A Soluble Form of α1,3-Galactosyltransferase Functions Within Cells to Galactosylate Glycoproteins*

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It has been assumed that membrane-bound glycosyltransferases function within the Golgi apparatus to glycosylate glycoproteins. We now report, however, that a truncated, soluble recombinant form of the murine α1,3-galactosyltransferase expressed in human 293 cells is highly efficient and comparable to the full-length enzyme in α-galactosylating both newly synthesized membrane-associated and secreted glycoproteins. Although the soluble enzyme was secreted by cells as expected, we also found that the full-length, membrane-associated form was secreted. Unexpectedly, both secreted forms are cleaved identically at two primary sites within the stem region by endogenous protease(s) at the indicated positions in the sequence 7KDWW↓FPS↓WFKNH. These results demonstrate that the soluble α1,3-galactosyltransferase is functional within the cell and that specific proteolysis occurs in the stem region. The widespread occurrence of different soluble glycosyltransferases secreted by cells suggests that normal glycoconjugate biosynthesis may involve cooperation between membrane-bound and soluble enzymes.

The synthesis of the carbohydrate groups in secreted and membrane-bound glycoconjugates occurs in the endoplasmic reticulum and Golgi apparatus and involves glycosyltransferases, which utilize sugar nucleotides imported into the lumen of these organelles by specific import proteins (1–4). Most of the cloned mammalian glycosyltransferases share many features in common, although their primary structures are often unrelated. The deduced amino acid sequences of the cDNA clones encoding many glycosyltransferases reveal that they are type 2 transmembrane proteins containing a short N-terminal cytoplasmic tail consisting of 4–24 amino acids, a membrane-spanning domain, and an extended stem region in the lumen that precedes the large C-terminal catalytic domain (4, 5). The transmembrane domain and flanking sequences of many glycosyltransferases may be important in retaining/targeting the enzymes in the Golgi apparatus (6–17). However, the exact mechanisms and the precise purpose of the targeting have not been elucidated. Because of their transmembrane nature, it has been presumed that Golgi glycosyltransferases function in the membrane-anchored form.

Numerous glycosyltransferases have been detected, however, as soluble forms in serum, milk, colostrum, and/or growth media from normal and transformed cell lines (18–23). The mechanism(s) of formation and secretion of soluble and active glycosyltransferases are not understood. N-terminal sequence analyses of soluble forms of the α2,6-sialyltransferase and β1,4-galactosyltransferase demonstrate that they arise through proteolysis within the stem region, thereby releasing the catalytic domain from the membrane (24, 25). The proteases responsible for cleaving the enzymes are not defined, although evidence suggests that a cathepsin D-like protease may be responsible for the cleavage of α2,6-sialyltransferase (26). The functions of the soluble glycosyltransferases are not known.

One enzyme that has recently been shown to be secreted from cultured cells is the murine α1,3-galactosyltransferase (α1,3GT)† (27, 28), which is responsible for the synthesis of terminal α-Gal sequences in Galα1→3Galβ1→4GlcNAc-R. The murine (29), bovine (30), porcine (31, 32), and New World monkey (33) cDNA encoding for α1,3GT have been identified. The α1,3GT is expressed in a variety of mammalian species, but it is not expressed in Old World monkeys, apes, and humans (34, 35). The expression of the α1,3GT in murine F9 teratocarcinoma cells is transcriptionally up-regulated by retinoic acid induction, but most of the newly synthesized enzyme is eventually secreted into the culture media of the cells (27). The α1,3GT is secreted by many cell types, since we have detected soluble forms of the α1,3GT in the culture media of all cell lines expressing the enzyme, and we have found enzyme activity in the sera of different animals expressing the functional α1,3GT gene (28).

The common occurrence of the α1,3GT in a soluble form led us to question whether it may have a function. One possibility is that the soluble enzyme may have a biosynthetic role within the Golgi apparatus. In this report we describe experiments showing that a truncated, soluble recombinant form of α1,3GT expressed in human 293 cells is highly efficient in α-galactosylating both newly synthesized membrane-associated and secreted glycoproteins and that it is comparable to the wild-type enzyme in its efficiency. Interestingly, both the truncated and full-length enzymes are secreted from 293 cells and both are cleaved by endogenous protease(s) at two primary sites in the stem region. These results demonstrate that a soluble glycosyltransferase can function within cells and suggest that normal proteolysis of membrane-bound glycosyltransferases may be important in generating enzymes that participate in glycoconjugate biosynthesis.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, UDP-Gal, raffinose, EDTA, N-acetyllactosamine, ATP, α-D-galactose-1-lactone, Tween 20, phenyl-

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1 The abbreviations used are: α1,3GT, UDP-Gal:α1,3-Gal β1,4-galactosyltransferase; HA, influenza hemagglutinin; TBS, Tris-buffered saline; GS-1-B4, Griffonia simplicifolia I-B4 isolecitin; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
methylsulfonyl fluoride, pepstatin, aprotinin, leupeptin, UDP-hexa-
olamine-Sepharose (4 μmol/ml resin), hors eradish peroxidase-conju-
gated Grifﬁoniam simplicifolia isoleucin 1-B (GS-1-B), and ﬂuorescein
isothiocyanate-conjugated GS-1-B (FITC-GS-1-B) were obtained
from Sigma. Mouse Engelbreth-Holm-Swann laminin was obtained
from Upstate Biotechnology Inc. (Lake Placid, NY). Restriction endo-
rase were purchased from Boehringer Mannheim. UDP-β-D-galactose
(15 Ci/mmol) was obtained from American Radiolabeled Chemicals
Inc. (St. Louis, MO). The BCA protein assay kit was purchased from
Pierce. Triton X-100 was obtained from Bio-Rad. The ECL Western blotting
kit was purchased from Amersham Corp. Tissue culture reagents
were obtained from Life Technologies, Inc. All other chemicals used were of
the highest grade available.

Cell Culture—Mouse teratocarcinoma F9 cells were cultured in Dul-
becco’s modiﬁed Eagle’s medium containing 15% fetal calf serum on
gelatin-coated tissue culture plates as described (36). For induction of
differentiation, cells were grown under identical conditions in media
supplemented with 10-7 m all-trans-retinoic acid for 5 days. Human 293
kidney cells (ATCC CRL 1573) were cultured in Dulbecco’s modiﬁed
Eagle’s medium containing 10% fetal calf serum. CHO-Tag cells, ex-
pressing the large T antigen, were cultured in α-minimal essential
media containing 10% fetal calf serum, ribonucleosides, and G418 (400
μg/ml). CHO-Tag cells were a generous gift of Dr. John B. Lowe
(University of Michigan, Ann Arbor, MI).

Construction and Transfection of Influenza Hemagglutinin Epitope-
Tagged Full-length α1,3GT Vector—A C-terminal fusion of the murine
α1,3GT to a 9-amin o acid peptide tag from influenza hemagglutinin
(HA) was generated by PCR using the 5′-primer 5′-GGGGGACACT-
CTGTGCCCCACATCCG-3′ and the 3′-primer 5′-ATGGTCTTAGAT-
AGCGTGATCGGTGCTATGAGACCATTCTTCTACAAATTAC-3′ as described.2 Briefly, the plasmid pCDM7-αGT, which contains
the cDNA of the murine α1,3GT (29), was used as the template for
the PCR reaction. The resulting PCR fragment (490 base pairs),
containing a SalI restriction site at the 5′-end and an XbaI restriction
site at the 3′-end, was subcloned into the vector PCR II and sequenced.
Wild-type α1,3GT cDNA in pcDNA1/Amp (Invitrogen, San Diego, CA)
was excised with SalI and XbaI, and the excised fragment was replaced
with the 490-base pair SalI/XbaI PCR fragment encoding the epitope
9. The 9-amin o acid HA epitope tag is speciﬁcally recognized by
the mouse monoclonal antibody 12CA5 (37).

This full-length construct, designated α1,3GT-HA, was co-trans-
ferred into 293 cells, along with the G418 selection plasmid PBKBF
(a gift from Dr. Kinji Fukudome, Oklahoma Medical Research Founda-
tion, Oklahoma City, OK), in a 4:1 molar ratio, using lipofectamine
reagents (Life Technologies, Inc.) according to the procedure recom-
mended by the supplier. Clonal cell lines were derived from the G418
(400 μg/ml)-resistant transfectant population using cloning cylinders.
Flow cytometric analyses were performed (27), and a representative
clonal line that stably expresses cell surface α-galactosylated glycocon-
jugates was selected and designated fl-α1,3GT-1.

Construction and Transfection of HA Epitope-Tagged Full-length
α1,3GT Vector Containing a Mutated Stem Region—A mutated form of
the full-length α1,3GT was generated in which the stem sequence was
mutated to contain Asp residues at three potentially critical sites for
specificity column according to the procedure recommended by the supplier.

The rabbit α1,3GT antiserum was raised by injecting an animal three
times with 1,3-Galactosylglycolipid with the murine α1,3GT used as the
template for the PCR reaction. The resulting PCR fragment was
ampliﬁed by using the 5′-primer 5′-ACCGGATCCCAACAGAATTCCA-
GAT-3′ and the 3′-primer 5′-AGCCTGCAGTTA-
9-AG-3′ primer of the enzyme was constructed in the mam-
mary expression vector RSV-PL4 (38). Speciﬁcally, the N-terminal cytoplasmic
and the transmembrane domain of the NKR-P1 were removed, and the
extracellular domain of the NKR-P1 was ampliﬁed by PCR using the 5′
and 3′ PstI restriction sites at the 5′-end and a
3′-end was
ligated into the vector RSV-PL4, resulting in a fusion protein of the soluble
NKR-P1 in-frame to the cDNA encoding the murine α1,3GT used as the template
for the PCR reaction. The PCR product was phosphoryl-
digested with XbaI and then ligated into the StuI/XbaI site of RSV-PL4
vector, resulting in a fusion protein of the soluble α1,3GT in-frame to the
transferrin signal sequence present in the vector. This construct was
transfected into human 293 cells using lipofectamine reagent (Life
Technologies, Inc.), and clonal selection was carried out in the presence
of G418 (400 μg/ml) as described above. Enzyme assays were performed
(27), and representative clonal lines that stably secreted enzyme into
media were selected and designated s-α1,3GT-1, s-α1,3GT-2, and s-α1,3GT-3.

The HPC4-NKR-P1 was constructed in the mammalian expression
vector RSV-PL4 (38). Speciﬁcally, both the N-terminal cytoplasmic
and the transmembrane domain of NKR-P1 were removed, and the
extracellular domain of the NKR-P1 was ampliﬁed by PCR using the 5′
and 3′ PstI/PstI restriction sites at the 5′-end and a
3′-end was
ligated into the vector RSV-PL4, resulting in a fusion protein of the soluble
NKR-P1 in-frame to the cDNA encoding the murine NKR-P1 used as the
template for the PCR reaction. A kind gift of Drs. Massimo Trucco and Roberto Giorda (Children’s Hos-
pital of Pittsburgh, Pittsburgh, PA). The PCR product was phosphoryl-
digested with XbaI and then ligated into the StuI/XbaI site of RSV-PL4
vector, resulting in a fusion protein of the soluble NKR-P1 in-frame to the
transferrin signal sequence present in the vector. This construct was
transfected into human 293 cells using lipofectamine reagent (Life
Technologies, Inc.), and clonal selection was carried out in the presence
of G418 (400 μg/ml) as described above. Western blot analysis
was performed using culture media of transfected cells, and representative
clonal lines that stably secreted NKR-P1 into media were selected.

Preparation of Cell Extracts, Microsomes, and Culture Media—Cell
extracts were prepared in the presence of the protease inhibitors phen-
ol-methylsulfonyl fluoride (1 μm), pepstatin (1 μg/ml), aprotinin (10
μg/ml), and leupeptin (10 μg/ml), and microsomes were prepared as
described previously (27, 39). To prepare cell culture media for enzyme
assays and immunoblot or lectin blot analyses, cells were grown in complete
media until they reached ~80% conﬂuency and were then subcultured in fresh
media with 0.25% trypsin at 37 °C for 1 h of culture and subjected to low speed centrifugation (300 × g for 10 min),
and the supernatant was centrifuged at high speed (100,000 × g for 1 h).
Media were concentrated in a Centricon-10 concentrator (Amicon Inc., Beverly, MA).

Antibodies—Monoclonal anti-HA (clone 16B12) ascites ﬂuid was pur-
chased from Berkeley Antibody Co. (Berkeley, CA) and was used at a dilution of 1:500. Monoclonal anti-α1,3GT antibody was a gift from
Dr. Charles Esmon (Oklahoma Medical Research Foundation) and was
used at a concentration of 10 μg/ml. To prepare rabbit α1,3GT anti-
serum against the murine α1,3GT, a fusion protein was constructed that
contains the putative luminal domain of the murine α1,3GT and a
6-histidine tag at the N terminus. Speciﬁcally, the luminal domain of
the α1,3GT comprising amino acid residues 61–326, was ampliﬁed by
PCR using the 5′-primer 5′-ACCGGATCCCAACAGAATTCCA-
GAT-3′ with a BamHI site and the 3′-primer 5′-AGCCTGCAGTTA-
9-GAG-3′ with a PstI site. The plasmid pCDM7-αGT, which contains
the cDNA of the murine α1,3GT (28), was used as the template for
the PCR reaction. The PCR product was digested with BamHI and PstI
and then ligated into the BamHI/PstI site of PQE-10 vector (QIAexpress, Qiagen), resulting in a 6-His-tagged fusion protein
of truncated α1,3GT. The resulting PCR fragment containing a BamHI
restriction site at the 5′-end and a PstI restriction site at the 3′-end was
cloned into the vector pBS II SK− and sequenced. This construct was
used to transform Escherichia coli strain M15 (pREP4), and the
expressed 6-His-α1,3GT was puriﬁed using a nitrotriacetic acid afﬁni-
ty column according to the procedure recommended by the supplier.
The rabbit α1,3GT antisera was raised by immunizing three
(10-day intervals) with the nickel afﬁnity-puriﬁed, bacterial
6-His-tagged fusion protein of the truncated α1,3GT. Sera were col-
lected and were used at a dilution of 1:500. The secondary antibody conju-
gates, horseradish peroxidase-conjugated goat anti-rabbit and horse-
radish peroxidase-conjugated sheep anti-mouse IgG antibody, were
used at dilutions of 1:500.
Purification and Microsequencing of the Secreted Form of α1,3GT—The catalytically active, secreted form of α1,3GT was purified from culture media of human 293 transfectants by affinity chromatography on a column of UDP-hexanolamine-Sepharose with some modification (40). Culture media from fl-1,3GT-1 and s-α1,3GT-2 cells were harvested when the cells were ~90% confluent and then dialyzed overnight at 4 °C against 20 mM HEPES, 5 mM MgCl₂, pH 7.0 (buffer A) with several changes of buffer. The dialyzed material was applied to a column containing UDP-hexanolamine-Sepharose (4 μmol/ml resin) and washed with buffer A. This was followed by additional washing with buffer A containing 0.75 mM NaCl until the absorbance of each fraction at 280 nm reached background levels. The bound enzyme was eluted with 5 mM UDP in buffer A, and enzyme was concentrated in Centricon-10. The purified enzyme was resolved on 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond-ECL Western, Amersham Corp.) and followed by lectin blot analysis. The standard ECL immunoblotting procedures were as follows. Blots were blocked with TBS (20 mM Tris, 150 mM NaCl, pH 7.5) containing 5% nonfat dry milk at room temperature for 1 h, and then analyzed by fluorescence-activated cell sorting as described previously (27).

Immunoblotting—The α1,3GT was purified by UDP-hexanolamine affinity chromatography from s-α1,3GT-2 culture media as described above. NKR-P1 was purified by immunofluorescence chromotography on immobilized anti-HPC4. The purified proteins were resolved in 10% SDS-PAGE. The separated proteins were electrophoretically transferred onto nitrocellulose membranes and then stained by standard ECL immunoblotting analysis. The standard ECL immunoblotting procedures are as follows. Blots were blocked with TBS (20 mM Tris, 150 mM NaCl, pH 7.5) containing 5% nonfat dry milk at room temperature for 1 h, and then analyzed by fluorescence-activated cell sorting as described previously (27).

Lectin Blotting—For the detection of a-galactosylated glycoproteins, 30 μg of the microsomal fraction and 100 μg of soluble proteins in culture media were prepared as described previously (27) and subjected to electrophoresis on 7.5% SDS-PAGE. The separated proteins were electrophoretically transferred onto nitrocellulose membranes (Hybond-ECL Western, Amersham Corp.) and followed by lectin blot analysis. The ECL lectin blotting was performed as described previously (27) except that the a-galactosylated glycoproteins were detected using 10 μg/ml horseradish peroxidase-conjugated secondary antibodies in dilution buffer. Blots were washed three times (15 min each) in TTBS followed by one wash in TBS and developed using the ECL Western blotting kit according to the manufacturer's instructions. For the detection of α1,3GT derived from fl-α1,3GT-1 cells, the microsomes and soluble protein in the culture media were resolved in 10% SDS-PAGE and analyzed by standard ECL immunoblotting using monoclonal anti-HA antibody.

Expression of Surface Gal1 → 3Gal Determinants by Both Soluble and Full-length Constructs of α1,3GT—Cell surface expression of terminal Gal1 → 3Gal determinants requires expression of the cognate α1,3GT (29). The stably expressing cell lines were tested for their ability to display cell surface-localized oligosaccharide product of enzyme using flow cytometry and FITC-GS-I-B₄. GS-I-B₄ is a plant lectin that specifically binds terminal Gal1 → 3Gal linkages (43). As expected, the parental 293 cell line does not display surface Gal1 → 3Gal determinants (Fig. 2). In contrast, 293 cells transfected with either α1,3GT-HA or HPC4-αGT stained brightly with FITC-GS-I-B₄ (Fig. 2). The levels of expression of surface Gal1 → 3Gal determinants in the stably expressing clones were comparable to the normal level observed in retinoic acid-differentiated F9 cells (Fig. 2). These observations demonstrate that the soluble α1,3GT can participate in biosynthesis of surface-localized glycoconjugates containing the Gal1 → 3Gal determinant. Based on these flow cytometric analyses and the level of staining with GS-I-B₄, the soluble α1,3GT appears to be comparable to the full-length enzyme in generating surface Gal1 → 3Gal determinants.

**RESULTS**

Construction of Full-length and Soluble Chimeras of Marine α1,3GT—To test the functionality of the soluble form of murine α1,3GT and to compare it to the full-length enzyme, fusion proteins were constructed as described under “Experimental Procedures” and illustrated in Fig. 1. A full-length chimeric α1,3GT was constructed in which the 9-amino acid influenza virus hemagglutinin (HA) epitope tag (37) was fused to the C-terminal end of full-length α1,3GT. This chimera is shown in Fig. 1A and is designated the α1,3GT-HA construct. A soluble form of the α1,3GT was constructed in which the fusion protein consists of the transferase signal peptide preceding an HPC4 epitope (38) and followed by amino acids 63–394 of the murine α1,3GT encoding the C-terminal catalytic domain. This chimera is shown in Fig. 1B and is designated the HPC4-αGT construct.

Human kidney 293 cells were transfected with either the α1,3GT-HA or HPC4-αGT, and stably expressing 293 cell lines were isolated as described under “Experimental Procedures.” One clone stably expressing α1,3GT-HA was designated fl-α1,3GT-1. Three clones stably expressing the HPC4-αGT were identified, and these were designated s-α1,3GT-1, s-α1,3GT-2, and s-α1,3GT-3.

**FIG. 1. Construction of full-length HA-tagged α1,3GT (α1,3GT-HA), HPC4-tagged, soluble α1,3GT (HPC4-αGT), and mutant full-length HA-tagged α1,3GT (α1-1,3GT-HA).** A, the 9-amino acid epitope tag from HA was generated by PCR and fused to the full-length α1,3GT cDNA in the pcDNAI/Amp vector. TM indicates the putative transmembrane segment. The amino acid sequence across the transmembrane junction is indicated, and two primary cleavage sites giving rise to soluble forms of α1,3GT are indicated by arrows. B, the vector HPC4-αGT consists of a DNA fragment coding for the transferase signal peptide followed by the HPC4 epitope and a factor Xa cleavage site (hatched area). After PCR amplification of the DNA sequences corresponding to amino acids 63–394 of the murine α1,3GT by appropriate primers, it was digested with SmaI and XbaI and ligated into the StuI and XbaI sites of RSV-PL4 for expression in 293 cells. C, three amino acid residues in the stem region of α1,3GT-HA were converted to Asp by PCR. The detailed descriptions of these constructs are presented in “Experimental Procedures.” Arrows indicate positions of proteolysis in the native construct.
were probed with peroxidase-conjugated GS-I-B4. As expected, the glycoproteins from parental 293 cell microsomes and serum-free media were not reactive with GS-I-B4 (Fig. 3, A and B). In contrast, significant amounts of GS-I-B4-reactive membrane-associated glycoprotein was present in microsomes of fl-α1,3GT-1, s-α1,3GT-1, and s-α1,3GT-2 cells, although the levels of expression appear somewhat higher in fl-α1,3GT-1 microsomes. However, no appreciable differences were observed in the overall patterns of α-galactosylated membrane-associated glycoproteins in microsomes between the clones expressing either full-length or truncated enzyme (Fig. 3A). In addition, both the levels and the pattern of GS-I-B4-reactive material in the serum-free media of all the clones were similar (Fig. 3B). In control experiments, GS-I-B4 bound well, as expected, to a commercial preparation of Engelbreth-Holm-Swarm laminin, which is known to contain terminal α1,3-galactosyl residues (44, 45). These results demonstrate that both the full-length and soluble α1,3GT cause the biosynthesis of high levels of α-galactosylated glycoproteins.

Enzyme Activities in Cell Extracts and Media from Cells Expressing Soluble and Full-length α1,3GT—To determine whether there are significant differences in the level of expression of the soluble versus the full-length α1,3GT and whether there are differences in secretion of the two forms, we measured the enzyme activity in cell extracts and culture media using N-acetyllactosamine as the acceptor and UDP-[3H]Gal as the donor. The results are shown in Table I. No activity was detected in extracts of the parental 293 cells. Significant α1,3GT activity was present in extracts of fl-α1,3GT-1 cells (49.0 nmol/h), but approximately two-thirds of the total activity, which represents activity in the cell extracts plus culture media, was recovered in the culture media (Table I). The activities of the α1,3GT in extracts of s-α1,3GT-1, s-α1,3GT-2, and s-α1,3GT-3 cells were slightly lower than that of fl-α1,3GT-1 cell extracts (Table I). As might be expected, the levels of activity of α1,3GT in media of s-α1,3GT-1, s-α1,3GT-2, and s-α1,3GT-3 cells were slightly higher than that in the media from fl-α1,3GT-1 cells.

Secreted α1,3GT Does Not Act Extracellularly to Add Galα1→3Gal Determinants to Cells—We considered the possibility

![Log Fluorescence Intensity](image)

**Fig. 2. Cell surface expression of the α-galactosylated glycoconjugates.** Cells were stained with GS-I-B4 directly conjugated with FITC (10 μg/ml) and subsequently analyzed by cytometry. The fluorescence histogram (shaded histogram) is compared with the fluorescence histogram of unstained cells (open histogram). The analyzed cell lines are indicated in each histogram: parental 293, s-α1,3GT-1 (293 transfected with soluble, truncated α1,3GT, low expresser), s-α1,3GT-2 (293 transfected with soluble, truncated α1,3GT, high expresser), fl-α1,3GT-1 (293 transfected with full-length α1,3GT), and retinoic acid-differentiated F9 cells (RA/F9).

![Fig. 3. GS-I-B4 blotting of glycoproteins from 293 cells transfected with either full-length α1,3GT or soluble, truncated α1,3GT.](image)
that the secreted α1,3GT might transfer α-galactosyl residues to surface glycoproteins after it was secreted from the cells, rather than from within, possibly using secreted UDP-Gal as the donor. Parental 293 cells were grown to ~80% confluency, and the media were replaced by freshly harvested culture media of s-α1,3GT-2 cells. The enzyme activity in the transfected media was 0.3 nmol/12.5 µl/h. Control parental 293 cells received media from parental 293 cells not expressing the α1,3GT. After 5 h of incubation with media either containing or lacking α1,3GT activity, the cells were harvested and tested for surface Galα1→3Gal determinants using flow cytometry and FITC-GS-I-B4. There was no expression of surface Galα1→3Gal determinants in cells exposed to the α1,3GT-containing media, and there were no detectable differences between control parental 293 cells, which had never been exposed to α1,3GT-containing media, and the treated cells (data not shown). These observations demonstrate that surface Galα1→3Gal determinants in cells expressing the α1,3GT arise from enzyme activity within the cells rather than from addition of the determinants by the secreted enzyme outside the cells.

Proteolysis of Full-length and Soluble Forms of α1,3GT and Recovery of Enzyme in Culture Media—To confirm that the full-length enzyme was released in a soluble form into the media, the α1,3GT in fl-α1,3GT-1 cells was analyzed by Western blot analysis using the monoclonal anti-HA antibody. The α1,3GT in microsomal preparations migrates as a diffuse band centered at ~53 kDa in reducing SDS-PAGE, whereas the apparent size of the α1,3GT from medium is ~50.5 kDa (Fig. 4A). These data demonstrate that the full-length α1,3GT is expressed by the cells and secreted as a lower molecular mass soluble form and that both forms retain the HA epitope at their C termini.

We also attempted to identify the soluble form of the α1,3GT expressed from s-α1,3GT-2 cells using Western blot analysis and anti-HPC4 monoclonal antibody. Unexpectedly, the soluble, secreted form of the enzyme from s-α1,3GT-2 cells did not react with the anti-HPC4 monoclonal antibody (Fig. 4B). The recombinant protein was present, as shown by its reactivity in a Western blot with rabbit polyclonal antibody prepared against bacterially derived murine α1,3GT. The secreted enzyme had an apparent Mr ~42,000. These results indicate that the recombinant α1,3GT had lost the HPC4 epitope during secretion of the enzyme.

We considered the possibility that the HPC4 determinant was cleaved from the protein by a protease(s) that might recognize in some way the foreign HPC4 determinant on the recombinant protein. To address this possibility, another HPC4-tagged recombinant glycoprotein was prepared and expressed in 293 cells. In this experiment 293 cells were stably transfected with cDNA encoding a chimeric form of the mouse NKR-P1 in which the HPC4 epitope was appended to the N terminus in a construct designated HPC4-NKR-P1, prepared as described under “Experimental Procedures.” NKR-P1 is a dimeric surface protein with ~30-kDa subunits expressed on murine natural killer cells (46). It is a type 2 transmembrane protein, and in this regard it resembles glycosyltransferases in its proposed topological orientation within the membrane. Media from cells expressing HPC4-NKR-P1 were reactive with anti-HPC4 monoclonal antibody (Fig. 4B). These results demonstrate that the HPC4 epitope is retained on this recombinant protein. Consistent with this finding that the HPC4 determinant is cleaved from the α1,3GT constructs but not from the HPC4-NKR-P1, we also observed that the recombinant α1,3GT in the culture media of s-α1,3GT-2 cells did not bind to immobilized anti-HPC4 in affinity chromatography, whereas the HPC4-NKR-P1 was recovered upon immunoaffinity chromatography (data not shown). These results demonstrate that the HPC4 epitope is selectively cleaved from the N terminus of the recombinant α1,3GT derived from HPC4-aGT. Direct peptide sequencing results described below support this conclusion.

To determine the generality of secretion of the α1,3GT from cells, we also transiently transfected CHO-Tag cells, which express the large T antigen, with the full-length α1,3GT-HA construct. The media harvested 48 h after transfection were assayed for α1,3GT enzyme activity, and protein in the media was used for a Western blot with the monoclonal anti-HA antibody. The media contained 45 nmol/h of total activity and the apparent molecular mass of secreted α1,3GT from CHO-Tag cells was ~50.5 kDa in reducing SDS-PAGE (data not shown), which is similar to that of secreted α1,3GT from fl-α1,3GT-1 cells. These observations confirm that secretion of α1,3GT from cells is a common phenomenon, which is consistent with recent studies showing that the enzyme is secreted from a large number of cell types (28).

Both the Full-length α1,3GT and the Recombinant, Soluble Form of the α1,3GT Are Cleaved in the Stem Region—To identify the N terminus of the α1,3GT secreted by fl-α1,3GT-1 cells, the enzyme was purified from cell culture media by affinity chromatography on UDP-hexanolamine. The purified enzyme was resolved on 10% SDS-PAGE and subjected to N-terminal sequencing as described under “Experimental Procedures.” Two soluble species were identified, with the predominant species being 3 amino acid residues longer than the minor species. The N termini of the two species reveal that soluble forms of α1,3GT are derived from the full-length form by proteolytic cleavage C-terminal to Trp<sup>76</sup> and C-terminal to Ser<sup>79</sup> in the putative stem region of the enzyme, as shown in Fig. 1A.

We then determined the N terminus of the secreted α1,3GT derived from s-α1,3GT-2 cells, which had lost the N-terminal HPC4 epitope as described above. The enzyme was purified from the culture media of the cells by affinity chromatography on UDP-hexanolamine and subjected to SDS-PAGE, on which it had an apparent size of ~42 kDa (Fig. 4B). The gel band containing the enzyme was subjected to N-terminal sequencing as described under “Experimental Procedures.” Again, two N-terminal sequences were identified, and these were identical to those observed in the soluble forms recovered in the culture media from fl-α1,3GT-1 cells (Fig. 1A). These results indicate that for both the soluble and full-length forms of the α1,3GT specific proteolysis occurs within the dipeptide sequence Trp<sup>76</sup>Phε<sup>77</sup> and Ser<sup>79</sup>Phε<sup>80</sup> (Fig. 1A).

These data indicate that proteolysis occurs within a discrete region of the stem of the α1,3GT. However, it is possible that other cleavage sites also exist. For example, other cleavage sites could be present N-terminal to the defined sites, with multiple proteolytic cleavages generating the two major final forms identified above. To address this possibility, we prepared a mutated form of the full-length α1,3GT in which the stem sequence was mutated to contain Asp residues at three potent-
tially critical sites for cleavage, as shown in Fig. 1C. These charged residues would presumably prohibit recognition by proteases that potentially require hydrophobic residues for cleavage. The plasmid encoding this mutant, termed st-1-α1,3GT-HA, was stably transfected into 293 cells. Media and microsomes from these cells were analyzed by Western blot using the monoclonal anti-HA antibody. Unexpectedly, the st-1-α1,3GT-HA was cleaved as efficiently as the wild-type protein, and the soluble, mutated enzyme was recovered in the culture media (data not shown). The results were comparable to those shown in Fig. 4A. The N terminus of the soluble, mutated enzyme is not yet defined. These data indicate either that proteolysis of the stem region can occur in other sites in addition to those identified above or that the mutated stem region in st-1-α1,3GT-HA may represent a new recognition site for a different protease(s).

**DISCUSSION**

These studies demonstrate that a soluble form of the α1,3GT, which is similar to that formed naturally through proteolysis of the membrane-associated full-length enzyme, is functional within the Golgi apparatus and is capable of generating quantitatively levels of Ga1→3Gal determinants in cell surface and secreted glycoproteins. These observations suggest the hypothesis that soluble glycosyltransferases, generated by specific proteolysis of the membrane-associated enzymes within the secretory apparatus, can participate in the biosynthesis of complex glycoconjugates.

Numerous studies have documented the existence of soluble glycosyltransferases in body fluids (18, 19, 23, 47–53) and in the growth media from normal and transformed cell lines (21, 22), although the functional significance of such soluble enzymes has not been explored. In addition, the mechanisms regulating formation of soluble glycosyltransferases have not been clearly defined. It has been shown that soluble forms of the rat liver α2,6-sialyltransferase and the bovine β1,4-galactosyltransferase are derived by proteolysis within the stem domain (24, 25). Cleavage of the membrane-associated α2,6-sialyltransferase may involve a cathepsin D-like protease (26) that recognizes peptides containing hydrophobic residues (54). Such an enzyme could participate in cleavage of the α1,3GT, since cleavages occurred adjacent to aromatic residues. However, cleavage also occurred in the mutant st-1-α1,3GT-HA, which contained mutations in three residues potentially recognized by cathepsin D-like proteases (Fig. 1C). These data indicate either that other cleavage sites exist in the stem region, in addition to those we have defined, or that the mutations in st-1-α1,3GT-HA created recognition sites for other proteases.

Many more experiments along these lines will be required to determine the mechanisms of cleavage of the stem region of the α1,3GT and the proteases responsible.

It has been reported that the α2,6-sialyltransferase is not efficiently secreted from transfected CHO cells (55), implying that CHO cells lack a protease capable of recognizing the stem region of the α2,6-sialyltransferase. As we found, CHO cells are capable of efficiently cleaving the stem domain of the murine α1,3GT and secreting the enzyme. Thus, proteolytic cleavages of glycosyltransferases in heterologous cells may vary. It has also been found that the human GDP-L-fucose:β-D-galactoside 2→α1-fucosyltransferase is not efficiently secreted from COS-1 cells transfected with the cloned full-length GDP-L-fucose:β-D-galactoside 2→α1-fucosyltransferase cDNA (56), although GDP-L-fucose:β-D-galactoside 2→α1-fucosyltransferase is found in abundant levels in human serum (57–59). It is likely that secretion of a glycosyltransferase from cells is determined by a combination of factors including the nature of the stem region and cognate endogenous proteases in cells expressing the enzyme.

Numerous studies have documented the importance of the transmembrane domains and flanking sequences of glycosyltransferases for enzyme retention in the Golgi apparatus (6–17). However, in some cases the transmembrane domain may not be sufficient or even necessary for Golgi targeting/retention of enzymes, and other domains of the enzymes may also be important. A soluble form of mannosidase II can interact with the membrane-associated form of N-acetylgalactosaminyltransferase II in a transmembrane-independent retention mechanism termed “kin recognition” (60). In this case, a membrane-associated enzyme might act as a docking protein for a soluble enzyme. In addition, a soluble form of the α2,6-sialyltransferase containing only the stem region and the catalytic domain is slowly secreted from COS-1 cells, further implying that the stem regions and/or the catalytic domains of that enzyme may contribute to Golgi retention (13). We observed that a significant amount of the soluble α1,3GT was retained in the cells and that it appeared to be slowly secreted. Whether a kin recognition-type mechanism operates for the α1,3GT and whether the enzyme contains luminal domains important for Golgi retention are not known, but experiments are in progress to test these interesting possibilities.

Since these results indicate that a soluble glycosyltransferase can be functional within the Golgi apparatus, what is the importance of the full-length form? It may be that the soluble and full-length forms of the enzymes have somewhat different but overlapping functions. The full-length forms of...
glycosyltransferases may be important in glycosylating membrane-associated acceptors such as glycosphingolipids and membrane-bound glycoproteins. We observed that the soluble α1,3GT as well as the full-length derived enzyme in glycosylating secreted proteins from 293 cells. However, the soluble enzyme appeared to be less efficient than the full-length derived enzyme in its action on endogenous membrane-associated glycoproteins (Fig. 3A). Thus, a cooperative mechanism may exist in the Golgi whereby both the membrane-bound and soluble enzymes function to fully glycosylate the repertoire of available acceptors.

The apparent molecular mass of secreted α1,3GT from α1,3GT-2 cells was ~42 kDa, which is consistent with the expected size of the cleaved polypeptide (37.5 kDa) plus two N-glycans of ~2.0 kDa each (Fig. 4B). However, the secreted α1,3GT from fl-α1,3GT-1 had an apparent size of ~51 kDa (Fig. 4A). The expected molecular mass of the soluble enzyme, including the HA epitope, generated by proteolysis of the full-length enzyme in fl-α1,3GT-1 is 38.6 kDa.) Assuming that the protein contains two N-glycans with predicted sizes of ~2.0 kDa each, the size would be expected to be 42.6 kDa, which is ~8 kDa lower than the observed size. The larger size of the secreted enzyme derived from the full-length form may be due to some type of unusual post-translational modification that preferentially occurs on the full-length, membrane-associated enzyme. Such a molecular difference was also observed in β1,4-galactosyltransferase purified from human milk (61). It has been reported that a truncated soluble form of β1,4-galactosyltransferase carried additional post-translational modifications compared with the Golgi-localized membrane-bound full-length β1,4-galactosyltransferase and that these modifications were added just prior to enzyme secretion (6). Therefore, additional post-translational modifications may occur in the full-length construct of α1,3GT as it goes through the biosynthetic secretory pathway, and these may be different from those that occur in the recombinant, soluble form of the enzyme. We are currently addressing this issue to determine the exact molecular mass of the secreted α1,3GT from fl-α1,3GT-1 and the post-translational modifications of the enzyme.

In summary, these studies have demonstrated the ability of a soluble enzyme to efficiently glycosylate glycoproteins during their biosynthesis within cells. Whether other glycosyltransferases also function as soluble forms within cells will need to be tested in the future. In addition, it will be interesting to determine whether the prevention of cleavage of the α1,3GT, either by further mutagenesis of the requisite sequence in the stem region or by inhibition of the protease(s) responsible for cleavage, has an effect on the efficiency or specificity of α-galactosylation by the enzyme. Finally, it will also be important to define whether the membrane-bound and soluble enzymes differ in their recognition of acceptor glycans, recognition of glycolipids versus glycoproteins, and recognition of specific N-glycosylation sites in proteins.

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