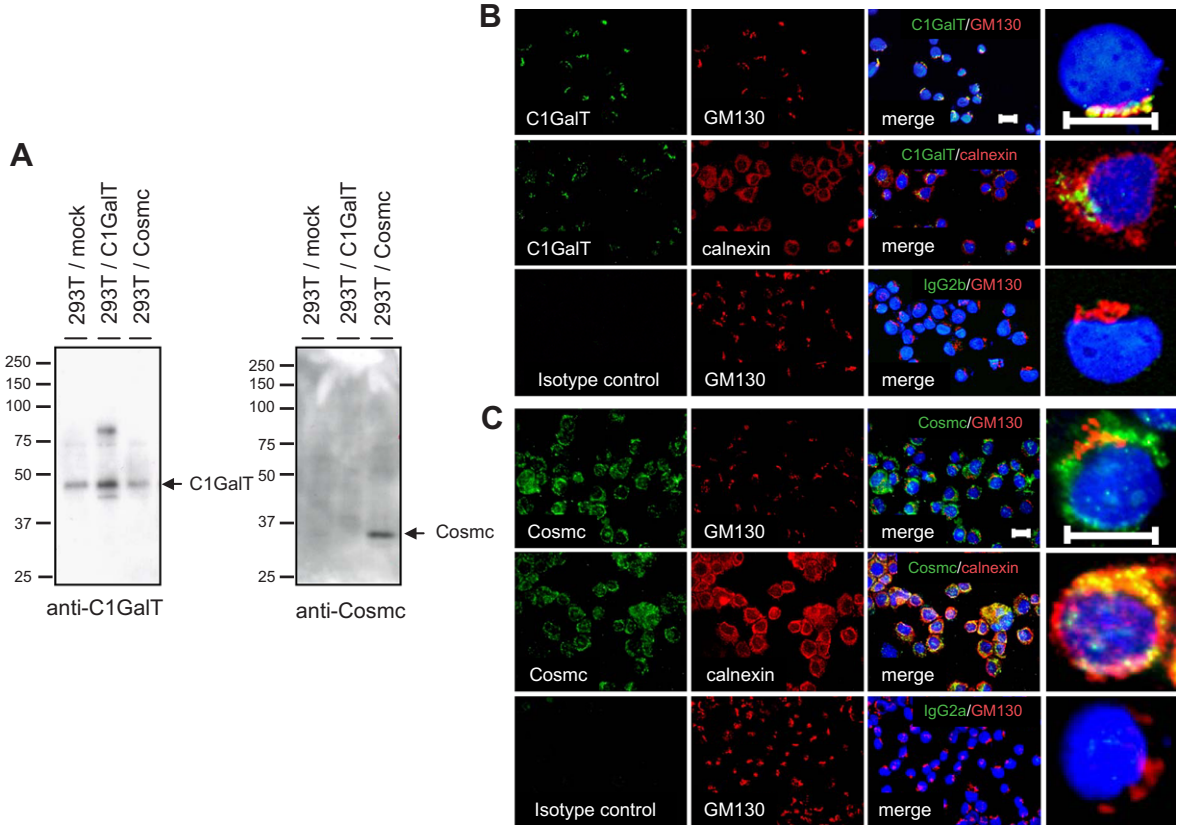

Fig. 1. (A) Western blotting of mock-, C1GalT- and Cosmc-transfected 293T cells. Approximately 5 μg of whole cell lysates were separated by 10% SDS–PAGE under reducing conditions. 4A2 mAb detected C1GalT in mock-, C1GalT-, and Cosmc-transfected 293T cells (left). 1E5 mAb detected Cosmc only in Cosmc transfectant (right). Molecular markers are shown in kDa. (B,C) Subcellular localization of C1GalT and Cosmic in LSB cells. (B) LSB cells were stained with 4A2 mAb (green) and either with anti-GM130 (red, top panels) or anti-calnexin (red, middle panels). Merged images were shown in yellow. (C) LSB cells were stained with 1E5 mAb (green) and either with anti-GM130 (red, top panels) or anti-calnexin (red, middle panels). Staining with isotype control IgG2b or IgG2a is shown in the bottom panel, and staining with Hoechst 33342 (blue) shows the location of nuclei. A scale bar is 10 μm.

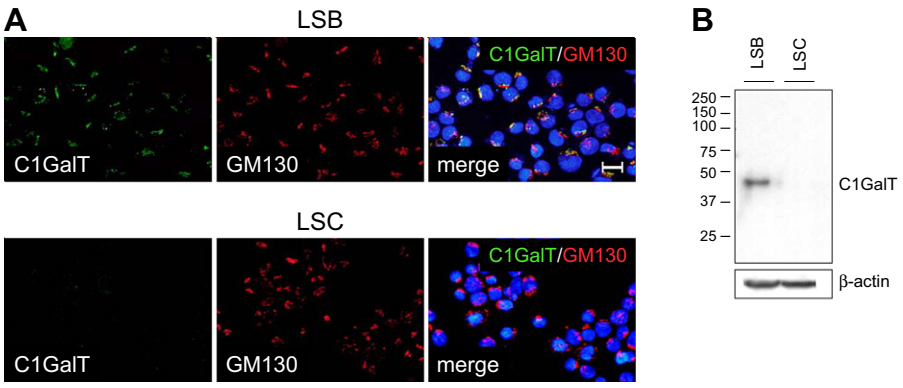

Fig. 2. LSC cells do not express C1GalT. (A) LSB and LSC cells were stained for the 4A2 mAb (green) and anti-GM130 (red). Yellow signals in merged image indicate endogenous C1GalT is detected in LSB cells. A scale bar is 10 μm. (B) Western blotting analysis of C1GalT in LSB and LSC cells. Approximately 10 μg whole cell lysate were separated by 10% SDS–PAGE under reducing conditions. The 4A2 mAb specifically detected C1GalT (48 kDa) in LSB cells but not in LSC cells.

sion of C1GalT and GM130, which were overlapped in Golgi apparatus. In contrast, 4A2 mAb did not show any signals in LSC cells, although anti-GM130 gave apparent positive signals for Golgi apparatus in LSC cells. This finding is further confirmed by Western blotting analysis as shown in Fig. 2B. A positive band below 50 kDa of molecular weight was detected by 4A2 mAb in LSB but not in LSC cells. Although C1GalT is also fully transcribed in LSC cells (data not shown), C1GalT protein could not be detected by immunocytochemistry or Western blotting in LSC cells. This indicated that C1GalT protein translated may be unfolded and rapidly degraded because of null function of Cosmc.

Transfection of Cosmc reconstituted C1GalT expression in Golgi apparatus in LSC cells

Next, we transfected a full-length *Cosmc* cDNA into LSC cells which lacked C1GalT protein due to functional ablation of Cosmc. The Cosmc-transfected LSC cells (LSC+Cosmc) obtained PNA-reactivity which indicates presence of T antigen and lost Tn- and sTn-reactivities after Cosmc-transfection. These reactivities of LSC+Cosmc cells are very similar to those of LSB cells (Fig. 3A and B).

As seen in Fig. 3B, LSC+Cosmc cells showed exclusive co-localization of C1GalT with GM130. Thus, C1GalT was recovered for its expression in Golgi apparatus by introduction of the functional *Cosmc* cDNA. This result raised a question as to whether C1GalT was proteolytically digested in proteasome in the absence of Cosmc. To test this possibility, we treated LSC cells with the proteasome inhibitor, lactacystin. Compared to the LSC+Cosmc cells, LSC cells treated with lactacystin showed a diffuse distribution of C1GalT in the cytoplasm, which is similar to the ER-staining pattern by anti-calnexin antibody (Fig. 3C). This is apparently different from the Golgi-staining pattern detected by anti-GM130 antibody. This result indicated that C1GalT is unfolded without the help of the specific chaperone, Cosmc, and proteolytically digested in proteasomes before being transported to the Golgi apparatus.

Cosmc determines distribution of C1GalT to Golgi apparatus

Co-transfection of C1GalT and Cosmc reportedly exerted a higher core 1 synthase activity than single transfection of each gene into 293T cells [2]. To visualize this phenomenon based on the core 1 synthase activity, we performed immunostaining with 4A2 on C1GalT-transfected, Cosmc-transfected, C1GalT- and Cosmc-co-transfected and mock-transfected 293T cells. As seen in Fig. 4A, endogenous C1GalT was generally detected in Golgi apparatus in mock-transfected 293T and Cosmc-transfected 293T cells. On the contrary, the diffuse distribution of C1GalT was seen in the cytoplasm of the C1GalT-transfected 293T cells. This diffuse staining pattern was similar to that of the anti-calnexin antibody staining and that of anti-C1GalT staining of LSC cells in the presence of lactacystin, indicating that C1GalT was not localized in the Golgi apparatus. In the co-transfected cells with C1GalT and Cosmc, overexpressed C1GalT recurred, in the Golgi apparatus.

We performed Western blotting analysis under non-reducing conditions to differentiate the monomer or dimer form of C1GalT from the aggregated one. As shown in Fig. 4B, we observed a macromolecule of aggregated protein in C1GalT-transfected 293T cells. In contrast, C1GalT was expressed mainly in forms of monomer and dimer in the co-transfected cells with C1GalT and Cosmc. These results demonstrated that the amount of Cosmc controls the C1GalT folding and distribution in the Golgi apparatus. In this non-reducing gel, endogenous C1GalT in the mock-transfected and Cosmc-transfected cells could not be detected because of the lower sensitivity compared to the reducing conditioned gel.

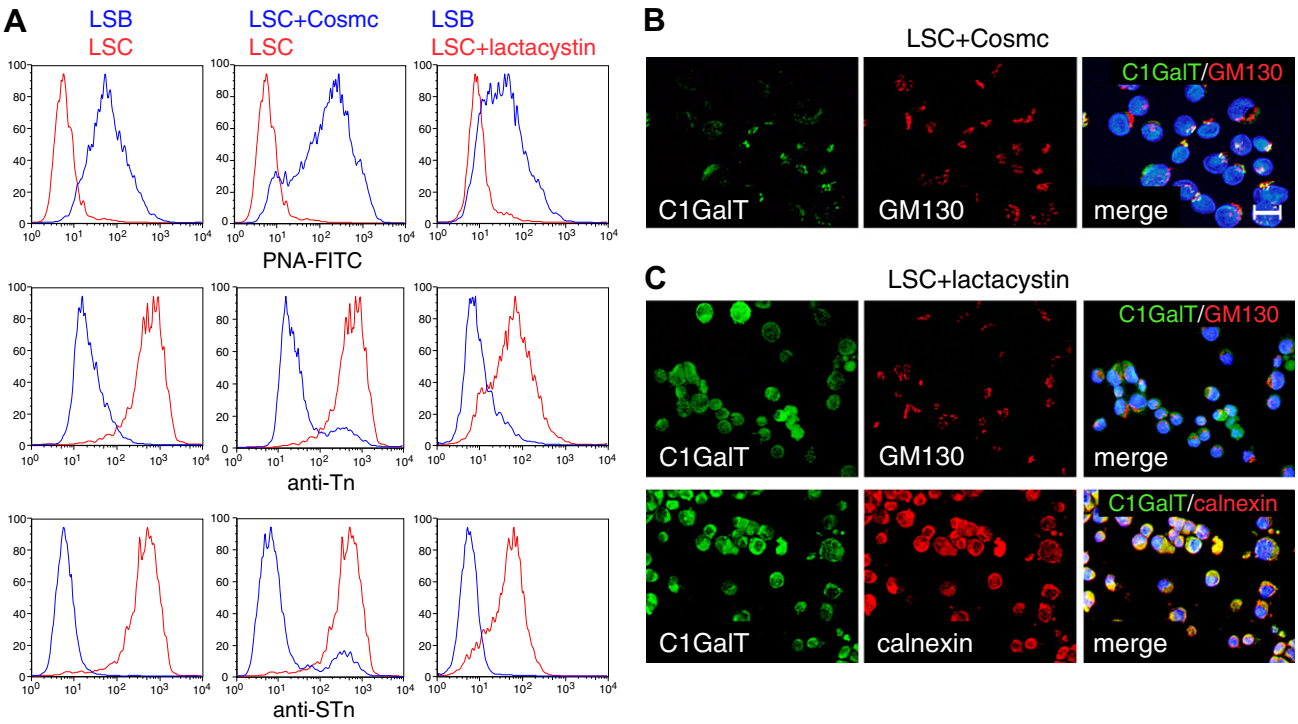

Fig. 3. C1GalT localize in Golgi apparatus. (A) FACS analysis of C1GalT in LSB, LSC, LSC transfected with Cosmc (LSC+Cosmc) and LSC treated with lactacystin (LSC+lactacystin) cells. Surface core 1 expression is detected by PNA-FITC in LSB and LSC+Cosmc (blue) but not in LSC (red) cells. The cells were also stained with anti-Tn and anti-STn. (B) Immunofluorescence images of L cells stained with 4A2 mAb. LSC+Cosmc cells were labeled with 4A2 mAb (green) and anti-GM130 (red, top panels). (C) LSC cells were incubated with lactacystin (10 μM) for 18 h, then cells were stained 4A2 mAb (green) and anti-GM130 (red, top panels) or anti-calnexin (red, bottom panels). A scale bar is 10 μm.

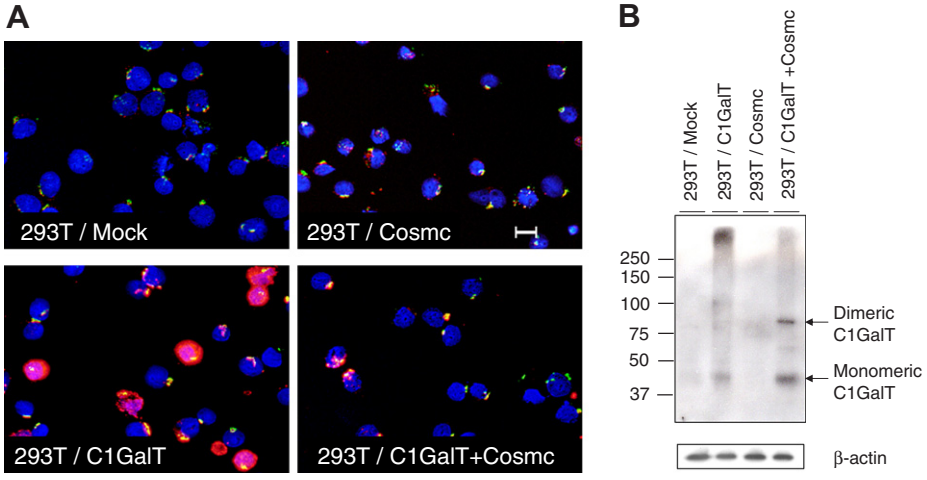

Fig. 4. Intracellular dynamics of C1GalT in mock-transfected, C1GalT-transfected, Cosmc-transfected and C1GalT- and Cosmc-co-transfected 293T cells (A) Immunofluorescence images of 293T transfectant stained with 4A2 mAb. 293T cells transfected with C1GalT and Cosmc were stained with 4A2 mAb (red) and anti-GM130 (green). Yellow signals in merged image indicate C1GalT is localized at Golgi apparatus. (B) Western blotting analysis. Approximately 5 μg of whole cell lysates were separated by 10% SDS–PAGE under non-reducing conditions. 4A2 mAb detected C1GalT a monomer at 42 kDa and a dimmer at 84 kDa in C1GalT and Cosmc co-transfected 293T cells. A scale bar is 10 μm.

Discussion

In the present study, we established 4A2 and 1E5 mAbs which specifically recognize C1GalT and Cosmc, respectively. Through immunological detection using these antibodies, we demonstrated exclusive localization of C1GalT in the Golgi apparatus of LSB cells that could express the core 1 structure.

In this study, the following four findings were substantiated to provide increased insights regarding the core 1

structure synthesis. (1) C1GalT was apparently localized in the Golgi apparatus in LSB cells, while Cosmc was distributed diffusely in the cytoplasm of LSB cells as same as calnexin, that is known as an ER protein. Both proteins showed a merged staining pattern in ER (Fig. 1B and C). (2) ClGalT protein was absent in LSC cells in spite of substantial amounts of *C1GalT* mRNA in the cells. This was due to the lack of Cosmc function (Fig. 2A). (3) C1GalT appeared in the Golgi apparatus in LSC cells by introduction of Cosmc. (4) Treatment of LSC cells with the proteasome inhibitor, lactacystin, resulted in diffuse appearance of C1GalT in the cytoplasm. These findings suggest that Cosmc regulates the intracellular dynamics of C1GalT from ER to Golgi apparatus that determines the core 1 synthase activity.

Other groups reported that expression of active C1GalT requires co-expression with Cosmc and they form a molecular complex when both proteins were co-overexpressed in 293T cells [2,15]. Our immunocytochemical study could not demonstrate a constitutive complex formation of C1GalT with Cosmc in the cells. When the present results are taken together with those of earlier studies, C1GalT would need the help of Cosmc in ER to be an active enzyme. C1GalT and Cosmc would temporarily form a complex in ER, and then only C1GalT would be sorted into the Golgi apparatus. C1GalT needs a molecular chaperone, Cosmc, to maintain or acquire correct folding and be distributed correctly to the Golgi apparatus. This intracellular dynamics is indispensable for C1GalT to obtain core 1 synthase activity.

Cosmc has been assumed to be a chaperone specific to C1GalT from its initially identified characteristics [2]. Our results support this idea by demonstrating that Cosmc was localized in ER in association with translocation of C1GalT to the Golgi apparatus. PNA binding and anti-Tn antibody staining did not change in the lactacystin-treated LSC cells in spite of elevated C1GalT expression because it was incorrectly localized in ER. However, introduction of Cosmc into LSC cells recovered C1GalT expression correctly in Golgi and augmented PNA binding was observed. These results suggest that Cosmc interacts with C1GalT in ER, and plays a role in facilitating C1GalT folding and its transport to Golgi. The precise mechanism of interaction of C1GalT with Cosmc in ER warrants further study.

The intracellular dynamics of C1GalT depending on Cosmc in this study leads support to the idea that Cosmc is a kind of chaperone specific to C1GalT. The basic paradigm of molecular chaperones is that they recognize and selectively bind to non-native, but not to native, proteins, and distribute the relatively stable proteins to the appropriate place [16].

Cosmc is possibly the only chaperone for C1GalT, as there are several chaperones for specific proteins, such as HSP47 that is a chaperone specific to collagen [17], and calmegin that is specific to alpha/beta fertilin [18]. Indeed, a higher level of core 1 synthase activity was reported when C1GalT was coexpressed with Cosmc in 293T cells, compared with the activity observed when only C1GalT or Cosmc was expressed [2]. In addition, our results suggest that the endogenous levels of Cosmc may be rate-limiting and that the amounts of Cosmc regulate the expression of C1GalT in the correct localization, i.e. in the Golgi apparatus, in 293T cells (Fig. 4).

Though substantial amounts of *C1GalT* mRNA were detected in LSC cells, C1GalT protein was not detected by the 4A2 mAb due to the lack of Cosmc function. As mutations of Cosmc have now been found in human cancer cell lines [2,3] and in patients with Tn syndrome [7], these antibodies would be very useful to measure the amounts of C1GalT protein and Cosmc in such cells having the *Cosmc* gene mutation. Down-regulation of transcription of Cosmc in B cells from an IgAN patient has been reported [8]. The antibodies would also be useful to detect diminished C1GalT protein in such patients. It remains to be confirmed whether or not anti-C1GalT 4A2 mAb can be used to evaluate such disease activity in pathological specimens.

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