Previously, it has been shown that glycoproteins with ~130-kDa molecular mass react with antisera from patients with renal vasculitis (Kain, R., Matsui, K., Exner, M., Binder, S., Schaffner, G., Sommer, E. M., and Kerjaschki, D. (1995) J. Exp. Med. 181, 585–597). To search for a molecule that reacts with the antibodies, we screened an AG11 human placental cDNA library. Two of the isolated clones were found to encode a putative counterpart of the rodent trans-Golgi network (TGN) glycoprotein 38, hTGN46, which has the tyrosine containing motif YQRL shared by mouse and rat TGN38. Moreover, reverse transcription-polymerase chain reaction analysis of hTGN46 transcripts and genomic analysis of a cDNA deposited as an expressed sequence tag in dbEST Data Base revealed that additional cDNAs exist that are produced by alternate usage of 3’-splice sites of intron III. Alternative splicing results in frame shifts and leads to novel larger translation products with one (for hTGN48) or two (for hTGN51) additional tyrosine-containing motifs. hTGN51 expressed in Chinese hamster ovary cells were localized to the trans-Golgi network, overlapping with β-1,4-galactosyltransferase even after mutating the tyrosine-containing motif common to hTGN46. In contrast, mutated hTGN48 and hTGN46 are no longer retrieved to the TGN. These results strongly suggest that hTGN51 may have a unique function compared with hTGN46 or hTGN48 in shuttling between the cell surface and the TGN.

The trans-Golgi network (TGN) is a tubulo-vesicular structure adjacent to the trans-most cisternae of the Golgi stack. The TGN is the major site where secretory and membrane proteins are sorted to the plasma membrane, lysosomes, endosomes, and secretory granules (1–3). The final stages of processing N-glycans and most likely O-glycans take place in the TGN, and in some instances such unique glycosylation plays a role in sorting to the correct destination (1–3).

TGN38, originally characterized in rat, is a type I integral membrane protein predominantly localized to the TGN (4). The rat cDNA encoding rTGN38 predicts a protein with 357 amino acid residues. The cytoplasmic domain consisting of 33 amino acids contains a YQRL sequence, which has been shown in other proteins to be a signal for the endocytic pathway and for lysosomal targeting (5–9) and a retrieval signal for rTGN38 from the plasma membrane (10, 11). Mutation of this motif resulted in constitutive expression of TGN38 at the plasma membrane (10, 11). It has also been demonstrated that the transmembrane domain of TGN38 plays a role as a retention signal in the TGN, making rTGN38 unique in having both retrieval and retention signals (12).

In rat, a cDNA encoding a translational product different from the original rTGN38 was isolated (13). This cDNA has an insertion of 5 bp, which 4 bp replace the counterpart of TGN38 cDNA in the region of the cytoplasmic tail. This insertion changes the reading frame such that the three C00H-terminal amino acids of TGN38 are replaced with a different sequence, and the cytoplasmic tail is 23 amino acid residues longer than that of TGN38. This variant, termed rTGN41, also contains the same tyrosine motif as rTGN38. On the other hand, the mouse homologue of rTGN38 has two forms due to allelic differences (14).

Previously, it has been shown that sera from patients with autoimmune vasculitis were found to react with glycoprotein(s) with a molecular mass of ~130 kDa (gp130s) (15). Monoclonal and polyclonal antibodies were raised against gp130s, and monoclonal antibody AG11 was found to show a staining profile identical to that of sera from patients. Attempts to isolate cDNA clones using AG11 to screen expression libraries have been unsuccessful. In contrast, one 130-kDa glycoprotein has been identified as LAMP-2 in a Agt11 library screen using the antibodies specific to gp130s (15). The subcellular distribution of LAMP-2 differs from that detected by the AG11 antibody, suggesting that LAMP-2 is only one of the members of gp130s (15).

By using the antibodies specific to gp130s in the present study, we screened a Agt11 library constructed from poly(A)+ RNA isolated from human placenta. In addition to cDNAs encoding LAMP-2, we first isolated cDNA encoding the human homologue of rat TGN38, designated human TGN46 (hTGN46). We then detected additional transcripts that encode larger proteins than hTGN46. These novel forms of hTGN46 are produced by different usage of 3’-splicing sites in intron III. The largest protein, hTGN51, is unique in having two additional tyrosine containing motifs and a dileucine motif (16, 17),
both of which together we showed to function as a retrieval signal to the TGN.

**EXPERIMENTAL PROCEDURES**

Preparation of Rabbit Polyclonal Antibodies Specific to gp130s—Rabbit polyclonal antibodies specific to gp130s were prepared as described previously (15). Polyclonal antisera specific to gp130s were passed through *Escherichia coli* lysates immobilized on CNBr-activated Sepharose 4B (Pierce) and purified on gp130s immobilized on CNBr-activated Sepharose 4B (300 μg/ml), as described previously (15).

Isolation of cDNAs Encoding Human TGN46—A *λgt11* cDNA library (18) was screened with affinity purified rabbit anti-gp130 antibodies. After screening 10^6 plaques, 9 positive clones were isolated. Among the clones analyzed, 7 clones were found to harbor cDNAs encoding lamp-2. Using a Blast Search (19), 2 clones were found to harbor cDNAs with no homology to human sequences reported at that time, but both were homologous to sequences encoding the cytoplasmic and transmembrane domains of rat TGN38 (4).

The nucleotide sequence of the obtained clones indicated that the 5'-end was missing. To obtain a complete cDNA clone, 5'-RACE (Life Technologies, Inc.) was carried out (20) using poly(A)~1 RNA from human placenta (CLONTECH Laboratories) as a template and an oligonucleotide complementary to nucleotides 658–675. hTGN46 was amplified by PCR using a 5'-anchor primer provided in the RACE kit and a 3'-PCR nested primer, 5'-TCCA GGC CGT TGG CTC AAG CT-3' (primer 20), which is complementary to nucleotides 133–153 (Fig. 1).

From this 5'-RACE product, cDNA sequence was amplified by PCR using two gene-specific sequences. The upstream primer used was primer 30, 5'-TAT GGA TCC CGC GAG AGC ATT AG-3' (BamHI site underlined; 244 to 246), and the downstream primer was primer 20. The 200-bp PCR product was subcloned into pBluescriptII/SK by T-A cloning.

In parallel the cDNA sequence of one of the clones (4a1) in pBluescript was linearized at an *Xho* I site and subjected to partial digestion with *Sty* I. A 1449-bp cDNA product was then ligated to the 3'-end of the 5'-RACE product described above at a common *Sty* I site. This cDNA was then subcloned into the BamHI and *Xho* I sites of pcDNA3 (Invitrogen), resulting in pcDNA3-hTGN46.

**Fig. 1.** The nucleotide and deduced amino acid sequences of hTGN46. Sequences of the exons and exon-intron boundaries are shown. The transcription initiation site is shown by an arrow with a vertical line. Deduced amino acids are shown under the coding sequence. The leader peptide and transmembrane domain are singly and doubly underlined, respectively. The tyrosine containing motif is indicated by a bold line; potential N-glycosylation sites are indicated by asterisks, and the tandem repeat sequences are shown by arrowheads. The region 1.5 kb downstream from nucleotide 1034, which is 3' to the stop codon, is identical to an EST sequence (accession number EST21065). A polyadenylation signal is denoted by a single underline.
Detection of Multiple Transcripts Encoding hTGN46, hTGN48, and hTGN51—PCR was carried out using various cDNA libraries as templates. For the first PCR, the 5′-primer (primer 11) corresponds to nucleotides 986–1003, and the 3′-primer (primer 10) is complementary to nucleotides 1345–1367 of hTGN46. For the second PCR, the nucleotide sequence encoding the luminal domain (nucleotides 137–1185) was amplified using primer 1 (nucleotides 137–153) and primer 2 (nucleotides 1188–1185).

Fluorescence In Situ Hybridization Analysis of Human TGN46 Gene—A human genomic P1 plasmid library was screened by PCR as described (21). The 5′- and 3′-primers for PCR correspond to the sequence of nucleotides 1245 to 1278 (primer 27) and nucleotides 1386 to 1369 (primer 10), respectively, of hTGN46 sequence. Purified DNA from one of the isolated P1 clones, clone 10508, was labeled with digoxigenin-dUTP by nick translation. Labeled probe was hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood leukocytes under the conditions as described (22).

Isolation of Genomic Clones Harboring the hTGN46 Gene—To isolate genomic clones of TGN46, the above P1 plasmid, 10508, was digested by BamHI and XhoI and cloned into pBluescript. The resultant colonies were then screened initially by hybridization with the cDNA clone 8c1 that encompasses nucleotides 28–2103 as a probe. Five positive clones were then screened by cDNA encompassing either nucleotides 45–153 or nucleotides 1369 to 1386. Three clones harboring the 5′-half of the genomic sequence and two clones harboring the 3′-half were isolated.

 Primer Extension Analysis and Promoter Activity Assays—5′-Primer extension analysis was carried out essentially as described (23). The primer was complementary to nucleotides +23 to +46 (46 is at the end of exon 1). Poly(A)+ RNA from HL-60 and human embryonic kidney 293 cells was annealed to the 5′-end-labeled oligonucleotide and incubated with Superscript II RNase H reverse transcriptase (Life Technologies, Inc.) as detailed previously (23).

Various 5′-ends of the hTGN46 gene were obtained by PCR using the P1 plasmid as template. A 2.7-kb fragment immediately upstream of nucleotide −44 and shorter genomic fragments spanning −1.2 kb to −340 bp were cloned in sense into the pGL3 basic vector (Promega). The shorter fragment was also cloned in the reverse orientation. These nucleotide sequences were determined by sequencing. NIH3T3 cells were transiently transfected with these constructs. Negative and positive controls for transcription were carried out using pGL3 vector itself and PTreLu (CLONTECH), respectively (24). Luciferase Assay System (Promega) and Micrometer LB96B lumimeter (EG&G Berthold) were used. The results were normalized to protein content.

 Isolation of cDNAs Encoding TGN48 and TGN51—cDNA was synthesized using reverse transcriptase, poly(A)+ RNA derived from fetal liver and oligonucleotide r3, shown in Fig. 4, corresponding to 3′-untranslated sequences. Using the cDNAs synthesized as templates, PCR was performed to amplify sequence from exon 3 to exon 4 under the conditions described (16). In the first PCR, the 5′-primer (primer 27) corresponds to nucleotides 1245–1278 and the 3′-primer (primer B) is complementary to nucleotides 1345–1372 of the hTGN51 sequence. This PCR reaction yielded 128- and 87-bp products derived from the transcripts for TGN51 and TGN48 (Fig. 2C). For isolation of cDNA encoding TGN51, another PCR was carried out to isolate the 3′-region of the sequence using a 5′-primer (primer A) corresponding to nucleotides 1301–1321 and a 3′-primer (primer 54) complementary to nucleotides 1518–1547. This PCR product and the 128-bp product described above were then used as a template for PCR using primers 27 and 54 as described (16), resulting in cDNA encoding nucleotides 1245–1547 of hTGN51.

For cloning of cDNA encoding TGN48, a second PCR was carried out to amplify a sequence between nucleotides 1350 and 1547. Using this PCR product and the 87-bp fragment obtained in the first PCR as templates, a final PCR was carried out using primers 27 and 54. Control experiments were performed omitting reverse transcriptase or after digestion of poly(A)+ RNA by ribonuclease A. These cDNA fragments were cloned into PmiI and XhoI sites of pcDNA3-hTGN46, replacing the corresponding sequence of hTGN46, which resulted in pcDNA3-TGN46 and pcDNA3-TGN51, respectively.

Mutation of Tyrosine Residue 430 in the Cytoplasmic Domain—Site-directed mutagenesis was carried out by PCR using pcDNAI-hTGN46 as a template as described (28). The 5′-primer for this PCR (primer 30) was used in the isolation of pcDNA3-TGN46. The 3′-primer was 5′-TCTGTATTAGGACTTCGTTCCCCAGGTGTTGTGACTCT-3′, complementary to nucleotides 1280–1318 in hTGN46, in which the tyrosine codon was changed to asparagine, as shown by double underlines. The PCR product was blunt-ended by vent DNA polymerase, digested with BamHI, and cloned into the BamHI and EcoRI sites of pcDNA3, resulting in pcDNA3-TGN46YN.

To isolate cDNA encoding mutated TGN48 and 51, cDNA fragments obtained by PmiI and XhoI digestion were separately cloned into pcDNA3-TGN46YN which had been digested with the same restriction enzymes, resulting in pcDNA3-hTGN46YN and pcDNA3-hTGN51YN.

Preparation of Anti-TGN46/48/51 Antibodies—The fragment of cDNA encoding part of the luminal domain of hTGN46 was excised from clone 4a1 with BamHI and HindIII (position 862) (see Fig. 1) and ligated in-frame into pMALc2 (New England Biolabs). Transformed E. coli DH5a were induced to express a soluble maltose binding fusion protein (15). Purified recombinant fusion protein was digested with factor Xa, resulting in precipitates that almost exclusively contained hTGN46 peptide. One hundred μg of this protein were used for the initial immunization and for further boosts. Sera were passed through a column of bacterial proteins produced in E. coli DH5a and then purified by adsorbing on protein A-Sepharose (Pierce) as described (15, 27).

The specificity of the antibodies was confirmed by Western blot analysis of TGN46 contained in fusion proteins and human tissues performed as described previously (15, 27).

Expression of TGN46, TGN48, TGN51, and Corresponding Mutant Forms in CHO Cells—To express hTGN46 and related glycoproteins, pcDNA3-hTGN46 was transfected into Chinese hamster ovary (CHO) cells grown on coverslips using LipofectAMINE (Life Technologies, Inc.) as described (27). In some experiments, TGN glycoproteins were co-expressed with human β1,4-galactosyltransferase. For this, pcDNA1-GalT (28) and pcDNAI-hTGN46, hTGN48, hTGN51, or corresponding mutant proteins were transiently transfected into CHO cells.
Rabbit anti-hTGN46 antibodies and a mouse monoclonal antibody specific to human β-galactosyltransferase (29) were used in the assay. Samples were visualized as described (27, 30). When cell surface proteins were to be detected, saponin was omitted throughout the procedure.

Immunocytochemical Staining for Electron Microscopy—Indirect immunoperoxidase staining was performed as described previously (15, 27, 31). For gold labeling, ultrathin frozen sections of paraformaldehyde/lysine/periodate-fixed normal human kidneys were incubated with purified rabbit anti-hTGN46 antibodies followed by goat anti-rabbit IgG-10-nm gold conjugate (1:20, Auroprobe, Amersham Corp), as described (15). Controls were performed either by omitting the primary antibody or by replacing it with rabbit preimmune serum.

RESULTS

Cloning of cDNA Encoding Human TGN46 Glycoprotein—A λgt11 cDNA expression library constructed from poly(A)⁺ RNA of human term placenta was screened with affinity purified rabbit anti-gp130s antibodies. After screening 10⁶ plaques, 9 positive clones were isolated. Among them, seven clones were found to harbor cDNAs encoding LAMP-2, whereas two other clones were found to have no homology to any human sequences in the data base at that time.

Further analysis of these two clones revealed that they contain sequences highly homologous to the cytoplasmic domain of rat TGN38, rTGN38 (4). We designated the protein encoded by these clones as human TGN46, hTGN46. Comparing rTGN38 to human cDNA sequences cloned, two clones, 4a1 and 8c1, contain almost all of the coding sequence but lack the extreme 5’-region. The sequence obtained by 5’-RACE reaction was ligated to one of the cDNA clones isolated (4a1) at a common StyI site to form a cDNA encoding the entire hTGN46 polypeptide, resulting in pcDNA3-hTGN46.

Sequencing the isolated cDNA in pcDNA3-hTGN46 revealed an insert of 2142 base pairs encoding a single open reading frame, predicting a protein of 437 amino acids (molecular weight, 45,724) (Fig. 1). In Fig. 1, we combine results obtained from cDNA and genomic clones described below. The hydropathy plot (data not shown) indicates that the amino acid sequence encompassing from residue 1 to 21 and that from 385 to 402 are hydrophobic, suggesting that they represent a leader peptide and a transmembrane domain, respectively. The luminal domain of hTGN46 contains nine potential N-glycosylation sites and a large segment enriched in proline, glycine, serine, and threonine residues, which likely serve as O-glycosylation sites. Notably, the sequence from residues 55 to 250 consists of 14 tandem repeats of 14 amino acid residues (see sequence between two horizontal arrows in Fig. 1). This sequence is followed by a domain enriched in acidic amino acids (see dotted line in Fig. 1), the transmembrane domain, and the cytoplasmic segment. It is noteworthy that there is no homology in the luminal domain between hTGN46 and rodent TGN38 except for the acidic region (4, 13, 14).

Characterization of cDNAs Related to hTGN46—To determine whether variants of hTGN46 are expressed in placenta and kidney, cDNA sequences of hTGN46 were amplified using various cDNA libraries as templates. When the sequence encoding the luminal domain was amplified by PCR, only one product (~1.0 kb), that expected from hTGN46 sequence, was obtained (Fig. 2B). In contrast, two bands were obtained when PCR was carried out with primers to the luminal domain and 3’-untranslated sequence (Fig. 2A). Of these products, the shorter one (402 bp) corresponds to the hTGN46 sequence (nucleotides 998–1399). The longer product is larger than this shorter product by approximately 60 bp.

The results suggest that there is more than one form of hTGN46 and that a novel form contains additional sequences between the acidic region and the COOH terminus.

The hTGN46 Gene Is Mapped to Chromosome 2 Band

![Fig. 3. Chromosomal localization of hTGN46 gene as revealed by fluorescence in situ hybridization.](image)

A, specific hybridizations are discernible by fluorescent staining (shown by arrows) on chromosome 2, which was labeled by propidium iodide. B, specific hybridization for the hTGN46 gene occurs on chromosome 2, band p11.2.
Different forms of hTGN46 described above could be produced by different genes or by alternative splicing of mRNA encoding hTGN46. To determine whether more than one gene encodes hTGN46 and related sequences, the hTGN46 gene was localized on chromosomes using fluorescence in situ hybridization procedures. These experiments demonstrated that hTGN46 gene is localized in chromosome 2. Measurement of 10 specific hybridizations of chromosome 2 demonstrated that 10508 is located at a position 10% the distance from the centromere to the telomere of chromosome 2 arm p, an area corresponding to chromosome 2, band p11.2 (Fig. 3). The results strongly suggest that only one gene encodes different forms of hTGN46, although tandemly arranged multiple genes would provide the same results.

Isolation of Genomic Clones Harboring the hTGN46 Gene—The above results suggest that different transcripts of hTGN46 are produced by alternate splicing. To determine whether variant transcripts are produced from the same gene, we isolated genomic clones from the P1 plasmid, as detailed in “Experimental Procedures.” Two representative genomic clones were obtained, and clone 6 contained exon 1 to intron III sequence, whereas clone 24 contained intron III to exon 4 sequence. The genomic organization of hTGN46 as shown in Fig. 1 is derived from this analysis.

Primer extension analysis showed that the transcription start site lies 62 bp upstream from the initiation methionine codon. In addition, the 5′-flanking sequence from 2.7 kb to 42 bp upstream from the initiation methionine codon showed strong promoter activity (data not shown). These results suggest that different transcripts are not produced by different initiation of transcription or from different genes.

Differential Usage of 3′-Splice Sites in Intron III—The results shown in Fig. 2, A and B, also indicate that a portion of the sequences present in introns II or III may be transcribed in longer forms of hTGN46. We were particularly interested in determining whether the 5′-splice or 3′-splice site of intron III differs in those larger transcripts. EST (expressed sequence tag) data bases were searched with the nucleotide sequences around the 5′- and 3′-splice sites of intron III, and one sequence (accession number H82891), which contains a 35-bp sequence upstream from the 3′-splice site of intron III (Fig. 4), was identified. There is a 3′-splice site (agl) 24 bp upstream from the 5′-end of the EST sequence (Fig. 4),
suggesting that agI is utilized in this cDNA as a 3'-splice site.

To determine whether such a transcript exists, RT-PCR was performed using a 5'-primer in exon 3 and a 3'-primer that includes intron III sequence immediately upstream from the 3'-splice site of intron III (primer B in Fig. 4). This PCR produced two fragments 128 and 87 bp in size (Fig. 2C). The size of the larger fragment is the one expected when the upstream 3'-splice site (agI marked by I in Fig. 4) is utilized. Notably, the transcript for the shorter form was barely detected in 293 and HL-60 cells (Fig. 2C). To confirm the presence of the larger transcript, PCR was performed using a 5'-primer (A) which includes the 5'-end of the sequence starting after agI (Fig. 4). The results clearly indicate that this 234-bp transcript exists in all cells tested (Fig. 2C). The expression of this transcript was confirmed by Northern blot analysis using probe 59, which corresponds to the sequence present between agI and agII (Fig. 4), as shown in Fig. 5. It is noteworthy that the transcript of TGN51 is more abundant in fetal lung and kidney than fetal brain and liver.

Isolation of cDNA Encoding Novel Forms of hTGN46—The above results indicate that cDNA encoding novel forms may be isolated by PCR amplification of sequences between exon 3 and exon 4. However, the majority of PCR products amplified by primers 10 and 11 shown in Fig. 1 was derived from hTGN46 mRNA, probably because the transcripts of the variants are present in low abundance. The sequence from nucleotides 1300 to 1547 was thus obtained by RT-PCR using primers A and 54 shown in Fig. 4. This PCR product, representing the 3'-half of the sequence, and the 128-bp PCR product shown in Fig. 2C (right lanes) were used as templates to obtain a cDNA sequence encompassing nucleotides 1245–1547 using primers 27 and 54. The cDNA fragment was digested with PflMI and XbaI and cloned into the corresponding portion of pcDNA3-hTGN46, resulting in pcDNA3-hTGN51 (see Fig. 4).

To clone hTGN48, the cDNA encompassing nucleotides 1350–1547 was obtained by PCR using primers 57 and 54 shown in Fig. 4. This PCR product and the 87-bp PCR product shown in Fig. 2C (right lanes) were used for templates to amplify the sequence containing nucleotides 1245–1308 and 1350–1547 (see Fig. 4) in a contiguous sequence. This PCR product was then cloned into PflMI and XbaI sites of pcDNA3-hTGN46, replacing the corresponding portion of hTGN46 and forming pcDNA3-hTGN48 (see Fig. 4).

hTGN48 and hTGN51 are produced by alternative usage of 3'-splice sites of intron III. These alterations in splice sites change the reading frame such that the COOH-terminal amino acid of hTGN46 is replaced with different and larger sequences resulting in pcDNA3-hTGN46, pcDNA3-hTGN48, and pcDNA3-hTGN51 (see Fig. 4).

Expression of hTGN46, hTGN48, and hTGN51—Rabbit antiserum was raised against the lumenal domain of hTGN46. TGN38 endogenously expressed in CHO cells did not react with the above prepared antiserum, consistent with the fact that hTGN46/48/51 and rodent TGN38/41 have no homology in the NH2 terminus region. CHO cells were thus used as recipient
cells for introducing cDNA encoding TGN46 and its variants.

In the first set of experiments, pcDNA1-human GalT (28) and cDNA encoding hTGN46, hTGN48, or hTGN51 were expressed together in CHO cells. The results, as shown in Fig. 6 (top panel), demonstrated that most of the β-galactosyltransferase staining overlaps with all three forms of human TGN glycoproteins in distribution, showing strong yellow staining.

To confirm the above results, immunoelectron microscopy was performed on sections of normal human kidney using antibodies specific to hTGN46 and its variants. As shown in Fig. 7A, TGN46 was detected in cisternae, which are at the far end of the Golgi complex with regard to the distance from centriole, indicating that TGN46 resides at the trans-Golgi cisternae. Similar results were obtained on kidney tubular epithelial cells (Fig. 7, B–D). These results support the above conclusion that hTGN46 and its variants are present in the trans-Golgi cisternae.

Mutation of the Tyrosine Motif at Residues 430–433—As shown in Fig. 4, TGN46, TGN48, and TGN51 share the same amino acid sequences up to residue 436, indicating that all forms of these related proteins have a common tyrosine-containing motif from residue 430 to residue 433. TGN46 and TGN51 contain one and two additional tyrosine motifs, respectively (Fig. 4).

To determine whether these additional tyrosine-containing sequences in hTGN46 and hTGN48 function as retrieval signals, tyrosine residue 430 was mutated to asparagine in TGN46, TGN48, and TGN51, and the mutated proteins were then expressed in CHO cells. As shown in Fig. 6 (lower panel), mutated TGN46 and TGN48 were localized at the plasma membrane, and a very small portion was retrieved to the TGN. In contrast, TGN51 was still retrieved to the TGN even after the tyrosine-containing motif at residues 430–433 was mutated (Fig. 6, lower panel). To confirm the results shown in Fig. 6, wild type and mutant TGN46, TGN48, and TGN51 were expressed in CHO cells, and those cells expressing the proteins were examined without permeabilization. As shown in Fig. 8A, C, and E, wild type TGN46, TGN48, and TGN51 were faintly expressed on the cell surface. In contrast, mutant TGN46 and TGN48 were substantially expressed on the cell surface, whereas only a small amount of mutant TGN51 was expressed on the cell surface (Fig. 8, B, D, and F). These results indicate that the tyrosine motif at residues 430–433 is critical for retrieving TGN46 and TGN48, and that Tyr-Ser-Ser-Gly (residues 444–447) in TGN48 does not function well as a retrieval signal.

In the present study, we have cloned cDNAs encoding human TGN46 (hTGN46) using antibodies reacting with glycoproteins of ~130 kDa. The deduced amino acid sequence of the isolated cDNA shows that the COOH terminus is highly homologous to rat and mouse TGN38 (4, 14). The subcellular distribution of hTGN46 still differs from AG11 (15), and it is not clear if this is a good candidate for autoimmune antigens in vasculitis. Human TGN46 was not cloned when we started our studies; however, a sequence almost identical to that described here but shorter on both the 5’- and the 3’-ends has been reported recently (32).

In the present study, we have also isolated novel forms of TGN46. The presence of novel forms was apparent when the sequence between the luminal domain and 3’-untranslated region was amplified by PCR using various cDNA libraries as templates (Fig. 2A). Genomic analysis and sequence comparison of the intron III sequence and EST data bases revealed that these novel transcripts are produced by alternative usage of the 3’-splice of intron III (Fig. 4). We show that the two novel forms are 58 and 17 bp larger than that of hTGN46. More importantly, these alternative usages of 3’-splice sites result in frame shifts resulting in one (TGN48) or two (TGN51) additional tyrosine-containing motifs.

Expression of hTGN46, hTGN48 and hTGN51 demonstrated that these proteins reside mostly in the TGN (Figs. 6 and 7). Although TGN46 is ubiquitously present in various tissues, TGN51 is more abundant in fetal lung and kidney, whereas TGN48 is barely present in embryonic kidney 293 and promyelocytic HL-60 cells (Figs. 2 and 5). We also demonstrated that the additional tyrosine-motif found in hTGN51 functioned as a retrieval signal to the TGN, since mutation of the tyrosine-motif shared by all three of these forms did not alter its traffic to the TGN. In contrast, the second tyrosine motif in hTGN48 functioned poorly as a retrieval signal (Figs. 6 and 8). The additional tyrosine motif in hTGN51 contains the amino acid sequences of Tyr-Val-Leu-Leu and Tyr-Ile-Pro-Leu, whereas that in hTGN48 is Tyr-Ser-Ser-Gly. The results obtained in mutant hTGN48 and hTGN51 are consistent with previous results demonstrating that a tyrosine motif for the endocytic pathway should contain a bulky hydrophobic amino acid at the fourth position from the tyrosine (5–11). Apparently, Tyr-Ser-Ser-Gly in hTGN48 does not function as a retrieval signal through an endocytic pathway.

In addition to tyrosine-containing motif, hTGN51 contains a Leu-Leu motif at residues 475–476 (Fig. 4). It has been shown that this motif also serves as an endocytic pathway and lyso-
somal targeting signal (16, 17, 33–35). It is thus possible that hTGN51 contains a fourth retrieval signal in addition to three tyrosine-containing motifs. In this regard, it is noteworthy that rat TGN41 also contains a Leu-Val motif (13). This motif worked as a lysosomal targeting signal when the last 9 amino acids of rat LimpII sequence was replaced with human mannose-6-phosphate receptor sequences containing a Leu-Val sequence (16). These results strongly suggest that Leu-Val and Leu-Val in hTGN51 and rTGN41, respectively, may work as retrieval signals for these proteins. On the other hand, it has been suggested that other amino acids such as acidic amino acids or phosphorylation sites are required in order for Leu-Val (Val) motif to function (36, 37). Further studies will be necessary to determine whether the Leu-Val and Leu-Val motif in hTGN51 and rTGN41 work as retrieval signals to the Golgi.

It is noteworthy that the transmembrane domains are also well conserved among human TGN46/48/51 and rodent TGN38/41. It has been shown that the transmembrane portion of TGN38 is critical as a Golgi retention signal (12). Similarly, the transmembrane domain was shown to be critical as a retention signal for Golgi resident proteins such as glycosyltransferases (28, 38–41). These results strongly suggest that the transmembrane domain of hTGN46, hTGN48, and hTGN51 play a role as a Golgi retention signal. In addition to the above two domains, the amino acid sequence upstream from the transmembrane domain (residues 314–384, Fig. 1) is also conserved well among human, rat, and mouse TGN glycoproteins. This sequence is enriched in acidic amino acid residues, but the role(s) of this acidic region is not known.

In human TGN46 variants, transcripts diverge three codons after the tyrosine-containing motif shared by all three forms of TGN glycoproteins. By comparing rat TGN38 and TGN41 sequences (4, 13), it is possible to infer that these two different transcripts start after one codon from the tyrosine motif, shared by rTGN38 and rTGN41. Although the genomic organization of rTGN38/41 has not been elucidated, it is possible that these two forms arise from different usage of the 3′-splice sites as demonstrated for human TGN46, -48, and -51 in the present study.

It has been reported that chicken Lamp-2 variants differ in the transmembrane domain and the cytoplasmic tails due to alternative splicing (42). These variations in the transmembrane and cytoplasmic domain were found to influence the rate of internalization of Lamp-2, resulting in different levels of cell surface expression exhibited by variants of Lamp-2 (43). These results strongly suggest that hTGN46 variants may also differ in the rates of retrieval to the TGN. Such a difference may suggest different functions for different forms of hTGN46 on the cell surface and the TGN.

It has been proposed that both rTGN38 and rTGN41 together associate with p62 and Rab6 and that this complex is involved in budding of exocytic vesicles (44). Other studies have shown that the tyrosine motif in rTGN38 associates with AP-1 present in clathrin-coated pits (45). Moreover, the tyrosine-containing and dileucine motifs are apparently recognized by different cytoplasmic carriers (46). These results point toward the possibility that hTGN48 and hTGN51 in particular may have unique function in association with hTGN46 in the process of both endocytic and exocytic pathways. The discovery of two novel forms of human TGN glycoproteins hTGN48 and hTGN51 will allow us to determine the roles that TGN glycoproteins play in these intricate intracellular movements.

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