The Transmembrane Domain of N-Glucosaminyltransferase I Contains a Golgi Retention Signal*

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The enzyme N-acetylglucosaminyltransferase I (NT, EC 2.4.1.101) is a resident type II transmembrane protein of the Golgi apparatus. To delineate the portion of its primary sequence that is responsible for the Golgi retention of this protein, we constructed chimeras containing different N-terminal portions of NT joined to a reporter sequence, the ectodomain of a type II surface membrane protein. These chimeric proteins were found to be retained in the Golgi apparatus as assessed by cell surface biotinylation and immunofluorescence. We found that the transmembrane domain of NT is sufficient to confer Golgi retention of the fusion proteins and propose that it contains the Golgi retention signal of the parent molecule.

Following their translocation into the endoplasmic reticulum (ER), proteins destined for the exocytic pathway are believed to move toward the plasma membrane by default unless retained by certain structural motifs (see Ref. 1 for review). Such retention signals have been identified for both the luminal (2) and the transmembrane (3) resident proteins of the ER. These retention signals have also been shown to be transplantable to reporter proteins, resulting in their retention in the ER (2,3).

The Golgi apparatus is the central organelle of the secretory pathway. It consists of an arranged stack of cisternae that facilitates sequential modification of carbohydrate moieties on various macromolecules in transit through the exocytic pathway (see Ref. 4 for review). The cis-Golgi (5) and the trans-Golgi (6) networks also play instrumental roles as sorting compartments for cellular traffic. Several Golgi specific proteins have been cloned. Most of these are glycosyltransferases with a type II orientation, i.e. with their short N terminus protruding into the cytoplasm and their large ectodomain in the lumen (for review, see Ref. 7).

The sorting or retention signal for the Golgi apparatus is not well established. Preliminary findings suggest that a common peptide stretch close to the hydrophobic domains of these proteins may be important for Golgi retention (8). Deletion of the first, but not the second and third, transmembrane domain of the Golgi-residing coronavirus El protein destroyed Golgi retention (9). More recently, it was shown that this first transmembrane domain of the avian coronavirus infectious bronchitis virus alone could retain two different plasma membrane proteins in the Golgi (10). However, studies performed with another coronavirus, the mouse hepatitis virus A59, gave contrasting results (11). It has also been proposed that the Golgi retention signal of a-2,6-sialyltransferase resides at the N terminus of the molecule (7).

In order to define the primary sequence of a Golgi protein that is responsible for its Golgi retention, different N-terminal portions of a medial Golgi protein N-acetylglucosaminyltransferase I (NT, EC 2.4.1.101) (12) were attached to the ectodomains of two type II membrane surface proteins, namely, dipeptidyl peptidase IV (D4) (13) and the transferrin receptor (TFR) (28). We found that the transmembrane domain of NT is sufficient to localize the fusion proteins to the Golgi apparatus and propose that it contains the Golgi localization signal of the molecule.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media, fetal bovine serum, diazoylated fetal bovine serum, and genetin (G418) were purchased from Gibco. [35S]methionine (>1000 Ci/mmol) was from Amersham Corp. Goat anti-mouse IgG and its fluorescein isothiocyanate-conjugated derivatives were from Calbiochem. Sulfo-NHS-biotin and streptavidin-agarose were from Pierce Chemical Co. Lectin-agaroses were from EY Laboratories. Transwells were from Costar. Monoclonal antibodies against rat dipeptidyl peptidase IV (D4) were generous gifts from Dr. D. L. Mendrick (Harvard Medical School). The Madin-Darby canine kidney (strain II) cell line was a generous gift from Dr. K. Simons (EMBL) and was maintained as previously described (13). The human cDNA clone of N-acetylglucosaminyltransferase I (NT) was a generous gift from Dr. P. Stanley (Albert Einstein College of Medicine). The human cDNA clone for transferrin receptor was obtained from the American Type Culture Collection, deposited by Dr. P. Ruddle (Yale University).

Construction of NT-D4 and NT-TFR Chimeras—Standard procedures for DNA manipulations, polymerase chain reaction, and transfection were followed (29). NT-D4 chimeras (Fig. 1) were constructed by ligating DNA fragments resulting from stepwise truncations of the N terminus of the NT cDNA to the ectodomain fragment of D4. In the case of the chimera NT*'-D4, the sequence of the 6-amino acid residue cytoplasmic domain of NT was replaced by that of D4. These fragments were generated by oligonucleotide-primed polymerase chain reactions and their sequences confirmed by DNA sequencing.

Both the NT-D4 and NT-TFR chimeras were inserted into the mammalian expression vector pRNS (13) and transfected by the calcium phosphate precipitation method into Madin-Darby canine kidney cells. Transfectants were selected and maintained in culture media containing 750 μg/ml of G418.

Indirect Immunofluorescence—This was done as previously described (13). Cells grown on coverslips were fixed with 2.7% paraformaldehyde and incubated with primary antibodies to D4 or TFR with (for total staining) or without (for cell surface staining) permea-
bilization with 0.1% saponin. The cells were subsequently incubated with rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate, mounted in 90% glycerol in phosphate-buffered saline containing 1 mg/ml p-phenylenediamine, and observed with the Axioskop microscope (Carl Zeiss).

**Metabolic Labeling of Cells and Immunoprecipitation**—As previously described (13), cells grown on tissue culture flasks were washed twice with Hank’s balanced salt solution and labeled for varying lengths of time with 1 μCi/ml [35S]methionine in methionine-deficient medium after a 45-min preincubation in the same medium. Cells grown on transwell filters were labeled with 1 μCi of the same labeling medium added to the basolateral (bottom) chamber after wash. Cells with Hank’s balanced salt solution containing 1 mM CaCl2 and 1 mM MgCl2. The cells were then chased for varying lengths of time in medium containing excess cold methionine (100 mg/liter). Labeled cells in flasks were then scraped off and washed in a hypotonic solution (10 mM Hepes, pH 7.5, 0.25 mM sucrose and 1 mM phenylmethylsulfonyl fluoride). Cell pellets or transwell filters were then extracted with lysis buffer (25 mM Tris-HCl, pH 7.8, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). The extract was preclarified with mouse serum-agarose (Sigma) and immunoprecipitated with protein-A-Sepharose beads precoated with rabbit anti-mouse IgG followed by the respective monoclonal antibodies to D4 and TFR. The immunoprecipitated proteins were eluted by boiling for 5 min in SDS sample buffer with or without β-mercaptoethanol for recovery of biontinylated proteins.

**Cell Surface Biotinylation**—This was carried out as described previously (13). Cells grown on transwell filters were pulsed and chased for varying time periods and incubated with 0.5 mg/ml sulfo-NHS-s-s-biotin in phosphate-buffered saline containing 1 mM CaCl2 and 1 mM MgCl2 for 15 min. The incubation was repeated with fresh biotin solution for another 15 min. The proteins were then immunoprecipitated and the biotinylated proteins recovered by binding to streptavidin-agarose after elution of the immunoprecipitate in SDS sample buffer. Lecin Affinity Chromatography—The use of lecin affinity chromatography has been described previously (20). Immunoprecipitated proteins were eluted and diluted in lectin buffer (40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2). An aliquot of this was incubated with the various lecin agarooses and eluted for SDS-PAGE by boiling in SDS sample buffer with β-mercaptoethanol.

### RESULTS

**Construction of NT-D4 and NT-TFR Chimeras**—Other results have hinted that the N terminus of type II glycosyltransferases contain the Golgi retention signal. To localize this signal within the NT molecule, varying lengths of the N-terminal sequences of NT were joined to the cytoplasmic domain of D4. NT5-D4 includes the cytoplasmic domain and the transmembrane domain of NT (Fig. 1). NT3-D4, NT29-D4, and NT50-D4 are constructs with a progressive 12-residue extension from the transmembrane domain of NT. To check if the transmembrane domain of NT itself is sufficient to confer Golgi retention, the 6 residues of the cytoplasmic domain of NT was replaced by the 6 residues of D4, thus creating the chimera NT5-29-D4. To confirm that the signal is functional in other molecular contexts, we also joined the cytoplasmic and transmembrane domain of NT to the cytoplasmic domain of the transferrin receptor, thus creating NT-TFR.

**The Transmembrane Domain of NT Contains a Golgi Retention Signal**—Pooled transfectants of D4 itself and the four constructs NT5-29-D4 to NT50-D4 were examined by immunofluorescence (Fig. 2). Unpermeabilized cells transfected with D4 (surface staining) showed a bright surface staining in accordance with the expression of the protein (13). Permeabilized D4 cells also stained brightly at cell borders with some perinuclear and peripheral staining. In contrast, very weak background staining was observed on the surfaces of all 4 of the NT-D4 chimeras. However, permeabilized counterparts of these cells showed intense perinuclear vesicular-tubular staining, which is characteristic of Golgi staining patterns (14–16). These results suggest that the cytoplasmic domain and the transmembrane domain of NT are sufficient to confer Golgi localization. To further narrow down the sequence of the Golgi retention signal, NT7-29-D4 was constructed, in which the transmembrane region of D4 was replaced by that of NT. Transfectants of this construct, too, showed strong perinuclear intracellular staining and weak background surface staining (Fig. 2e). The transmembrane domain of NT, therefore, contains a signal for Golgi retention and is by itself sufficient to retain a polypeptide in the Golgi.

**Total and Surface Expression of D4 and the Various Fusion Proteins**—To estimate the fractions of expressed D4 and the various fusion proteins that were located on the cell surface, cells grown on transwells were biotinylated from both surfaces and the proteins immunoprecipitated. One-tenth of the total immunoprecipitate was removed. The remaining immunoprecipitate was then incubated with streptavidin-agarose, and the captured biotinylated proteins were eluted by boiling in SDS sample buffer. These were loaded alongside their corresponding one-tenth of total immunoprecipitate on a 5% polyacrylamide gel (Fig. 3A). It can be seen that wild-type D4 had very high cell surface expression. In contrast, most of the NT chimeras had low or undetectable surface expression. It is interesting that both the constructs NT50-D4 and NT7-29-D4 had significantly higher surface expression than NT-D4. The 12-amino acid residue extension from the transmembrane domain therefore seems to enhance the retention efficacy of the fusion protein. Fig. 3B shows the respective percentages of surface and total expression when the surface expression of wild-type D4 was arbitrarily defined as 100%. These results confirm the data obtained by indirect immunofluorescence in that the transmembrane domain of NT is able to confine the chimeric fusion proteins within the cell and further revealed the importance of amino acid residues immediately adjacent to it in enhancing the retention efficacy.

**The Targeting Signal Functions in a Different System**—To see if the Golgi retention signal would function in a heterologous system, the first 29 amino acid residues of NT were joined to another reporter molecule, the ectodomain of the TFR. Cells transfected with the cytoplasmic tail-deleted TFR showed strong surface staining (Fig. 4). In contrast, cells transfected with NT-TFR showed weak background surface

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2 W. Hong, unpublished data.
Retention of a Golgi Membrane Protein by the Transmembrane Domain

Fig. 2. Subcellular localization of D4 and its fusion constructs with NT by immunofluorescence. Cells grown on coverslips were incubated with monoclonal antibody against D4 followed by rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate. Cells were either permeabilized with 0.1% saponin for total staining (B, D, F, H, J, and L) or unpermeabilized for surface staining (A, C, E, G, I, and K). The plates show surface and total stainings for D4 (A and B), NT29-D4 (C and D), NT19-D4 (E and F), NT35-D4 (G and H), NT45-D4 (I and J), and NT55-D4 (K and L).

Fig. 3. A, quantitation of total and surface expression of D4 and its fusion constructs with NT by cell surface biotinylation. Cells grown on transwells were pulsed for 30 min, chased for 2 h, and then biotinylated on both surfaces. The proteins were immunoprecipitated. One-tenth of the total immunoprecipitate was removed, and biotinylated proteins were recovered (Surf) from the rest of the immunoprecipitate by binding to streptavidin-agarose and loaded alongside one-tenth of the total immunoprecipitate (1/10 T) in an SDS-PAGE 9% gel. B, quantitation of the data in panel A. The surface expression of D4 is arbitrarily set as 100% (A), and the respective surface expression of the various fusion proteins are shown as a percentage of this. B, NT29-D4; C, NT19-D4; D, NT35-D4; E, NT45-D4; F, NT55-D4.

Fig. 5. Lectin Binding Profile of the Chimeric Proteins—N-Acetylgalcosaminyltransferase I has been localized to the medial cisternae of the Golgi stack (17). To gain an insight as to whether the chimeric proteins bearing the targeting signal of NT were also targeted to a similar compartment, the terminal carbohydrate modification of NT7-29-D4 and NT-TFR were examined by lectin affinity chromatography. The lectins used include concanavalin A (specific for α-linked mannose structures), succinylated wheat germ agglutinin (specific for N-acetylgalosamine), Erythrina cristagalli lectin (ECA; specific for galactose residues), Sambucus nigra lectin (specific for α-2,3-sialic acid linked to galactose), and Maackia amurensis lectin (specific for α-2,3-sialic acid linked to galactose). D4 is known to undergo only N-linked glycosylation (26), whereas both N- and O-linked oligosaccharides can be found on the transferin receptor (27). It would be particularly interesting to know if the carbohydrate termini of the fusion proteins had been modified by sialic acid linkages and what type of linkages they might have acquired. As shown in Fig. 5, both staining and intense intracellular perinuclear staining when permeabilized. These results, taken together, suggest that the transmembrane region of NT is important for Golgi retention. Furthermore, this sequence can function in different fusion protein environments and is thus transferable from its parent molecule to heterologous systems.

Lectin Binding Profile of the Chimeric Proteins
under "Experimental Procedures." Cells were either unpermeabilized on coverslips were incubated with monoclonal antibody against trans-structure with
its cytoplasmic domain deleted for surface staining and
were eluted by boiling in SDS sample buffer and run on a 9% SDS-PAGE gel. The lectin-bound proteins were examined by their affinity for different lectins (concanavalin A, S. nigra lectin (SNA), and M. amurensis lectin (MAA)).

The terminal carbohydrate modifications of these fusion proteins have therefore undergone modifications by sialyltransferases. a-2,3-Sialylation activity can be found in Madin-Darby canine kidney cells, and D4 itself binds to M. amurensis lectin. Binding to S. nigra lectin but not M. amurensis lectin suggests that the chimeric proteins bearing the Golgi targeting signal of NT are targeted to the same brefeldin A-sensitive compartment as a-2,6-sialyltransferase. The location of a-2,3-sialyltransferase is therefore distal to that of the fusion proteins. Due to lack of information on the exact cisternal partitioning of the sialylation enzymes, no firm conclusion on the location of the fusion proteins can, however, be drawn.

DISCUSSION

The present study provides evidence showing that the transmembrane domain of a type II Golgi resident protein contains a Golgi retention sequence that could by itself retain the ectodomain of a surface membrane protein in the Golgi. Intracellular confinement of the chimeric proteins was demonstrated by the loss of surface expression and by intracellular immunofluorescence staining, which revealed characteristic Golgi staining patterns.

Several models are possible to rationalize the ability of the membrane spanning region of a protein molecule to function as a retention signal. One possibility is that the transmembrane region is recognized and specifically retained by a receptor molecule. Such receptors do exist, albeit in the form of the signal sequence receptor (21), which functions in the translocation of polypeptide chains across the endoplasmic reticulum. The transmembrane domain may also be a retention signal analogous to the KDEL sequence at the C terminus of luminal ER proteins (2), and proteins bearing this sequence are retrieved by an analogous system. This would help explain the sialic acid modifications of the chimeras and the Golgi to ER retrograde pathway manifested during brefeldin A treatment (22).

Another possibility is that the transmembrane region also functions as the site within the molecule where oligomerization or aggregation of proteins of identical or similar structure may occur. Such oligomers or aggregates would then be too large to enter Golgi transport vesicles. A previous report on the preferential association between the synthetic transmembrane region of glycophorin A and native glycophorin (23) supports this possibility.

During the course of the preparation of this manuscript, Nilsson et al. (24) and Munro (25) reported that the transmembrane regions of β-1,4-galactosyltransferase (GT) and α-2,6-sialyltransferase contain their respective Golgi retention signals. These type II proteins, like NT, are glycosyltransferases located in the trans-Golgi and the trans-Golgi network, respectively. These reports, in conjunction with our results, suggest that the Golgi retention signal of type II glycosyltransferases reside in their transmembrane domains. Fusion proteins expressed from chimeric constructs containing the β-1,4-galactosyltransferase transmembrane domain have been localized by immunoelectron microscopy to the trans-cister- nae of the Golgi stack (24). It remains to be seen whether fusion constructs of α-2,6-sialyltransferase, NT, and E1 (10) can be localized to the trans-Golgi network, the medial-Golgi, and the cis-Golgi, respectively. Should the cisternal location of the fusion proteins turn out to be the same as the parent molecule, it would imply that the transmembrane domain Golgi retention signals are sufficiently subcompartment-specific. The fact that the NT chimeras have α-2,6-sialylation but not α-2,3-sialylation, whereas α-2,6-sialyltransferase chimeras with identical reporter sequences (30) have both types of sialylations (binding to both S. nigra lectin and M. amurensis lectin), provides preliminary evidence for the subcompartment specificity of their respective Golgi retention signals. The luminal or membrane environments of subcompartments of the Golgi apparatus must be sufficiently different from each other for the manifestation of subcom-

Fig. 4. Subcellular localization of transferin receptor with its cytoplasmic domain deleted (A and B) and its fusion construct with NT (C and D) by immunofluorescence. Cells grown on coverslips were incubated with monoclonal antibody against transferin receptor and processed for immunofluorescence as described under "Experimental Procedures." Cells were either unpermeabilized for surface staining (A and C) or permeabilized with 0.1% saponin for total staining (B and D).

Fig. 5. Lectin binding profiles of NT-[29]-D4 and NT-TFR. The terminal carbohydrate modifications of these fusion proteins were examined by their affinity for different lectins (concanavalin A (ConA), succinylated wheat germ agglutinin (sWGA), E. cristagalli lectin (ECA), S. nigra lectin (SNA), and M. amurensis lectin (MAA)). Cells were pulsed for 30 min and chased for 2 h. Immunoprecipitated proteins were then divided into equal aliquots and then bound to various lectins coupled to agarose. The lectin-bound proteins were eluted by boiling in SDS sample buffer and run on a 9% SDS-PAGE gel. One-tenth of the total immunoprecipitated proteins without further treatment was loaded as controls.

NT-[29]-D4 and NT-TFR bound to concanavalin A, E. cristagalli lectin, and S. nigra lectin but not to succinylated wheat germ agglutinin or M. amurensis lectin. These chimeric proteins have therefore undergone modifications by sialyltransferase to acquire α-2,6-sialic acid linkages, but not α-2,3 linkages. Sialyltransferases are known to reside at the trans-Golgi cisternae and the trans-Golgi network. However, certain medial-Golgi proteins have also been shown to be sialylated (18-19). Recent studies indicate that transacted N-linked α-2,6-sialyltransferase resides in a brefeldin A-sensitive compartment. α-2,3-Sialylation activity can be found in Madin-Darby canine kidney cells, and D4 itself binds to M. amurensis lectin. Binding to S. nigra lectin but not M. amurensis lectin suggests that the chimeric proteins bearing the Golgi targeting

3 B. L. Tang, unpublished results.
4 W. Hong, unpublished results.
partmental specificity in protein retention. Investigations on
the underlying mechanisms of this specificity will undoubt-
edly provide insights on the ways of generating subcompart-
mental diversity itself.

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