The SXYQRL Sequence in the Cytoplasmic Domain of TGN38 Plays a Major Role in Trans-Golgi Network Localization

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The trans-Golgi network (TGN) plays a central role in protein sorting/targeting. TGN38 is an integral membrane protein confined mainly to the TGN. When the cytoplasmic 32-residue sequence of TGN38 was fused to the ecto- and transmembrane domains of glycophorin A (a surface protein), the resulting chimeric protein was localized to the TGN. Detailed mutagenesis of the 32-residue sequence revealed that the Ser, Tyr, and Leu residues at positions 23, 25, and 28, respectively, are essential for TGN localization. Further studies demonstrate that the sequence SXYQRL can by itself confer significant TGN localization.

The signals and mechanisms that govern the selective localization of proteins at various intracellular (sub-)compartments or distinct surface domains are being extensively investigated (Hong and Tang, 1993 and references therein). ER luminal proteins bear a C-terminal -Lys-Asp-Arg-Glu-Leu (KDEL) (HDEL in yeast) sequence and are retained in the ER by a retrieval mechanism from the cis-Golgi (Hong and Tang, 1993, Hsu et al., 1992; Lewis and Pelham, 1990, 1992; Lewis et al., 1990; Pelham, 1990, 1991; Semenza et al., 1990; Tang et al., 1993). The signal that mediates the ER localization of type I membrane proteins has been revealed to be the C-terminal -Lys-Lys-X-X (KKXX) or -Lys-X-Lys-X-Lys (KKXK) sequence (Jackson et al., 1990; Shin et al., 1991), although the mechanism remains to be established. Recently, it was demonstrated that the signal responsible for Golgi localization of several glycosyltransferases (Aoki et al., 1992; Burke et al., 1992; Coley et al., 1992; Munro, 1991; Nilson et al., 1991; Russo et al., 1992; Teasdale et al., 1992 Tang et al., 1992a; Wong et al., 1992) resides mainly in the transmembrane domain. These three glycosyltransferases are most likely confined to the Golgi stack (Burke et al., 1992; Nilson et al., 1991; Russo et al., 1992; Tang et al., 1992b). In addition, the first of the three transmembrane regions of coronavirus E1 glycoprotein has been shown to be sufficient to confer Golgi localization (Swift and Machamer, 1991). Signals have also been identified that mediate selective targeting to the lysosomal/endosomal system (Dahms et al., 1989; Fukada, 1991; Johnson et al., 1990; Johnson and Kornfeld, 1992; Letourneur and Klausner, 1992; Mathews et al., 1992; Ookayamoto et al., 1992; Peters et al., 1990; Williams and Fukada, 1990) and to various surface domains of polarized epithelial cells (Brewer and Roth, 1991; Hunziker et al., 1991; Le Bivic et al., 1991, Matter et al., 1992; Mostov et al., 1992, and references therein).

Although the trans-Golgi network (TGN) plays a pivotal role in sorting proteins to various post-Golgi structures, its structure and composition are poorly defined. Similarly, the signal and mechanism that mediate protein localization in the TGN has not been revealed. The cation-independent mannose-6-phosphate receptor, which mediates lysosomal enzyme targeting, is present in the TGN as well as in the late endosomes (pre-lysosomal compartment) (Dahms et al., 1989; Wood et al., 1991). The best characterized TGN marker is a type I membrane glycoprotein named TGN38 (Ladinsky and Howell, 1992; Lippincott-Schwartz et al., 1991; Luzio et al., 1990; Reaves and Banting, 1992; Reaves et al., 1993). Its TGN localization has been established by both electron microscopy (Ladinsky and Howell, 1992; Luzio et al., 1990) and its unique response to the fungal metabolite brefeldin A (BFA). When expressed in transfected COS cells, TGN38 is localized to the Golgi and this Golgi localization is abolished by deleting the cytoplasmic domain, suggesting that the cytoplasmic domain of TGN38 is necessary for Golgi localization (Luzio et al., 1990). These results could be interpreted in two different ways. One possibility is that, like glycosyltransferases (Hong and Tang, 1993, and references therein), the transmembrane domain of TGN38 may contain the Golgi localization signal and that the function of this signal depends on the presence of a cytoplasmic tail. Alternatively, the TGN localization signal could be present in the cytoplasmic domain.

In this report, we demonstrate that the 32-residue cytoplasmic domain of TGN38, when appended to the ecto- and transmembrane domains of glycophorin A (GA), is sufficient to mediate TGN localization. Furthermore, Ser, Tyr, and Leu residues in the context of SDYQRL play the major role in TGN localization.

EXPERIMENTAL PROCEDURES

Materials

Cell culture media, fetal bovine serum, Geneticin (G418), and lipofectin were purchased from Life Technologies, Inc. Goat anti-mouse IgG was from Dako (Denmark). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was from Boehringer Mannheim Corp. Pyrococcus furiosus DNA polymerase was purchased from Stratagene Cloning Systems. Brefeldin A (BFA) was from Epicentre Technologies. Monoclonal antibody (6A7M) to and cDNA clone for glycophorin A were obtained from American Type Culture Collection...
FIG. 1. The cytoplasmic 32-residue sequence of TGN38 is sufficient for Golgi localization. a, both TGN38 and GA are type 1 integral membrane proteins with an N-terminal ecto- or luminal domain followed by a single transmembrane (TM) domain and a C-terminal cytoplasmic (Cyto) tail. The chimeric protein G/T38 contains the ecto- and the transmembrane domains of GA fused to the 32-residue cytoplasmic tail of TGN38. b, immunoblotting analysis of control cells (lane 1) and cells transfected with cDNA encoding GA (lane 2) or G/T38 (lane 3). As shown, specific polypeptides could be detected in transfected but not control cells using monoclonal antibodies against the ectodomain of GA. The open circles indicate the dimeric form of the respective protein. c, immunofluorescence microscopy of cells expressing GA (panels a and b) or G/T38 (panels c-f). GA gave a staining pattern typical of a surface protein in both unpermeabilized (panel a) and permeabilized (panel b) cells. No surface staining was observed for G/T38 (panel c). In permeabilized cells, G/T38 was confined to a perinuclear structure (panel d) typical of the Golgi apparatus. The perinuclear staining of G/T38 (panel e) co-localized well with that of Golgi mannosidase II (panel f).

Oligonucleotides

The sequence of the oligonucleotides used are listed and all are read from 5' to 3': 1, GTCTCGAGCACCATGTATGGAAAAATAA TCTTTG; 2, GGCAAAAGCAATAATCTTTCGT; 3, CGAAAGATTATTGCTTTTGCC; 4, GTCTCGAGTCAAAGCTTTAGGTTCAAACG; 5, GTCTCGAGTCAGGCCTTTAGGTTCAAACGTTGGCGTCACTGG; 6, GTCTCGAGTCAAAGCTTTAGGTTCAAACG; 7, GTCTCGAGTCAAAGCTTTAGGGCCAAACG; 8, GTCTCGAGTCAAAGCTTTAGGTTCAAACG; 9, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAG; 10, GTCTCGAGTCAAAGCTTTAGGTTCAAACG; 11, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGG; 12, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 13, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 14, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 15, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 16, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 17, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 18, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 19, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 20, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 21, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 22, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 23, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 24, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 25, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 26, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 27, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 28, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 29, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 30, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 31, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG; 32, GTCTCGAGTCAAAGCTTTAGGTTCAAACGTTGGTAGTCACTGGCCTTGGAACTGGAAC TGGAACTACTGG.

Native and Chimeric cDNAs

GA—A 730-base pair EcoRI cDNA fragment encoding for GA in the pH2L vector was obtained from ATCC. Plasmid pH2L was digested with EcoRI and blunted by the Klenow fragment of DNA polymerase I. The 730-base pair EcoRI/blunt fragment was isolated and then inserted into the SalI/blunt site of the expression vector pRIN (Low et al., 1991).

TGN38—The cloning and sequencing of TGN38 has been described previously (Luzio et al., 1990).
FIG. 2. G/T38 is localized to the TGN as revealed by its similarity of brefeldin A response to that reported for TGN markers. Cells expressing G/T38 were treated with BFA for various periods of time as indicated and then fixed with paraformaldehyde. Fixed cells were permeabilized and processed for immunofluorescence microscopy.

reaction using oligonucleotides 1 and 4, resulting in a chimeric cDNA fragment encoding the fusion protein G/T38.

G/T38Y-A—Oligonucleotides 5 (in which the codon for Y has been changed into that for A residue) and 3 were used to retrieve the cDNA sequence encoding for the cytoplasmic domain of TGN38. The PCR product was gel purified and mixed with the PCR product of oligonucleotides 1 and 2 (see above) prior to PCR again using oligonucleotides 1 and 5.

L(32j-A—Oligonucleotides 6 and 1 were used to modify G/T38 cDNA by PCR (G/T38 cDNA as the template). This process created a point mutation at the C terminus of G/T38 cytoplasmic tail (Leu at position 22 to Ala).

K(31)-A—Oligonucleotides 7 and 1 were used to modify G/T38 cDNA by PCR (G/T38 cDNA as template), creating a Lys to Ala mutation at residue number 31 of G/T38 cytoplasmic tail.

L(30)-A—This was constructed as above using oligonucleotides 8 and 1. Residue number 30 (Leu) of G/T38 cytoplasmic tail was mutated to Ala residue.

D(29)-A—Oligonucleotides 9 and 1 were used to construct cDNA encoding N(29)-A, in which the Asp residue at position 29 of G/T38 cytoplasmic tail was mutated to Ala.

E(26)-A—This construct was obtained using oligonucleotides 12 and 1. The Glu residue at position 26 of G/T38 cytoplasmic tail was changed to Ala.

Df24/-A—Oligonucleotides 13 and 1 were used to construct this chimeric cDNA using methods similar to the above. The Asp residue at position 24 of G/T38 cytoplasmic tail was mutated to Ala.

S(23)-A—This was created as above using oligonucleotides 14 and 1. In this construct, the Ser residue at position 23 of G/T38 cytoplasmic tail was mutated to Ala.

D/17-20—The PCR product obtained using oligonucleotides 19 and 1 (G/T38 cDNA as the template) was mixed with the PCR product from oligonucleotides 20 and 4 (G/T38 cDNA as the template). This mixture was used as a template in another PCR reaction using oligonucleotides 1 and 4 to create the construct encoding D/17-20. Four amino acid residues at positions 17-20 of G/T38 cytoplasmic tail were deleted.

D/13-16—The PCR product resulted from oligonucleotides 21 and 1 was mixed with that from oligonucleotides 22 and 4. G/T38 cDNA was used as the template for both PCR reactions. The mixture of the two PCR products was used as the template in another PCR reaction utilizing oligonucleotides 1 and 4 to obtain the construct encoding D/13-16. Four amino acid residues at positions 13-16 of G/T38 cytoplasmic tail were deleted.

D/9-12—This was constructed using the mixture of PCR products obtained from oligonucleotides 23/1 and 24/4 as template in a PCR reaction utilizing oligonucleotides 1 and 4. Four amino acid residues at position 9-12 of G/T38 cytoplasmic tail were deleted.

D/3-8—This was constructed as above using oligonucleotides 25/1, 26/4, and 1/4. The final PCR product encodes D/25-30, in which 6 amino acid residues at position 3-8 of G/T38 cytoplasmic tail were deleted.

G/S—Oligonucleotides 15 and 1 were used to construct a chimeric cDNA containing 12 amino acid residues of the C terminus of TGN38 plus 10 additional Ser residues before the transmembrane domain (cDNA G/T38 as template). The PCR product was gel purified and
TGN Localization Mediated by a Tyrosine-containing Sequence

Transfectants were pooled and maintained in minimal essential Eagle's media with 2500 µg/ml of Geneticin (G418).

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed as described previously (Low et al., 1991; Wong et al., 1992; Tang et al., 1992, 1993).

Western Blotting (Immunoblotting) Analysis

CHO cells in T25 tissue culture flasks were induced overnight in medium containing sodium butyrate (10 mM), scraped, and washed twice with PBS. Cells were then suspended in minimal amount of PBS and lysed by boiling in an equal volume of 2 × SDS sample buffer. One-third of the lysed cells was resolved by SDS-PAGE, and transferred to a nitrocellulose filter prior to sequential incubation with primary antibodies, goat anti-mouse IgG (6 µg/ml), 125I-protein A (0.1 μCi/ml), and autoradiography. Incubation of the filter with primary and secondary antibodies was done in PBS containing 5% skim milk and 0.05% Tween 20. Washing of filters was done in PBS containing 0.05% Tween 20.

RESULTS

The 39-Residue Cytoplasmic Domain of TGN38 Is Sufficient for Golgi Localization—In order to examine whether the cytoplasmic domain of TGN38 is sufficient for Golgi localization, we attached its cytoplasmic domain to the ecto- and the transmembrane (TM) domains of glycophorin A (GA). The choice of GA is based on several reasons. First, GA is a well established surface protein with the same membrane topology as TGN38 (type I), having an N-terminal ectodomain, a single TM domain, and a C-terminal cytoplasmic domain (Fig. 1a) (Siebert and Fukuda, 1986); second, like TGN38 (Luzio et al., 1990), GA is heavily O-glycosylated (Morrow and Rubin, 1987; Remaley et al., 1991); and finally, the cDNA for GA and monoclonal antibodies against GA are readily available. The cDNA fragment encoding the cytoplasmic domain of TGN38 was fused to that encoding the ecto- and TM domains of GA, resulting in a chimeric cDNA encoding a fusion protein (G/T38, Fig. 1a) that links the ecto- and TM domains of GA to the cytoplasmic domain of TGN38.

The cDNAs encoding GA and G/T38 were inserted into the mammalian expression vector (pRSN) (Low et al., 1991), and transfected into CHO cells. Stable transfectants were first examined for the expression of the desired proteins by immunoblotting analysis using monoclonal antibodies against the ectodomain of GA (Fig. 1b). In control cells (untransfected or transfected with unrelated cDNAs), no specific antigen was detected (lane 1). A protein of about 42 kDa was detected in cells transfected with GA cDNA (lane 2), this size is consistent with that reported for GA (Morrow and Rubin, 1987; Remaley et al., 1991). In cells transfected with G/T38 cDNA, a specific protein of about 38 kDa was revealed (lane 3). Interestingly, the size of G/T38 is significantly smaller than that of GA, although they share identical ecto-/luminal and TM domains with the cytoplasmic domain of G/T38 being smaller than that of GA by 3 amino acids only. The reason for this observation could be due to the fact that the net charge of the GA cytoplasmic domain is −2 while that of TGN38 is +9.

Indirect immunofluorescence microscopy (Fig. 1c) revealed strong surface staining for GA (panel a) but not for G/T38 (panel c). In cells permeabilized with saponin, strong perinuclear staining, characteristic of the Golgi, was observed for G/T38 (panel d). The perinuclear staining of G/T38 (panel e) colocalized well with the Golgi apparatus as revealed by costaining with antibodies against Golgi mannoside II, a well-established Golgi marker (Klausner et al., 1992, and references therein) (panel f). In permeabilized cells, GA staining is typical for a surface protein (panel b). GA is therefore

Cell Culture

CHO cells were obtained from ATCC. Cells were cultured in minimal essential Eagle's media supplemented with 10% fetal bovine serum, and antibiotic-antimycotic. The medium was changed daily. Expression of transfected genes was induced by culturing cells overnight in medium containing 10 mM sodium butyrate.

Transfection

Transfection of CHO cells was performed using liposomes (lipofectin) as described by the manufacturer (Life Technologies, Inc.).

FIG. 3. TGN localization of G/T38 may be saturable. Cells were either permeabilized (panels a and b) or not (panel c) and then processed for immunofluorescence microscopy. In cells expressing G/T38 at low to moderate levels (marked with I), G/T38 is confined to the Golgi. G/T38 is also mainly localized to the Golgi with some spotty localization in cells expressing higher levels of the protein (marked with II). In over-expressing cells (marked with III), G/T38 is predominantly localized to the surface and the strong surface staining was obvious in non-permeabilized cells (panel c).
FIG. 4. The Tyr residue at position 25 of the TGN38 cytoplasmic tail is essential for Golgi localization. a, the cytoplasmic 32-residue sequence of G/T38 is shown and the numbering is from the beginning of the cytoplasmic tail, which corresponds to residue 309 of mature TGN38. The unique Tyr residue at position 25 was mutated to Ala residue. b, control cells (lane 3) or cells transfected with cDNAs encoding G/T38 (lane 1) or G/T38 with Y to A mutation (G/T38Y-A) were analyzed by immunoblotting. Specific protein was detected in transfected cells but not in control cells. c, in contrast to Golgi staining of G/T38 (panels a and b), strong surface staining was observed for G/T38Y-A both non-permeabilized (panel c) or permeabilized (panel d) cells, demonstrating that this unique Tyr residue is essential for TGN localization.

expressed at the cell surface, while G/T38 is specifically localized to the Golgi apparatus, establishing that the cytoplasmic domain of TGN38 is sufficient to confer Golgi localization.

The Cytoplasmic Domain of TGN38 Is Sufficient for TGN Localization—Since the Golgi apparatus is composed of several distinct subcompartments (Hong and Tang, 1993; Klausner et al., 1992; Rothman and Orci, 1992) and TGN38 is confined to the TGN (Ladinsky and Howell, 1992; Lippincott-Schwartz et al., 1991; Luzio et al., 1990; Reaves and Banting, 1992), it would be of interest to examine if the majority of G/T38 is similarly present in the TGN. One of the best ways to distinguish the TGN from the rest of the Golgi is by its unique response to BFA (Ladinsky and Howell, 1992; Lippincott-Schwartz, 1991; Reaves and Banting, 1992; Reaves et al., 1993; Wood et al., 1991). Transfected CHO cells were treated with BFA for various periods of time and then fixed for indirect immunofluorescence microscopy (Fig. 2). In control cells, typical Golgi staining was observed (panel a). Short tubules could clearly be detected to emanate from the Golgi after 5 min of BFA treatment (panel b). By 10–15 min of BFA treatment, an extensive tubular network was observed (panels c and d). The tubular network became significantly collapsed after 30 min of BFA treatment (panel e). Compact stainings were detected after treating the cell with BFA for 60 min. The dynamic response of G/T38 to BFA is similar to that of TGN markers (Ladinsky and Howell, 1992; Lippincott-Schwartz, 1991; Reaves and Banting, 1992; Reaves et al., 1993; Wood et al., 1991), but totally different from that of proteins present in the Golgi stack (Klausner et al., 1992, and references therein; Tang et al., 1992b) or the CGN (Klausner et al., 1992, and references therein; Tang et al., 1993). Under identical conditions, mannosidase II was redistributed back to the ER as observed previously (Klausner et al., and references therein) (data not shown). G/T38 is thus mainly confined to the TGN, and the cytoplasmic domain of TGN38 is sufficient to confer TGN subcompartment localization.

A Saturable Mechanism May Be Involved in TGN Localization of G/T38—In cells expressing low to moderate levels of G/T38, only Golgi staining was observed (Figs. 1, 2, and 3, cells marked with I). When the expression levels were higher, some spotty staining was also detected throughout the cytoplasm in addition to the major Golgi staining (Fig. 3, cells marked with II). In these cells, no significant surface staining could be detected. The nature of the spotty staining is unknown but could be endocytotic or exocytotic intermediates because TGN38 is known to recycle between the TGN and the surface (Ladinsky and Howell, 1992). When G/T38 was expressed at very high levels, strong surface staining was observed and the majority of G/T38 seemed to be present on the cell surface (Fig. 3, cells marked with III). The bright surface staining was clearly observed in few non-permeabilized cells (Fig. 3c), while the majority of the cells do not show any surface staining. These results demonstrate that the majority of G/T38 is present in the Golgi in cells that express...
The Unique Tyr Residue in the Cytoplasmic Domain Is Necessary for TGN Localization—Examining the sequence of the cytoplasmic domain of TGN38 (Fig. 4a) revealed the presence of a unique Tyr residue at position 25 (numbering from the beginning of the cytoplasmic tail, which corresponds to residue 309 in the mature TGN38). Since a Tyr residue has been shown to be essential for signals involved in several aspects in the peripheral membrane system, including rapid endocytosis via clathrin-coated vesicles and lysosomal targeting of type I membrane proteins (Dahms et al., 1989; Fukuda, 1991; Johnson and Kornfeld, 1992; Letourneur and Klausner, 1992; Mathews et al., 1992; Okamoto et al., 1992; Peters et al., 1990; Williams et al., 1990), it was of great interest to examine if this Tyr residue is important for TGN localization. This Tyr was changed into an Ala residue (Fig. 4a), and the cDNA encoding this mutant form (G/T38Y-A) was similarly transfected into CHO cells. Immunoblot analysis (Fig. 4b) revealed the expression of G/T38Y-A (lane 2) in the stably transfected cells, which is of the same size as G/T38 (lane 1). While G/T38 was localized to the Golgi (Fig. 4c, panels a and b), G/T38Y-A showed typical staining for a surface protein (panels c and d). These results demonstrate that mutation of this Tyr abolished TGN localization, leading to the surface expression. The Tyr residue at position 25 thus plays a central role in
mediating TGN localization and the TGN localization signal may be present in the region containing this Tyr residue.

Ser at Position 23 and Leu at Position 28 Are Also Necessary for TGN Localization—Ala scanning mutagenesis was used to identify other important residues that are also necessary for the TGN localization. The last 10 residues (23-32, underlined in Fig. 4a) were individually mutated into an Ala residue, and the cDNA encoding each mutant form was inserted into the expression vector and transfected into CHO cells. Immunoblot analysis (data not shown) demonstrated that each mutant form was expressed in the transfected cells at comparable levels and has the same size as G/T38. Indirect immunofluorescence microscopy (Fig. 5) was used to localize each mutant form. When the Leu at position 28 (panels k and l) and the Ser position 23 (panels s and t) were each mutated into Ala, the TGN localization was severely reduced, resulting in significant surface staining. The predominant TGN localization was not significantly affected in other mutants. These observations demonstrate that Leu at position 28 and Ser at position 23 are also necessary for TGN localization.

Deletions in Other Cytoplasmic Regions Have No Major Effect on TGN Localization—In order to examine if any other regions in the cytoplasmic domain are also involved in the TGN localization, constructs with internal deletions in G/T38 were prepared (Fig. 6a). D/3-8, D/9-12, D/13-16, and D/17-20 have the internal residues 3-8, 9-12, 13-16, and 17-20, respectively, deleted. cDNAs encoding these mutated forms of G/T38 were transfected into CHO cells. Stable transfectants were shown to express the desired polypeptides as assessed by immunoblotting analysis (data not shown). Indirect immunofluorescence microscopy was employed to examine the subcellular localization of the expressed proteins (Fig. 6b). As shown, each of them was mainly confined to the Golgi, demonstrating that residues 3-20 play no major role in TGN localization. These results, in conjunction with those described in the above sections, suggest that the TGN localization signal is located in the last 12-residue (residues 21-32) sequence of TGN38 cytoplasmic tail.

SXYQRL Is Sufficient for Significant TGN Localization—We next examined whether short sequences containing the essential Ser, Tyr, and Leu residues could confer any TGN localization. KASDYQRLN (residues 21-29) or YQRL (residues 25-28) was each attached to a derivative of GA (G/S) (Fig. 7a), which contains the ecto- and transmembrane domains of GA with a cytoplasmic sequence of RRSSSSSSSSSS, resulting in the construction of chimeric cDNA encoding G/S(21-29) or G/S(25-28), respectively (Fig. 7a). The choice of using G/S to test the function of KASDYQRL and YQRL sequences is based on two main reasons; the first is that a cytoplasmic sorting signal (e.g. that for rapid endocytosis) can function only when it is placed a certain distance (e.g. 7 residues) away from the transmembrane domain (Collawn et al., 1990); second, a Ser-based cytoplasmic tail has been shown previously not to interfere with the
FIG. 7. SXYQRL is sufficient for significant Golgi localization. a, the sequence of the cytoplasmic tail of various chimeric proteins, which contain the same ecto- and transmembrane domains of GA. b, localization of each of them by immunofluorescence microscopy. G/S (panel a) is localized mainly to the surface. Addition of KASDYQRLN (panel b) or YQRL (panel c) to the C terminus of G/S resulted in significant perinuclear staining, which colocalized well with that of Golgi mannosidase II (panels d and e).

TABLE I

Compiling some of the known Tyr-containing sequences involved in sorting/targeting in the peripheral membrane system

| Targeting pathway | Marker | Sequence |
|-------------------|--------|----------|
| Basolateral surface | PShNGFR | NSLYSSL |
|                   | HAY    | SLQYR1CI |
|                   | FcRII-B2 | TITYS1L |
|                   | LDLR   | FDNPVYQKTTEDEVH |
|                   | LDLR   | QDGYPSRQMVSELDVVA |
| Lysosome/endosome | LEP100 and h-lamp-1 | HAGYQTL |
|                   | LAP    | PPGYRHV |
|                   | lamp-2 | HAGYEQF |
|                   | lamp-3 | RSGYEVM |
|                   | M6PR/46 | PAAYRGB |
|                   | M6PR/275 | YKYSKV |
|                   | CD3γ  | EQLYQPL |
| Rapid endocytosis | LDLR   | FDNPVYQKT |
|                   | InsulinR | GPIYNPEY |
|                   | ASGPR  | TKEYOQDF |
|                   | IgAR   | DLAYS1F |
|                   | THR    | PLSYTRF |

cytoplasmic ER localization sequence (KKXX/KXXKXX) of type I membrane proteins (Jackson et al., 1990). In both G/S(21-29) and G/S(25-28), the essential Ser, Tyr, and Leu residues are present in the context of SXYQRL (X = D, A, or S) separated from the transmembrane domain by a 14- and 20-residue sequence, respectively. The cDNAs encoding for G/S, G/S(21-29), and G/S(25-28) were individually transfected into CHO cells. Immunoblotting analysis showed that the transfected cells expressed the desired polypeptide (data not shown). When examined for subcellular localization by
indirect immunofluorescence microscopy (Fig. 7b), G/S gave a staining pattern typical of a surface protein (panel a), and a significant amount of G/S(21-29) (panel b) and G/S(25-28) (panel c) were present in the perinuclear region in addition to the cytoplasmic spotty staining. The perinuclear staining of G/S(25-28) was confirmed to be the Golgi apparatus by its colocalization with the Golgi mannosidase II (panels d and e). When cells were treated with BFA, G/S(25-28) behaved similar to TGN38 (Lippincott-Schwartz et al., 1991) and G/T38 (Fig. 2) (data not shown). These results, taken together, demonstrate that the SXYQRL sequence can confer significant TGN localization.

**DISCUSSION**

The TGN Localization Signal Is Similar to Sorting Signals Acting in the Peripheral Membrane System But Distinct from Those Mediating Golgi Localization of Glycosyltransferases—The identification of a cytoplasmic Tyr-containing signal for TGN localization is of great interest. This TGN signal bears similarity to other Tyr-containing signals involved in other sorting/targeting processes, including rapid endocytosis via the clathrin-coated vesicles, targeting to the lysosomal/endosomal system, and selective targeting to the basolateral surface of polarized epithelial cells (Dahms et al., 1989; Fukuda, 1991; Hunziker et al., 1991; Johnson and Kornfeld, 1992; Le Bivic et al., 1991; Letourneur and Klausner, 1992; Matter et al., 1992; Mathews et al., 1992; Mostov et al., 1992; Okamoto et al., 1992; Peters et al., 1990; Williams et al., 1990). This similarity implies that similar but distinct mechanisms may be involved in recognizing these Tyr-containing sorting/targeting signals, although much of the detail remains to be investigated. Previous studies from our laboratory (Wong et al., 1992; Tang et al., 1992a) and others (Aoki et al., 1992; Burke et al., 1992; Colley et al., 1992; Munro et al., 1991; Nilson et al., 1991; Russo et al., Teasdale et al., 1992) have shown that the transmembrane domain of three glycosyltransferases is the signal for Golgi localization. The Tyr-containing TGN localization signal is thus very different from those transmembrane domain signals for the Golgi glycosyltransferases in that it is located in a short cytoplasmic sequence while the signal for the Golgi glycosyltransferases is composed of hydrophobic transmembrane sequence. This implies that very different mechanisms may be used for TGN as opposed to Golgi localization of glycosyltransferases. This difference may be due to the fact that the Golgi glycosyltransferases are resident proteins of the Golgi system while TGN38 recycles between the TGN and the cell surface with a steady-state TGN localization (Ladinsky and Howell, 1992; Reaves et al., 1993).

A Saturable Mechanism for TGN Localization—An important observation is that TGN localization is highly specific under low to moderate levels of expression of the signal-containing proteins, but is abolished by overexpression of the signal-containing proteins. A saturable mechanism implies the existence of a highly specific receptor for this signal. It also suggests that the putative receptor for this TGN localization signal is present at a defined level. The molecular nature and its working mechanism is currently unknown. As mentioned earlier, Tyr-containing signals may use similar but distinct mechanisms for selective targeting. The best known system of a Tyr-containing signal is that for rapid endocytosis. A Tyr-containing endocytotic signal is recognized by a unique form (α-adaptin) of the adaptin family (Pearse and Robinson, 1990). α-Adaptin, like other members of the family, is a cytosolic protein, and its binding to the endocytotic signal promotes the assembly of clathrin-coated vesicles. It is possible that the Tyr-containing TGN localization signal is recognized by a novel member of the adaptin family, and this interaction could mediate the TGN localization by an undefined mechanism. The TGN-localized γ-adaptin (Robinson, 1990) might be one candidate that may interact with this TGN signal. Alternatively, a new undiscovered member of the adaptin family may be involved. TGN38 is known to recycle between the TGN and the surface via the endocytotic and/or the exocytotic pathway (Ladinsky and Howell, 1992; Reeves et al., 1993). Preliminary observations suggest that chimeric proteins containing the TGN localization signal could also shuttle between the TGN and the surface (data not shown).

An important question related to this is whether newly made TGN proteins are selectively retained in the TGN, and the shuttling between the TGN and the surface is just one of the properties of the TGN-retained proteins. In this case, cycling between the surface and the TGN plays no essential role in TGN localization. Alternatively, newly made TGN proteins could be transported initially to the surface, where they are selectively retrieved back to the TGN. In this case, the shuttling between the surface and the TGN is an obligatory step required for TGN localization. These issues will be explored by further investigations.

Tyr-containing Signals: Where Is the Specificity?—The identification of Tyr-containing signals involved in diversified targeting processes, including rapid endocytosis, lysosomal/endosomal targeting, basolateral targeting, and TGN localization, raised a number of questions as to how these signals achieve selective targeting/sorting. Compiling some of these Tyr-containing sequences (Table I) revealed that the primary sequence is distinct among sequences for each sorting/targeting process and even among each individual protein, although they share the same important property of containing a Tyr residue. It is thus tempting to speculate that the primary sequence (and the derived secondary structure) and/or post-translational modifications around the essential Tyr residue plays an important role in determining the specificity. The identification of an important Ser residue in the TGN localization signal is in support of this conclusion. Detailed mutagenesis of each residue in these Tyr-containing signals will, undoubtedly, shed more light on this important issue.

During the preparation of this article, a paper with similar results was published (Humphrey et al., 1993). Using electron microscopy, Bonifacino and colleagues have shown that fusion proteins bearing the cytoplasmic region of TGN38 were localized to the TGN. In our studies, we have demonstrated that fusion proteins with the same cytoplasmic region behave similarly to TGN38 upon BFA treatment. These results thus firmly established that the cytoplasmic region of TGN38 is sufficient for TGN localization. One major discrepancy is that mutation of serine residue at position 23 to alanine severely reduced TGN localization in our study, while identical mutation resulted in no effect on the TGN localization in the reported study (Humphrey et al., 1993). The reason for this discrepancy is currently unknown. One possible explanation could be due to the fact that different cell types and different expression systems were used, which may result in a difference in the expression levels of the fusion proteins and/or a difference in the host cell's capacity in recognizing the signal. Further experiments will be required to resolve this issue.

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