A unique molecular chaperone Cosmc required for activity of the mammalian core 1 β3-galactosyltransferase

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Human core 1 β3-galactosyltransferase (C1β3Gal-T) generates the core 1 O-glycan Galβ1-3GalNAcα1-Ser/Thr (T antigen), which is a precursor for many extended O-glycans in animal glycoproteins. We report here that C1β3Gal-T activity requires expression of a molecular chaperone designated Cosmc (*co*re 1 β3-Gal-T-*s*pecific *m*olecular *c*haperone). The human *Cosmc* gene is X-linked (Xq23), and its cDNA predicts a 318-aa transmembrane protein (≈36.4 kDa) with type II membrane topology. The human lymphoblastoid T cell line Jurkat, which lacks C1β3Gal-T activity and expresses the Tn antigen GalNAcα1-Ser/Thr, contains a normal gene and mRNA encoding C1β3Gal-T, but contains a mutated *Cosmc* with a deletion introducing a premature stop codon. Expression of Cosmc cDNA in Jurkat cells restored C1β3Gal-T activity and T antigen expression. Without Cosmc, the C1β3Gal-T is targeted to proteasomes. Expression of active C1β3Gal-T in Hi-5 insect cells requires coexpression of Cosmc. Overexpression of active C1β3Gal-T in mammalian cell lines also requires coexpression of Cosmc, indicating that endogenous Cosmc may be limiting. A small portion of C1β3Gal-T copurifies with Cosmc from cell extracts, demonstrating physical association of the proteins. These results indicate that Cosmc acts as a specific molecular chaperone in assisting the folding/stability of C1β3Gal-T. The identification of Cosmc, a uniquely specific molecular chaperone required for a glycosyltransferase expression in mammalian cells, may shed light on the molecular basis of acquired human diseases involving altered O-glycosylation, such as IgA nephropathy, Tn syndrome, Henoch-Schönlein purpura, and malignant transformation, all of which are associated with a deficiency of C1β3Gal-T activity.

The O-glycans in human glycoproteins and mucins are important in many aspects of cellular metabolism and cellular interactions, including those involved in leukocyte trafficking (1, 2). The biosynthesis of mucin-type O-glycans in animal mucins and other glycoproteins is orchestrated by a family of N-acetylgalactosaminyltransferases that transfer GalNAc to specific Ser/Thr residues to generate GalNAcα1-Ser/Thr, also known as the Tn antigen (3). Subsequently, this precursor is the acceptor for core 1 β3-galactosyltransferase (C1β3Gal-T or T-synthase) to generate the core 1 disaccharide O-glycan Galβ1-3GalNAcα1-Ser/Thr (4, 5), also known as the T antigen or Thomson–Friedenrich antigen. Unlike most glycosyltransferases, which occur in gene families, a single human gene on chromosome 7p14-p13 encodes the C1β3Gal-T (4), although there is a pseudogene for C1β3Gal-T on chromosome 5 (unpublished data). Other core structures for mucin-type O-glycans are known, but core 1 is the common core structure found on human erythrocytes and most lymphocytes, and it serves as a precursor for the branched core 2 O-glycan core Galβ1-3(GlcNAcβ1-6)GalNAcα1-Ser/Thr found on human leukocytes and which are required to generate ligands for P-selectin in leukocyte adhesion (2, 6). The factors regulating expression of core 1 O-glycans and T-synthase activity are of interest because Tn and sialyl Tn antigens are recognized as tumor-associated antigens for breast and colon carcinomas (7, 8), and the inability to synthesize core 1 O-glycan is a potentially contributing factor to several autoimmune diseases, including IgA nephropathy (9), Tn-syndrome (10), and Henoch-Schönlein purpura (11).

Intriguingly, the human T leukemic cell line Jurkat cells lack C1β3Gal-T activity, lack core 1 O-glycans, and generate the Tn antigen (12, 13). Thus, an alteration in the expression of the C1β3Gal-T is predicted to cause global changes in O-glycan structures in multiple glycoproteins. In exploring the factors that regulate C1β3Gal-T activity in Jurkat cells, we identified a protein that associates with C1β3Gal-T and is required for its activity. We have designated this protein, which has the properties of a chaperone, Cosmc (*co*re 1 β3-Gal-T-*s*pecific *m*olecular *c*haperone). Here, we show that a mutation in Cosmc in Jurkat cells causes loss of C1β3Gal-T activity and targeting of the inactive protein to the proteasome. Thus, mutations in Cosmc may contribute to expression of Tn antigen in tumor cells and human autoimmune diseases.

Materials and Methods

RT-PCR, PCR, Cloning of the RT-PCR Product, and Sequencing. Total RNA and genomic DNA from 5×10^7 cells of Jurkat (clone E6-1, ATCC TIB 152), Molt-4, and K562 cells were isolated by using the total RNA isolation kit (The RNA Company, Austin, TX) and QIAgen DNA mini kit (Qiagen, Valencia, CA). For RT-PCR of Cosmc, the forward primer was 5′-CTCCATAGAGGAGTTGTTGC-3′, and the reverse primer was 5′-TCACGCTTTTCTACCACTTC-3′. RT-PCR was performed in one step in 25 μl of reaction containing 500 ng of total RNA, RT/Tag mix, and primers (SuperScript One-Step RT-PCR kit, Invitrogen). The RT-PCR product was analyzed on a 1% Tris-acetate EDTA (TAE) agarose gel, and the expected 1,218-bp band was excised; the DNA was then extracted from the gel by using a QIAquick gel extraction kit (Qiagen). One-tenth of the product was cloned into PCR3.1(+) (Invitrogen) by TA cloning and sequenced. To examine the sequence of *Cosmc*, PCR was performed by using genomic DNA as the template. Because human *Cosmc* contains a single exon, the same primer pair was used for both RT-PCR and PCR of *Cosmc*. The PCR product was analyzed on a 1% TAE agarose gel, and the expected 1,218-bp band was excised, purified, and directly sequenced.

Construction of an Expression Vector Encoding C-Terminal HPC4 Epitope-Tagged Human C1β3Gal-T. A mammalian expression vector of pcDNA4 (Invitrogen) encoding C-terminal HPC4 epitope-

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Abbreviations: Tn antigen, GalNAcα1-Ser/Thr; T antigen, Galβ1–3GalNAcα1-Ser/Thr; C1β3Gal-T, core 1 UDP-Gal:GalNAc-α-R β1,3-galactosyltransferase; Cosmc, core 1 β3-Gal-T-specific molecular chaperone; PNA, peanut agglutinin; mCosmc, mutated Cosmc, wtCosmc, wild-type Cosmc; ER, endoplasmic reticulum.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AA578739 (human EST clone for Cosmc), AC011890 (human PAC clone RP4-655L22), and NP_067525 (mouse gene for Cosmc)].

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tagged human C1b3Gal-T was constructed by using PCR for introducing the HPC4 epitope into the cDNA. The forward primer was 5′-GCGGATCCATGGCCTCTAAATC-3′. The reverse primer containing sequence, encoding 12 aa of HPC4 epitope (EDQVDPRLIDGK) immediately following the C-terminal proline of human C1b3Gal-T, was 5′-GGAAGATCTACTTGCCGTCGATCAGCCTGGGGTCCACCTGGTCCTCAGGATTTCCTAACTTCACTTTTG-3′. The expected 1,144 bp of PCR product was purified on 1% TAE agarose gel and digested by *Nco*I and *Bgl*II. The expected 1,128-bp DNA fragment was purified and cloned into *Nco*I (partially digested)/*Bam*HI sites of pcDNA4 and its sequence was confirmed. For construction of an insect cell expression vector, the PCR product was digested with *Bam*HI and *Bgl*II, and the 1,134-bp DNA fragment was purified and cloned into the *Bam*HI site of pVL1393 (PharMingen). Thus, a baculovirus transfer vector encoding a C-terminal HPC4 epitope-tagged human C1b3Gal-T in pVL1393 was constructed.

Construction of an Expression Vector Encoding Human C-Terminal His-6-Tagged Cosmc. A cDNA encoding Cosmc with a C-terminal His-6 tag was generated by introducing the His-6 tag into Comsc cDNA by using PCR and EST AA578739 (Genome Systems) as a template. The PCR product was subcloned into pcDNA3.1(+) by using *Bam*HI/*Xba*I sites. Similarly, a baculovirus transfer vector encoding human C-terminal His-6-tagged Cosmc was constructed through subcloning of the Cosmc cDNA into pVL1393.

Construction of an Expression Vector Encoding Human Mutated Cosmc (mCosmc). An expression vector encoding mCosmc was constructed by replacing the *Hin*dIII/*Xba*I fragment of wild-type Cosmc (wtCosmc) in pCDNA3.1(+) with a mutated fragment of mCosmc in PCR3.1 vector generated by TA cloning of the RT-PCR of mCosmc from Jurkat cells. A baculovirus transfer vector encoding human mCosmc was constructed by subcloning the mCosmc cDNA from pcDNA3.1(+) into pVL1393.

Preparation of Baculovirus. Sf-9 insect cells were cultured in 5 ml of Sf-900II SFM with 10% FBS at 27°C and cotransfected with pVL1393 vector and baculovirus DNA by using the BaculoGold transfection kit (PharMingen). The media containing the baculovirus were harvested 4 days posttransfection. For large-scale preparation of baculovirus, Sf-9 cells in 25 ml of media were infected with 0.25 ml of baculovirus obtained above. The baculovirus (media) was collected 4 days postinfection and used for expression of human C1β3Gal-T and Cosmc.

Preparation of Cell Extracts. Transfected or infected cells were resuspended in an appropriate volume of 25 mM Tris·HCl buffer (pH 7.5) containing 150 mM NaCl and proteinase inhibitor mixture (Roche Molecular Biochemicals) and homogenized by sonication on an ice-bath for 5 s four times. The postnuclear supernatants were obtained by centrifugation of homogenate at 700 $\times$ *g* for 10 min, and the extracts were obtained by adding 1% Triton X-100 to the supernatant and solubilizing on ice for 30 min.

Expression of Human C1β3Gal-T and Cosmc in 293T Cells. Human 293T cells were transiently transfected with expression vectors encoding a C-terminal HPC4 epitope-tagged human C1β3Gal-T and/or the expression vector encoding human C-terminal His-6-tagged Cosmc or mCosmc, by using FuGENE 6 (Roche Molecular Biochemicals). Cells were harvested 72 h posttransfection, and a cell extract was prepared. One portion was assayed for C1β3Gal-T activity by using GalNAcα1-*O*-phenyl (Sigma) as the acceptor (5), and the other portion was used for capture of Cosmc on Ni-NTA Superflow and capture of C1β3Gal-T on HPC4 beads, prepared as described (4), and Western blot.

Expression of Human C-Terminal HPC4 Epitope-Tagged C1β3Gal-T and C-Terminal His-6-Tagged Cosmc and mCosmc in Hi-5 Cells. Hi-5 insect cells were cultured in EX-CELL 405 media at 27°C. For infection or coinfection of human C1β3Gal-T, Cosmc, and mCosmc, 0.75 ml of baculovirus was used. At 5 days postinfection, the cells were harvested, and extract was prepared for assaying C1β3Gal-T activity, capture of Cosmc on Ni-NTA, C1β3Gal-T capture on HPC4 beads, and Western blot.

Transfection of Jurkat Cell with Human HPC4 Epitope-Tagged C1β3Gal-T and Cosmc. Jurkat cells were transiently transfected with expression vectors encoding a human HPC4 epitope-tagged C1β3Gal-T and/or the expression vector encoding human Cosmc by using GENEPORTER transfection reagent and BOOSTER (Gene Therapy Systems, San Diego). Cells were harvested at 72 h posttransfection, and a cell extract was prepared. Using these transfection protocols, we also identified and established stably transfected Jurkat cells expressing human wtCosmc. These cells were tested for the ability of wtCosmc to complement the expression of core 1 O-glycans. Either mock-transfected Jurkat cells or Jurkat cells stably transfecting wtCosmc (3–5 $\times$ 10^5 cells) were washed with PBS and resuspended in 250 μl of RPMI 1640 complete media and either treated or not treated with 50 milliunits of neuraminidase (from *Arthrobacter ureafaciens*, Sigma) at 37°C for 1 h. The cells were washed twice with PBS and incubated with 5 μg/ml of fluorescently labeled ALEXA488-labeled peanut agglutinin (PNA) for 1 h at room temperature. The cells were washed with PBS twice, and a portion of the cells was transferred to an 8-well chambered slide and visualized under either a phase-contrast or fluorescent microscope.

Capture of Human HPC4 Epitope-Tagged C1β3Gal-T on HPC4 UltraLink. HPC4-tagged C1β3Gal-T in 500 μl of cell extract was captured with HPC4 UltraLink beads (100 μl) as described (4).

Capture of Human Cosmc on Ni-NTA Superflow. The Ni-NTA Superflow beads (100 μl, Qiagen) equilibrated with the Ni-NTA washing buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, and 0.1% Triton X-100 (pH 7.8) were incubated with the cell extracts overnight at 4°C on a rotator. The beads were washed five times with 1 ml of the washing buffer. One portion of the beads was removed for C1β3Gal-T enzyme assay. The bound material in the other portion was eluted with 100 μl of eluting solution (50 mM NaH_2PO_4/300 mM NaCl/250 mM imidazole/0.1% Triton X-100).

Western Blot of Human HPC4 Epitope-Tagged C1β3Gal-T. Ten to 20 microliters of cell extract, unbound material, and 25 μl of Ni-NTA bound material were electrophoresed on an SDS/PAGE (4–20%) under reducing conditions and transferred to a nitrocellulose membrane (Bio-Rad). The samples were Western-blotted for the HPC4 epitope by using 10 μg/ml HPC4 mAb at room temperature for 1 h, as described (4).

Results

Identification of Cosmc. The human T-leukemic cell line Jurkat is deficient in C1β3Gal-T activity and expresses truncated O-glycans bearing the Tn antigen (12, 13). We first considered the possibilities that the lack of C1β3Gal-T activity might be caused by either a mutation in the C1β3Gal-T gene or transcriptional regulation of C1β3Gal-T expression. We found, however, that the transcript level for the C1β3Gal-T in Jurkat cells is much higher than that in the human lymphoblastoid cell line MOLT-4, which has relatively high levels of C1β3Gal-T activity (data not shown), and that the cDNA

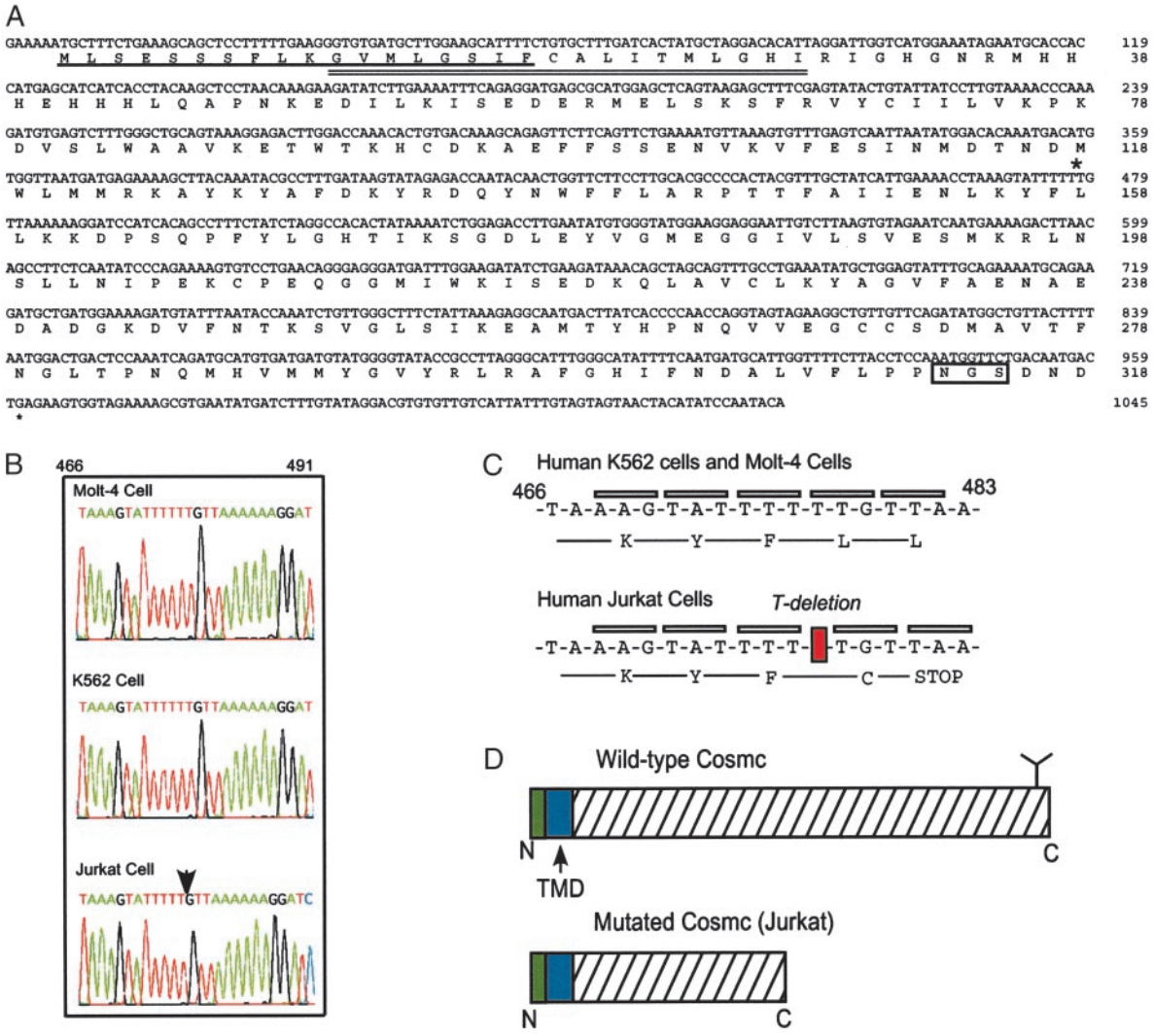

Fig. 1. Human Cosmc cDNA and deduced protein sequence and mutation of Cosmc in human Jurkat cells. (*A*) The nucleotide and deduced amino acid sequences of human Cosmc are shown. The cDNA predicts a 318-aa protein with a type II topology. The putative transmembrane domain is double underlined. The portions of the sequence that correspond to the identified N-terminal sequence copurified with the purified rat liver C1β3Gal-T (5) are indicated by the single underlining. The potential N-glycosylation site is boxed. The asterisk denotes the position of the T deletion described below. (*B*) The cDNA sequence of Cosmc was obtained by RT-PCR using total RNA from Jurkat, Molt-4, and K562 cells. The arrow indicates that portion of the sequence with a T deletion at base pair 478. (*C*) The T-deletion mutation at base pair 478 in Cosmc from Jurkat cells is indicated by the red box causing a truncation and introducing a stop codon. (*D*) Diagram of the domain organization of wtCosmc and mCosmc from Jurkat cells.

sequence for the C1β3Gal-T derived from Jurkat cells was normal. Thus, there is no mutation in the C1β3Gal-T gene in Jurkat cells, and the cells have normal transcripts, yet lack C1β3Gal-T enzyme activity. Human C1β3Gal-T is an ≈42-kDa subunit, dimeric enzyme, which is unusual in lacking common posttranslational modifications, such as addition of N-glycans (4). Surprisingly, we also found that expression of a recombinant, epitope-tagged form of the enzyme in Jurkat cells, as discussed below, did not result in any detectable recombinant protein. Together, these results demonstrated that some other factor(s) may posttranscriptionally regulate expression of the C1β3Gal-T protein and enzyme activity.

A clue to this other factor(s) was obtained by examining protein sequence data obtained during the purification of the C1β3Gal-T from rat liver. N-terminal sequences of a partially purified C1β3Gal-T, which migrated as a monomer at ≈42–43 kDa and a dimer at ≈84–86 kDa, were obtained (5). Two amino acid sequences of different proportions were observed for the ≈84- to 86-kDa material at each sequence cycle in the partly purified preparation. One sequence (ASKSWLNFL) was identified as corresponding to the now defined C1β3Gal-T (4, 5), whereas the other sequence (MLSESSSFLKGVMLGSIF) was derived from an unknown protein(s). BLASTP search of the National Center for Biotechnology Information EST database by using this N-terminal peptide sequence identified a human EST, AA578739, which contains one ORF of 954 bp (Fig. 1*A*). A BLAST search using this sequence identified a human P-1-derived artificial chromosome clone RP4-655L22 (GenBank accession no. AC011890). This clone, derived from chromosome Xq23, contained a full-length ORF in a single exon. The gene present in RP4-655L22 is predicted to encode a 318-aa protein with type 2 membrane topology, a short cytoplasmic N terminus, a single transmembrane domain, a large C-terminal domain (≈36.4-kDa polypeptide size), and one N-glycosylation sequon at Asn-313. Interestingly, the mature form of Cosmc retains the N-terminal Met residue. A homolog of this gene was found in mice, as discussed below, but no homologs were found in *Caenorhabditis elegans* or *Drosophila*. We considered whether

the protein encoded by this gene was a potential accessory protein of unknown function to the C1β3Gal-T. Based on studies described below, this protein was designated Cosmc.

Mutation of Cosmc in Jurkat Cells. We sequenced the cDNA for Cosmc from Jurkat cells and identified a T deletion at nucleotide position 478. This deletion causes a frameshift and the introduction of a stop codon (Fig. 1 *B* and *C*), resulting in a predicted mCosmc protein lacking most of the C-terminal domain (Fig. 1*D*). The *Cosmc* gene is encoded by a single exon, and we also sequenced the DNA after PCR. The DNA from Jurkat cells also encoded a single T deletion at the same position as seen in the cDNA. Jurkat cells were originally derived from a 14-year-old male (14), and because Cosmc is X-linked, we would expect the cells to have only a single copy of the *Cosmc* gene.

Chaperone Function of Cosmc. The mutation in Cosmc in Jurkat cells and the lack of expression of C1β3Gal-T by these cells led us to test the possible chaperone function of Cosmc. For this we exploited our observation that expression of the human C1β3Gal-T in Hi-5 insect cells anomalously resulted in a recombinant form of the enzyme lacking activity. Insect cells have very low levels of endogenous C1β3Gal-T activity (15). For these studies, we prepared epitope-tagged recombinant forms of C1β3Gal-T and Cosmc. A C-terminal His-6 tag chimeric form of wtCosmc was generated (bound by Ni-NTA columns), and a C-terminal 12-aa HPC4 epitope-tagged chimeric form of C1β3Gal-T was generated. The HPC4 epitope is recognized by the Ca^{2+}-dependent mAb HPC4 (16, 17). Coexpression of wtCosmc with the C1β3Gal-T in Hi-5 cells resulted in a substantial recovery of total enzyme activity, whereas coexpression with mCosmc only slightly enhanced enzyme activity (Fig. 2*A*). These results demonstrate that expression of Cosmc is required for the activity of human C1β3Gal-T. We also examined the potential role of Cosmc expression in human 293T cells, which have an endogenous, functional C1β3Gal-T and Cosmc. Interestingly, activity of recombinant C1β3Gal-T was enhanced by coexpression with Cosmc but not with mCosmc (Fig. 2*D*). The higher level of activity observed when recombinant C1β3Gal-T was coexpressed with Cosmc in 293T cells, compared with the activity observed when only recombinant C1β3Gal-T was expressed (Fig. 2*D*), raises the possibility that the endogenous levels of Cosmc may be rate-limiting, but this will need to be further explored.

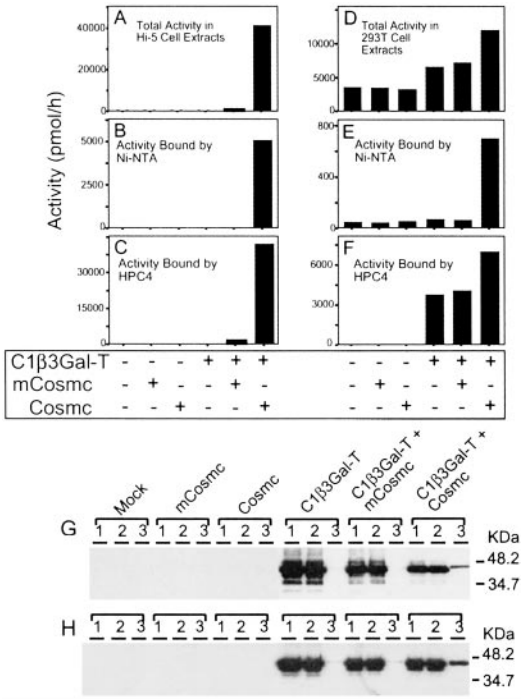

Fig. 2. Requirement of wtCosmc, but not mCosmc, for the activity of human C1β3Gal-T. (*A* and *D*) The human C-terminal HPC4 epitope-tagged C1β3Gal-T was expressed in Hi-5 insect cells with a baculovirus vector (*A*) or human 293T cells by transient transfection (*D*) with or without the coexpression of wtCosmc and mCosmc, as indicated. Infected Hi-5 cells were harvested 5 days postinfection. Extracts of the cells were prepared, and total activity of C1β3Gal-T was determined. (*B* and *E*) Extracts were incubated with Ni-NTA Superflow, and the total activity of bound C1β3Gal-T was determined. (*C* and *F*) Extracts were incubated with HPC4 beads, and the total activity of bound C1β3Gal-T was determined. The cell extracts from the above transfections of both Hi-5 cells (*G*) or human 293T cells (*H*) were analyzed by SDS/PAGE and Western blot with mAb to the HPC4 epitope present at the C terminus of the recombinant C1β3Gal-T. Lane 1 represents total cell extracts; lane 2 represents the material not bound by Ni-NTA; lane 3 represents material bound by Ni-NTA. Molecular weight markers are indicated. The sets of lanes 1–3 from each of the cell extracts derived from different transfections (or from mock-transfected cells) from *A–F* are indicated.

Association of Cosmc and C1β3Gal-T. To directly examine whether Cosmc can associate with C1β3Gal-T, we asked whether some C1β3Gal-T activity could be bound by Ni-NTA when the HPC4-tagged C1β3Gal-T was coexpressed with His-6-tagged wtCosmc. The results show that some of the active C1β3Gal-T is cobound with wtCosmc on Ni-NTA, but not when coexpressed with mCosmc in either Hi-5 cells (Fig. 2*B*) or 293T cells (Fig. 2*E*). We also found that active HPC4-tagged C1β3Gal-T was recoverable from Hi-5 cells on HPC4 beads when the enzyme was coexpressed with wtCosmc, but much less activity (≈2%) was detectable when coexpressed with mCosmc (Fig. 2*C*). Whereas expression of the HPC4-tagged C1β3Gal-T in 293T cells gave rise to active enzyme captured by the anti-HPC4 column, coexpression with the wtCosmc, but not mCosmc, enhanced this activity (Fig. 2*F*). These results demonstrate that Cosmc associates with C1β3Gal-T and that expression of active C1β3Gal-T requires coexpression with wtCosmc.

To confirm that Cosmc and C1β3Gal-T can associate, we performed Western blots on the HPC4-tagged C1β3Gal-T expressed in Hi-5 cells and 293T cells with or without His-6-tagged wtCosmc or mCosmc. When HPC4-tagged C1β3Gal-T was expressed in Hi-5 cells and 293T cells in the absence or presence of wtCosmc or mCosmc, a considerable amount of protein was generated (Fig. 2 *G* and *H*), but the enzyme lacked activity except when coexpressed with wtCosmc (Fig. 2 *A–C*). More importantly, HPC4-tagged C1β3Gal-T was present with His-6-tagged wtCosmc when the latter was captured on Ni-NTA (Fig. 2 *G* and *H*). By contrast, coexpression with mCosmc did not result in coisolation of HPC4-tagged C1β3Gal-T (Fig. 2 *G* and *H*). There was a slight amount of HPC4-tagged C1β3Gal-T detected in the Ni-NTA bound material in the absence of His-6-tagged wtCosmc from 293T cells (Fig. 2*H*). Remarkably, this could result from cobinding of HPC4-tagged C1β3Gal-T to endogenous Cosmc, as the N-terminal domain of Cosmc contains the sequence HHHEHHH (Fig. 1*A*), which may have weak binding to Ni-NTA. This finding might explain why there was a low level of C1β3Gal-T activity (Fig. 2*E*) in the material bound by Ni-NTA in the absence of recombinant Cosmc expression.

Complementation of Jurkat Cells by wtCosmc. We next tested whether wtCosmc could complement the mutation of the gene observed in Jurkat cells. The C-terminal, HPC4-tagged full-length C1β3Gal-T was transiently expressed in Jurkat cells along with wtCosmc, and we measured the total C1β3Gal-T activity in cell extracts and HPC4-bound C1β3Gal-T from cell extracts. Expression of wtCosmc in Jurkat cells enhanced the activity of the endogenous C1β3Gal-T (Fig. 3*A*), whereas expression of the HPC4-tagged C1β3Gal-T in the absence of wtCosmc only

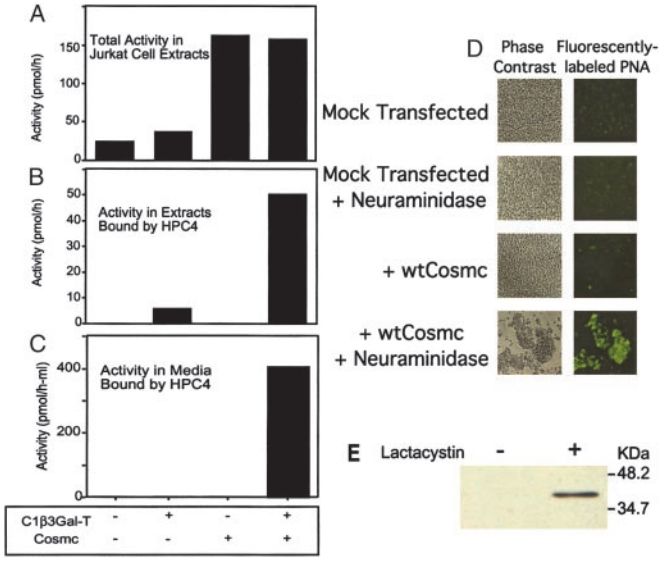

Fig. 3. Complementation of mCosmc in Jurkat cell with wtCosmc. (*A*) Jurkat cells were transiently transfected with expression vectors encoding the full-length human C-terminal HPC4 epitope-tagged C1β3Gal-T and/or the expression vector encoding human wtCosmc. At 72 h posttransfection, cell homogenates were prepared, and a portion was removed for assaying activity of C1β3Gal-T. (*B*) A portion of the extracts was incubated with HPC4 beads, and the total activity of bound C1β3Gal-T was determined. (*C*) Jurkat cells were stably transfected with a soluble, N-terminal HPC4 epitope-tagged form of C1β3Gal-T. The media from the cells were removed and incubated with HPC4 beads, and the total activity of bound C1β3Gal-T was determined. (*D*) Jurkat cells were either mock-transfected or stably transfected with cDNA encoding wtCosmc. Some cells were treated with *A. ureafaciens* neuraminidase. Both treated and untreated cells were incubated with fluorescently labeled Alexa488-PNA. The phase-contrast images of the cells were then monitored by phase contrast and fluorescence microscopy. (*E*) Jurkat cells stably expressing the soluble, N-terminal HPC4 epitope-tagged form of C1β3Gal-T (4) were incubated with or without lactacystin (10 μM) for 12 h. Cell extracts were then prepared and then separated by SDS/PAGE, and the level of the HPC4 epitope-tagged C1β3Gal-T was examined by Western blot with the HPC4 mAb.

slightly enhanced the total cellular activity (Fig. 3*A*). These results demonstrate that Cosmc can complement the defective mCosmc in Jurkat cells. Expression of full-length HPC4-tagged C1β3Gal-T in the absence of coexpressed wtCosmc somewhat elevated the cellular amount of HPC4-tagged C1β3Gal-T captured on HPC4 beads, but coexpression with wtCosmc considerably enhanced the cellular content of HPC4-tagged C1β3Gal-T captured on HPC4 beads (Fig. 3*B*). We then examined the activity of a stably expressed, soluble, N-terminally HPC4-tagged C1β3Gal-T in Jurkat cells. No activity of the soluble HPC4-tagged C1β3Gal-T was detectable in media when expressed in Jurkat cells in the absence of coexpressed wtCosmc (Fig. 3*C*), whereas coexpression with wtCosmc caused the production of significant levels of C1β3Gal-T activity captured on HPC4 beads (Fig. 3*C*). We further tested whether expression of wtCosmc in Jurkat cells could restore expression of core 1 O-glycan. To this end, we transfected Jurkat cells with cDNA encoding wtCosmc and tested the cells for their binding and agglutination by PNA. PNA binds well to the T antigen but does not recognize the Tn antigen (18, 19). To test for exposure of T antigen, cells were desialylated by treatment with neuraminidase, as PNA will not bind the sialylated T antigen. As shown in Fig. 3*D*, PNA was unable to agglutinate mock-transfected Jurkat cells, but it quantitatively agglutinated desialylated Jurkat cells stably expressing wtCosmc. In control experiments, this PNA-induced agglutination of desialylated Jurkat cells expressing wtCosmc was completely inhibited by lactose, demonstrating that the PNA-induced agglutination was carbohydrate-dependent (data not shown). These results demonstrate that the mutation of Cosmc in Jurkat cells can be complemented by wtCosmc causing elevation of endogenous C1β3Gal-T activity, restoration of the biosynthesis of core 1 O-glycans, and generation of an active, soluble, secreted form of HPC4-tagged recombinant C1β3Gal-T.

We noted that the recombinant HPC4 epitope-tagged C1β3Gal-T protein does not accumulate in Jurkat cells in the absence of coexpressed wtCosmc (Fig. 3*E*). Unfolded proteins in the endoplasmic reticulum (ER) are targeted for degradation by the proteasome (20). To test whether the inactive C1β3Gal-T in Jurkat cells is degraded in the ER, we treated Jurkat cells stably expressing the soluble HPC4 epitope-tagged C1β3Gal-T with the proteasome inhibitor lactacystin (21). Treatment of these Jurkat cells with lactacystin causes a significant increase in accumulation of the tagged protein (Fig. 3*E*), although the recombinant protein was still an inactive enzyme (data not shown). These results indicate that in the absence of Cosmc expression, the C1β3Gal-T is inactive and targeted for proteasome degradation.

Discussion

We have shown that expression of the active form of C1β3Gal-T (T-synthase) requires the coexpression of a unique molecular chaperone Cosmc. *Cosmc* contains a single exon encoding a predicted protein of ≈36.4 kDa. *Cosmc* is mutated in human Jurkat cells and generates a cDNA containing a T deletion resulting in a

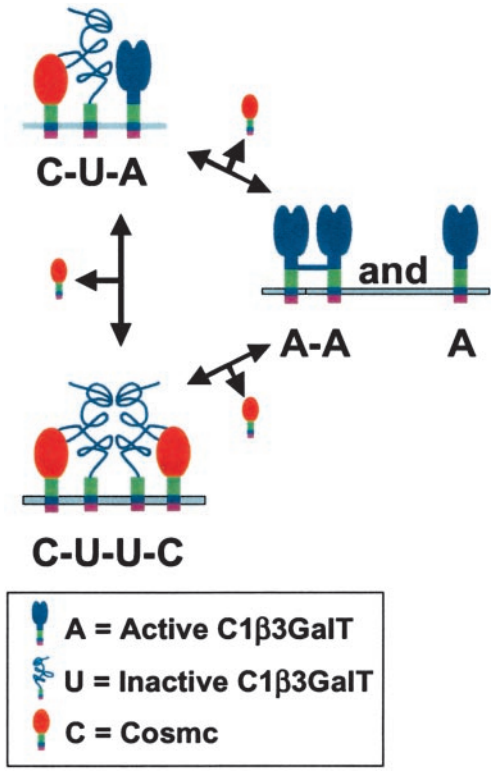

Fig. 4. Model of interactions between Cosmc and C1β3Gal-T in generation of active enzyme. Cosmc (C) is predicted to have a chaperone function in associating with inactive C1β3Gal-T (U) in complexes that may also contain an active form of the enzyme (A). Potential associations of oligomeric complexes are indicated as CUA and CUUC, which may copurify as shown in Fig. 2. After potential rounds of binding and dissociation between Cosmc and enzyme, stable active forms of the enzyme, either dimeric (AA) or monomeric (A), are generated. Other potential chaperones not yet defined may also be involved in formation of active enzyme.

frameshift and predicted truncation of the mutated protein. Furthermore, our studies show that Cosmc can associate with C1β3Gal-T and that wtCosmc can complement the defect in Jurkat cells and restore core 1 O-glycan biosynthesis. These results suggest a model of how Cosmc might function in the biosynthesis of the active form of C1β3Gal-T (Fig. 4). Cosmc may associate with folding intermediates of C1β3Gal-T, but whether this is a large oligomeric complex containing both native and partially unfolded forms of C1β3Gal-T (CUA), as opposed to complexes only of Cosmc and unfolded C1β3Gal-T (CUUC), is not yet defined. However, the presence of some C1β3Gal-T activity associated with Cosmc (Fig. 2) argues for the occurrence of some mixed complexes, such as CUA (Fig. 4). It is possible that other proteins, e.g., other chaperones, may also be present in complexes with Cosmc and C1β3Gal-T. Cosmc is not associated with the soluble active form of the recombinant C1β3Gal-T (data not shown), so Cosmc is not a subunit of C1β3Gal-T, which is consistent with our observation that highly purified rat liver C1β3Gal-T was also devoid of Cosmc (5). Thus, Cosmc seems to be a required cofactor for the generation of an active form of C1β3Gal-T, as complementation of the mutated Cosmc in Jurkat by wtCosmc restores C1β3Gal-T activity, and only inactive protein can be generated in insect cells in the absence of Cosmc coexpression. Such a cofactor function is consistent with a chaperone-assisted folding function of Cosmc. C1β3Gal-T occurs as a disulfide-bonded dimer, but monomeric forms of the enzyme may also be active (5). It is also likely that Cosmc occurs in a dimeric form, based on preliminary observations. Thus, Cosmc might associate with the C1β3Gal-T during its folding in the ER, although we have no direct evidence at present as to the subcellular localization of Cosmc. In this light it is especially interesting that recombinant HPC4 epitope-tagged C1β3Gal-T protein does not accumulate in Jurkat cells unless coexpressed with wtCosmc. The recombinant HPC4 epitope-tagged C1β3Gal-T protein can be rescued from degradation by the proteasome inhibitor lactacystin, indicating that Cosmc may function in facilitating C1β3Gal-T folding/stability and trafficking out of the ER. Of course, other chaperones or cochaperones may also be involved in the folding or stability of the human C1β3Gal-T.

It is noteworthy that Jurkat cells have many other glycosyltransferases whose activities seem normal because the only primary glycosylation phenotype identified to date in these cells is the deficiency of the C1β3Gal-T activity (13). The results strongly suggest that Cosmc is a specific chaperone for C1β3Gal-T, although future studies are required to determine whether Cosmc associates with proteins other than C1β3Gal-T. Northern blot analysis of Cosmc expression in human tissues reveals that Cosmc expression mirrors that observed for C1β3Gal-T (ref. 4 and data not shown), consistent with a potential specific role of Cosmc in regulating C1β3Gal-T expression. Several such protein-specific chaperones have been identified for other ligands, including HSP47, a collagen-specific molecular chaperone (22), calmegin, which is specific for alpha/beta fertilin (23), the copper chaperone for superoxide dismutase (CCS-1) (24), and many others (25), although Cosmc is a molecular chaperone known to be required for a specific glycosyltransferase. Some molecular chaperones, such as HSP40, HSP60, and HSP90, interact with proteins to stabilize intrinsically unstable folding intermediates (26, 27). The precise mechanism of function of Cosmc awaits further study. It is interesting that the mouse gene (GenBank accession no. NP_067525), which we believe encodes the ortholog of human *Cosmc*, was one of several genes studied by screening a cDNA library for genes causing the growth suppression of *Escherichia coli* (28). The authors of that study found that the protein encoded by NP_067525 had ATP binding activity, consistent with a possible ATP-dependent chaperone function for the human Cosmc. The protein encoded by mouse *Cosmc* is predicted to contain 316 aa, two residues shorter than the human ortholog, with 91.5% identity between the human and mouse *Cosmc*.

It is significant that *Cosmc* is located on the X chromosome, which may be relevant to understanding some human diseases that are associated with or result from acquired or inherited deficiencies in C1β3Gal-T activity. For example, the Tn and sialyl Tn antigens are common markers in many types of tumors (7, 8), indicating that alterations in expression of Cosmc and consequently the C1β3Gal-T may be contributing factors for altered glycosylation by tumor cells. A loss of activity of C1β3Gal-T caused by a mutation in Cosmc could result in increases in both Tn and sialyl Tn antigens. IgA nephropathy (Berger's disease) is characterized by a decreased galactose content of O-glycans of IgA1 (29–31), which has five potential O-glycosylation sites in the hinge region. It has been proposed that the galactose deficiency in IgA1 of these patients results from a deficiency in C1β3Gal-T activity (32). Interestingly, IgA nephropathy exhibits a 2:1 male predominance (33), suggesting a possible X-linkage. Our results suggest that mutations in *Cosmc*, either genetically inherited or occurring randomly in a precursor stem cell, might be associated with decreased C1β3Gal-T activity in select B-cell populations responsible for IgA1 production. Whether defects in Cosmc expression or in the direct expression of the C1β3Gal-T are involved in other diseases associated with decreased C1β3Gal-T activity and expression of Tn antigen, such as Tn syndrome and Henoch-Schönlein purpura, should also be investigated.

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