Localization of TGN38 to the *trans*-Golgi Network: Involvement of a Cytoplasmic Tyrosine-containing Sequence

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Abstract. Protein localization to the TGN was investigated by examining the subcellular distribution of chimeric proteins in which the cytoplasmic and/or transmembrane domains of the TGN protein, TGN38, were substituted for the analogous domains of the plasma membrane protein, Tac. Using immunofluorescence and immunoelectron microscopy, the COOH-terminal cytoplasmic domain of TGN38 was found to be sufficient for localization of the chimeric proteins to the TGN. Deletion analysis identified an 11-amino acid segment containing the critical sequence, YQRL, as being sufficient for TGN localization. TGN localization was abrogated by mutation of the tyrosine or leucine residues in this sequence to alanine, or of the arginine residue to aspartate. In addition to specifying TGN localization, the 11-amino acid segment was active as an internalization signal, although the property of internalization alone was insufficient to confer TGN localization. Overexpression of chimeric proteins containing TGN localization determinants resulted in their detection at the plasma membrane and in intracellular vesicles, and abolished detection of endogenous TGN38. These results suggest that discrete cytoplasmic determinants can mediate protein localization to the TGN, and reveal a novel role for tyrosine-based motifs in this process.

THE Golgi complex plays a central role in the intracellular transport, processing and sorting of proteins in the secretory pathway. Morphological and functional studies have established that the Golgi complex of higher eukaryotes is organized into at least three contiguous but distinct regions: the *cis*-Golgi network (CGN)[1], the Golgi stack and the TGN (reviewed by Mellman and Simons, 1992; Rothman and Orci, 1992). The CGN is a collection of tubules connected to the *cis*-Golgi cisternae which serves as the entry site for proteins transiting from the ER into the Golgi complex. Itinerant proteins entering the CGN are either recycled back to the ER or routed forward into the Golgi stack (Huttner and Tooze, 1989; Hsu et al., 1991; Pelham, 1991). The Golgi stack is composed of a variable number of flattened cisternae, in which an array of resident carbohydrate-modifying enzymes sequentially process the oligosaccharide chains of glycoproteins (Farquhar and Palade, 1981; Paulson and Colley, 1989). Immediately apposed to the *trans*-most aspect of the Golgi stack is the TGN (Roth et al., 1985; Geuze et al., 1985), a tubulo-reticular structure where proteins undergo further carbohydrate modifications and, in some cases, proteolytic processing (Duncan and Kornfeld, 1988). In addition to having a role in biosynthetic protein processing, the TGN is a major sorting compartment for proteins destined to more distal organelles of the secretory pathway, such as lysosomes, endosomes, secretory granules, and different domains of the plasma membrane (Griffiths and Simons, 1986).

The differentiated structure and function of the various Golgi regions is likely the result of the ordered localization of specific proteins to successive compartments of the system. Maintenance of such an organized arrangement of resident proteins requires mechanisms that retain certain proteins at defined locations while allowing other proteins to flow through the Golgi complex. Additional mechanisms must also exist to sort itinerant proteins along the different routes emanating from the Golgi complex. Both protein residence and sorting in the Golgi complex are thought to be mediated by specific structural signals. Recent studies have investigated the nature of retention signals of proteins that are specifically localized to the Golgi stack, including the avian coronavirus E1 glycoprotein and several glycosyltransferases (reviewed by Machamer, 1991). For all of these proteins, information necessary for Golgi localization was contained within the membrane-spanning domains. No obvious homology was noticed among the different transmembrane sequences involved in Golgi localization, suggesting a retention process that relies on a physical property of the membrane-spanning domains rather than on receptor-mediated recognition of a specific sequence motif. This lack of homology and the inability to saturate the retention mechanism by

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1. *Abbreviations used in this paper*: CGN, *cis*-Golgi network; DPAP A, dipeptidyl aminopeptidase A; TGN, *trans*-Golgi network.

overexpression of these proteins have suggested that localization to the Golgi stacks may be caused by formation of transport-incompetent assemblies involving transmembrane domain interactions (Machamer, 1991; Swift and Machamer, 1991; Munro, 1991; Nilsson et al., 1991).

In contrast to the growing amount of information available on structural determinants for retention in the Golgi stacks, very little is known about signals for localization to the TGN. Two isoforms of a rat type I integral membrane protein, known as TGN38 and TGN41, have recently been shown to be specifically localized to a membrane-bound compartment with the characteristics of the TGN (Luzio et al., 1990; Reaves et al., 1992). Mature TGN38 has an apparent molecular weight of 85,000-95,000, of which 38,000 are contributed by the core polypeptide and the rest by N- and O-linked oligosaccharide chains. TGN38 is organized into a 286-amino acid NH_2-terminal lumenal domain, a 21-amino acid membrane-spanning sequence and a 33-amino acid COOH-terminal cytoplasmic tail (Luzio et al., 1990). TGN41 is identical to TGN38, except that the last three amino acids of the TGN38 cytoplasmic tail are replaced by an additional 23-amino acid sequence (Reaves et al., 1992). Since both TGN38 and TGN41 have been localized to the TGN by morphological studies (Luzio et al., 1990; Reaves et al., 1992), the COOH-terminal polypeptide extension in TGN41 is not likely to play any role in TGN localization. Deletion of the cytoplasmic tail of TGN38 results in increased expression of the truncated protein at the cell surface, suggesting that this topologic domain contains information necessary for intracellular localization of the protein (Luzio et al., 1990). The exact nature of the structural information contained within the TGN38 cytoplasmic tail and the possible contribution of other topologic domains of the protein to TGN localization remain to be established.

In the present study, we have sought to identify specific sequences that are involved in localizing TGN38 to the TGN, as a first step to examine the molecular mechanisms that determine protein localization and sorting in this compartment. The results of our experiments demonstrate that the cytoplasmic tail of TGN38 can confer TGN localization on another protein which normally resides at the plasma membrane. Thus, this cytoplasmic tail contains information that is not only necessary but also sufficient for TGN localization. The sequence YQRL, near the COOH-terminal end of the cytoplasmic tail, is essential for TGN localization. Overexpression of chimeric proteins containing the TGN38 tail results not only in detection of the chimeras at the cell surface, but also in disappearance of endogenous TGN38 from its normal intracellular location. These observations suggest that, unlike retention in the Golgi stacks, localization to the TGN may be mediated by specific recognition of a cytoplasmic determinant containing a critical tyrosine-based motif.

Materials and Methods

DNA Recombinant Procedures

A DNA fragment encoding the transmembrane and cytoplasmic domains of TGN38 (Luzio et al., 1990) was cloned from rat liver RNA by RNA-PCR, performed according to the manufacturer's guidelines (Perkin-Elmer Cetus, Norwalk, CT). The PCR product was cloned into a BglII-XbaI cut pCDM8-Tac plasmid, containing a sequence encoding the Tac lumenal domain (Rutledge et al., 1992). All subsequent Tac-TGN38 chimeric and mutant DNAs were constructed by PCR, according to the procedure of Higuchi et al. (1988) and also cloned into pCDM8. The sequence of all the recombinant constructs was confirmed by the dideoxy chain-termination method.

Antibodies

The following antibodies were used: 7G7, mouse mAb to human Tac (Rubin et al., 1985*a*); anti-Tac, mouse mAb to human Tac (Uchiyama et al., 1981; provided by T. Waldmann, National Institutes of Health, Bethesda, MD); R3134, rabbit polyclonal antibody to human Tac (Sharon et al., 1986; provided by W. Leonard, National Institutes of Health); anti-mannosidase II, rabbit polyclonal antibody to rat α-mannosidase II (a gift of K. Moremen, University of Georgia, Athens, GA); and anti-TGN38, rabbit polyclonal antibody raised against a rat TGN38 fusion protein (Luzio et al., 1990; Reaves et al., 1992; a gift of G. Banting, University of Bristol, Bristol, U.K.). The rabbit anti-TGN38 antibody recognizes epitopes of the lumenal domain of TGN38 and does not react with the Tac-TGN38 chimeras or mutants used in our studies (Reaves et al., 1992; and data not shown). Fluorescently labeled donkey anti-mouse IgG and donkey anti-rabbit IgG suitable for multiple labeling experiments were purchased from Jackson ImmunoResearch (West Grove, PA).

Cells

All cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DME (Biofluids, Rockville, MD) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium).

Stable Transfectants

CV-1 or NRK cells were plated at a density of 3×10^5 cells/100-mm culture dish and grown overnight at 37°C. 3 h before transfection, cells were fed with 10 ml of fresh complete medium. Cells were incubated 16 to 20 h with 20 μg recombinant plasmid DNA and 2 μg pFNeo (Saito et al., 1987) precipitated by calcium phosphate (Graham and van der Eb, 1973; as modified by Gorman et al., 1983). Cells were then washed with PBS, treated with 10% DMSO in ice-cold PBS for 5 min, washed with PBS, and then incubated in complete medium for 24 h before selection in 1 mg/ml (for CV-1 cells) or 2 mg/ml (for NRK cells) active geneticin (G418; GIBCO/BRL, Gaithersburg, MD) in complete medium. Stably transfected clones were identified by immunofluorescence microscopy as below.

Immunofluorescence Microscopy

Cells grown to 40–50% confluence on glass coverslips were transiently transfected with 5 μg of DNA by the calcium phosphate precipitation method, as described above. After 36 to 60 h, cells were fixed for 15 min at room temperature in 2% formaldehyde in PBS and washed in PBS. The cells were then incubated for 1 h with primary antibodies in PBS containing 0.1% BSA and 0.2% saponin, washed in PBS, incubated with fluorescently labeled secondary antibodies for 30 min, washed again in PBS, and then mounted onto glass slides with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Samples were examined under a Zeiss inverted microscope equipped with a 63× lens (Carl Zeiss, Oberkochen, Germany). The same immunofluorescent labeling procedure was used for staining stably transfected cells.

Metabolic Labeling and Immunoprecipitation

Cells grown to 70 to 90% confluence in 100-mm dishes were transfected by the calcium phosphate precipitation method (Graham and van der Eb, 1973) using 20 μg of DNA per transfection. 36 h after transfection, cells were washed twice in PBS and incubated for 10 min at 37°C in 15 ml of methionine-free DME containing 15 mM EDTA to release cells from the dish. Cells were then washed twice in methionine-free DME, and incubated for 30 min at 37°C in 2 ml of 0.5 mCi/ml [^{35}S]methionine (Tran ^{35}S-Label, ICN Radiochemicals, Irvine, CA) in methionine-free DME containing 5% dialyzed FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Pulse-labeled cells were chased for different periods at 37°C in complete medium. Cell pellets were solubilized in 1 ml lysis buffer (0.5% [wt/vol] Triton X-100, 0.3 M NaCl, 50 mM Tris-HCl buffer, pH 7.4). Specific proteins were isolated from detergent-solubilized cells and from culture supernatants using protein A-bound antibody to Tac (7G7) as described (Bonifacino et al., 1990). Proteins were resolved by one-dimensional SDS-PAGE under

reducing conditions on 10% acrylamide gels. Quantitation was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Antibody Internalization

CV-1 cells were plated at 60% confluence in 100-mm tissue culture dishes and transfected with 20 μg of DNA by the calcium phosphate precipitation method. At 48 h after transfection, cells were trypsinized and replated in sterile 100-mm Petri dishes. At 60 h after transfection, cells were incubated for 5 to 10 min in complete medium containing 10 mM Hepes/NaOH, pH 7.3, and 10 mM EDTA to release the cells into suspension. Cells were washed twice with ice-cold DME at 4°C and incubated for 30 min at 4°C with ^{125}I-labeled anti-Tac antibody at 1×10^7 cpm/ml (~1 μg/ml) as described (Weissman et al., 1986). The labeled anti-Tac antibody was spun in a Beckman Airfuge (Beckman Instruments, Inc., Fullerton, CA) at 25 psi for 45 min immediately before use to remove antibody aggregates. Cells were washed three times in ice-cold DME, resuspended in cold complete medium and incubated in a 37°C water bath to allow antibody uptake. At the indicated times, the cells were placed on ice, pelleted, and incubated in 250 μl of DME with or without 1 mg/ml proteinase K for 30 min at 4°C. Cells were then centrifuged through a 200-μl cushion of FBS. Radioactivity of cell pellets was measured in a Beckman 5500 gamma counter (Beckman Instruments, Inc.). All experiments were performed in duplicate.

Immunoelectron Microscopy

EM of ultrathin cryosections of cells was performed as previously described (Peters et al., 1991*a*). Briefly, cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 37°C, washed in PBS containing 0.15 M glycine, and then embedded in 10% gelatin. Small gelatin blocks were incubated overnight in 2.3 M sucrose at 4°C and then frozen in liquid nitrogen. 80-nm thin cryosections were made with a cryo-ultramicrotome (Ultracut S; Reichert Jung, Vienna) using a Diatome diamond knife (Slot et al., 1988) and incubated at room temperature with a polyclonal antibody to Tac (R3134) for 30-60 min followed by incubation with protein A–gold complexes (Slot and Geuze, 1985) for 20 min at room temperature. Purified rabbit anti-mouse immunoglobulin (at 2 μg/ml) was used after the mAb and before protein A–gold. In the case of double labeling with anti-TGN38 antibodies, after the first incubation with gold-conjugated protein A, sections were incubated with 1% glutaraldehyde for 10 min and then with PBS containing 0.15 M glycine to prevent interaction between both immunoreagents (Slot et al., 1991). Before double-labeled sections were evaluated, the specificity of antibody staining was ensured by examining single-labeled sections and by reversing the order of incubations, as previously described (Peters et al., 1991*b*). Irrelevant control antibodies did not give any labeling. Sections were examined with a Phillips CM 10 transmission electron microscope.

Results

The Cytoplasmic Domain of TGN38 Contains Information That Is Sufficient for TGN Localization

To determine whether defined regions of the TGN38 molecule contained information that was sufficient for TGN localization, chimeric proteins were constructed by exchanging domains between TGN38 and the Tac antigen (interleukin-2 receptor α chain, Leonard et al., 1984) (Fig. 1, *top*). The Tac antigen is a type I integral membrane protein with a topological organization similar to that of TGN38 (Fig. 1, *top*). Unlike TGN38, however, Tac is primarily localized to the cell surface (Leonard et al., 1984; Weissman et al., 1986; Bonifacino et al., 1990).

In an initial construct, the Tac lumenal domain was fused to the transmembrane and cytoplasmic domains of TGN38 to create the chimeric protein, T-G-G (Tac lumenal-TGN38 transmembrane-TGN38 cytoplasmic) (Fig. 1, *top*). Plasmids encoding Tac and T-G-G were transfected separately into the rat fibroblast line, NRK, and the subcellular distribution of both proteins was examined by immunofluorescence microscopy of fixed, permeabilized cells using an antibody to a Tac lumenal epitope (7G7) (Rubin et al., 1985*a*). We observed that whereas normal Tac was expressed on the plasma membrane (Fig. 1 *a*), the T-G-G chimera was localized to a juxtanuclear, tubulo-vesicular structure in the majority of positively stained cells (Fig. 1 *c*). This distribution was consistent with localization to the TGN, as demonstrated by the similar pattern of immunofluorescent staining observed for endogenous TGN38 in the same cells (Fig. 1 *d*). Approximately 90% of the positively stained NRK cells showed localization of the chimeric protein to the TGN, whereas the remaining 10% also showed localization to the plasma membrane. In addition, ~15% of the cells displayed staining of cytoplasmic vesicles. No changes in the localization of the T-G-G chimera were noticed upon a 3-h incubation of the cells in 10 μg/ml cycloheximide, suggesting that the localization of the protein to the TGN was not a transient phenomenon. In addition to NRK cells, the localization of T-G-G was examined after transient expression in various other cell lines, including cells of monkey (CV-1, COS-1), bovine (MBDK), and human origins (RD4 and HeLa). In all cases, we observed prominent staining of a tubulo-vesicular, and sometimes distinctly reticular, structure characteristic of the TGN (data not shown). These observations suggested that the TGN localization of proteins containing sequences from TGN38 was conserved among several mammalian species. A variable proportion of the cells also showed staining of cytoplasmic vesicles and the plasma membrane, the appearance of which seemed to correlate with higher levels of expression in those cells.

To establish the localization of T-G-G at an ultrastructural level, stably transfected CV-1 cells (Fig. 2 *a*) were examined by immunoelectron microscopy of frozen sections using an antibody to Tac and protein A–gold particles. Deposition of gold particles was confined to tubules and vesicles on the *trans*-most face of the Golgi complex (Fig. 2 *b*), consistent with previous morphological descriptions of the TGN (Griffith and Simons, 1986; Geuze and Morre, 1991). Very little staining of the plasma membrane was apparent in these cells (Fig. 2 *b*), although binding studies with ^{125}I-labeled anti-Tac revealed the presence of a small amount of T-G-G on the surface of the stably transfected cells (~60-fold lower than in cells stably expressing Tac; data not shown). Taken together, the immunofluorescence and immunoelectron microscopy studies of cells expressing the T-G-G chimera suggested that the 54-amino acid residues comprising the transmembrane and cytoplasmic domains of TGN38 contained sufficient information for localization to the TGN.

To further define the sequences responsible for TGN localization, an additional chimeric protein was constructed in which sequences encoding the lumenal and transmembrane domains of the Tac antigen were fused to sequences encoding the cytoplasmic domain of TGN38 (T-T-G, for Tac lumenal-Tac transmembrane-TGN38 cytoplasmic) (Fig. 1, *top*). The T-T-G chimera was expressed by transient transfection of NRK cells, and its subcellular distribution studied by immunofluorescence microscopy. Like T-G-G, the T-T-G chimera was found to colocalize with endogenous TGN38 (Fig. 1, *e* and *f*). Thus, a determinant sufficient for TGN localization was contained within the 33-amino acid cytoplasmic tail of TGN38.

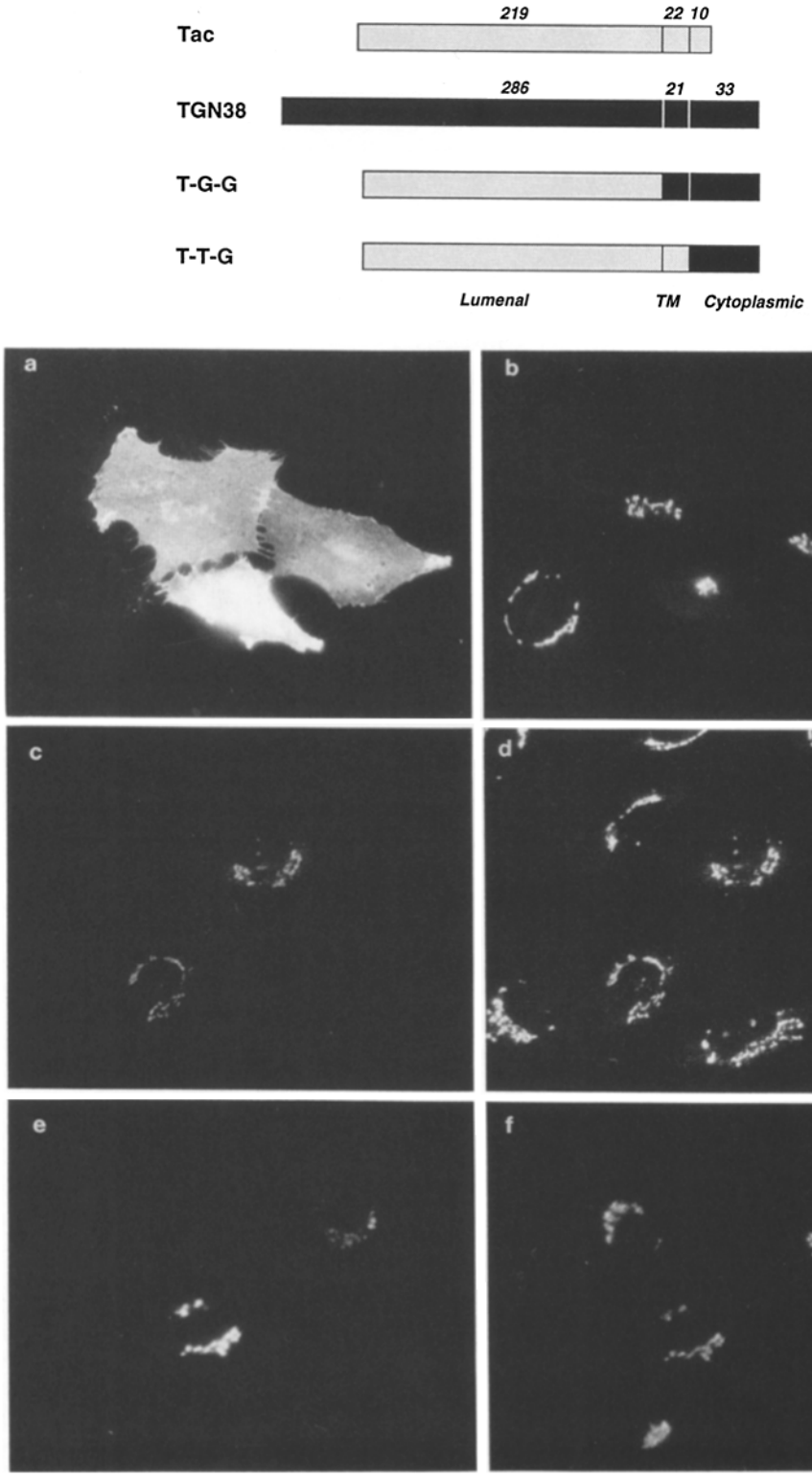


Figure 1. The cytoplasmic tail of TGN38 has sufficient information for protein localization to the TGN. (*Top*) Schematic representation of the Tac and TGN38 proteins, and of the Tac-TGN38 chimeras, T-G-G and T-T-G. The number of amino acids in each topological domain, corresponding to the mature polypeptide, is indicated. (*Bottom*) Immunofluorescence localization of Tac and Tac-TGN38 chimeric proteins in transfected NRK cells. Transiently transfected NRK cells expressing Tac (*a* and *b*), T-G-G (*c* and *d*), or T-T-G (*e* and *f*) were fixed, permeabilized, and stained simultaneously with a mouse antibody to Tac (7G7) and rabbit antibodies to endogenous TGN38, followed by rhodamine-conjugated donkey antibodies to mouse IgG and fluorescein-conjugated donkey antibodies to rabbit IgG. (*a*, *c*, and *e*) Rhodamine channel, Tac staining; (*b*, *d* and *f*) fluorescein channel, TGN38 staining. Notice the presence of normal Tac at the plasma membrane (*a*) and the colocalization of T-G-G (*c* and *d*) and T-T-G (*e* and *f*) with endogenous TGN38. Bar, 10 μm.

Decreased Shedding of Soluble Tac in Cells Expressing Tac-TGN38 Chimeras

As an additional test for the intracellular localization of proteins having the TGN38 cytoplasmic domain, we examined the production of a soluble form of Tac that is normally shed from the cell surface into the culture medium (Rubin et al., 1985*b*; Robb and Kutny, 1987; Cullen et al., 1987). Soluble Tac arises from cleavage of the extracellular domain by a proteolytic activity presumably localized to the plasma membrane (Rubin et al., 1985*b*; Robb and Kutny, 1987; Cullen et al., 1987). COS-1 cells expressing Tac or the Tac-TGN38 chimeric proteins were metabolically labeled and chased for different periods of time. Labeled Tac products were isolated from the cells and from the medium by immunoprecipitation. After 4 h of chase, Tac-expressing cells were found to

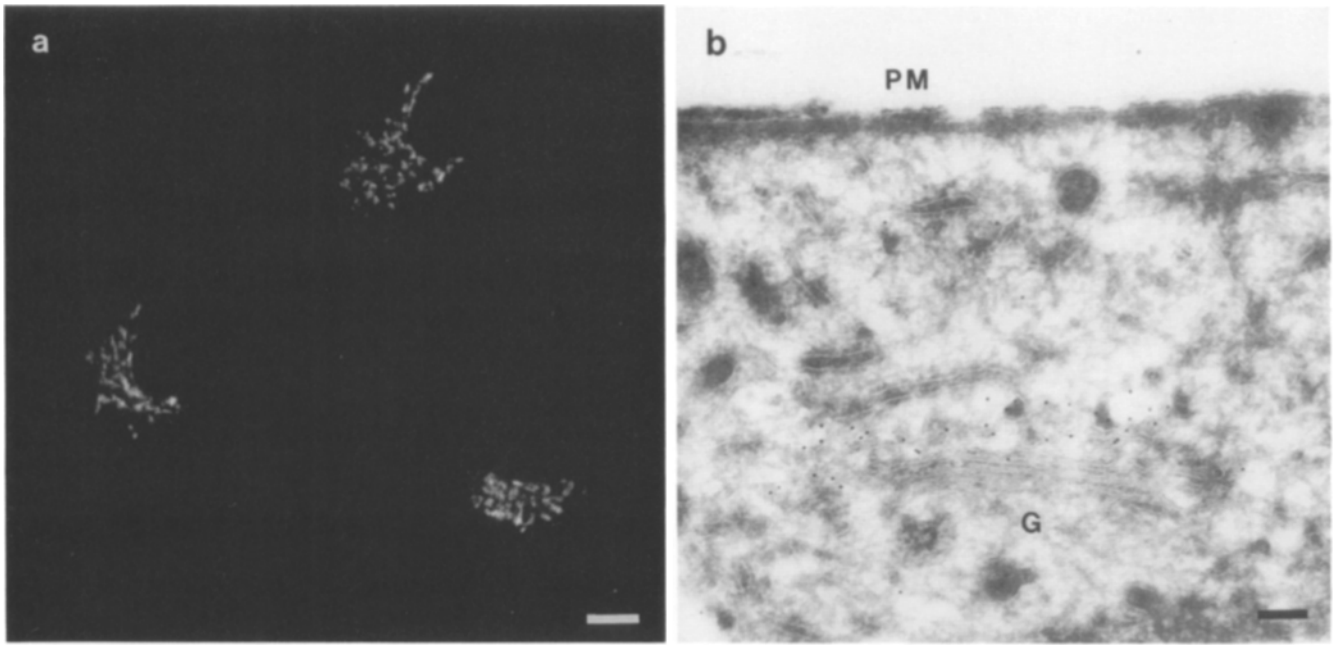


Figure 2. Immunolocalization of the T-G-G chimeric protein in stably transfected cells. (*a*) Immunofluorescence microscopy of CV-1 cells stably transfected with a plasmid encoding the chimeric protein T-G-G. Cells were fixed, permeabilized, and stained with a mouse antibody to Tac (7G7) and a rhodamine-conjugated donkey antibody to mouse IgG. Staining of a Golgi-like structure was observed in all stably transfected cells. (*b*) Immunoelectron microscopy of an ultrathin cryosection from a stably transfected CV-1 cell expressing T-G-G. Thin frozen sections were stained with a rabbit polyclonal antibody to Tac (R3134) and 15-nm gold-conjugated protein A. The chimeric protein was detected in tubules and vesicles on the *trans*-most aspect of the Golgi stack (*G*). Note the absence of gold particles on the plasma membrane (PM). Similar observations were made by electron microscopy of horseradish peroxidase-stained cells performed as described by Yuan et al., 1987 (data not shown). Bars: (*a*) 10 μm; (*b*) 0.2 μm.

shed 27% percent of the initially labeled protein, whereas cells expressing T-G-G and T-T-G shed only 5% (Fig. 3). The decreased shedding of the Tac extracellular domain in cells expressing Tac-TGN38 chimeras is thus consistent with the morphological analyses showing intracellular localization of the chimeras to the TGN. The residual shedding observed in these cells could be due to some level of proteolytic activity in intracellular compartments, or to the surface expression of the chimeric proteins in a fraction of the transiently transfected COS-1 cells.

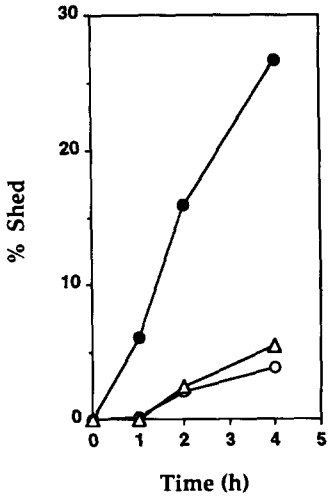


Figure 3. Shedding of a proteolytically cleaved form of Tac from cells expressing Tac and Tac-TGN38 chimeras. Transiently transfected COS-1 cells expressing normal Tac (●), T-G-G (○), or T-T-G (△) were pulse labeled with [^{35}S]methionine for 30 min at 37°C and chased for 0, 1, 2, or 4 h in complete medium. Tac proteins were isolated from detergent-solubilized cells and from culture media by immunoprecipitation with a mAb to Tac (7G7). Immune precipitates were analyzed by SDS-PAGE on 10% acrylamide gels. The amount of soluble Tac released into the medium was quantitated and expressed as a percentage of the amount of Tac synthesized during the pulse.

Identification of Cytoplasmic Residues Critical for TGN Localization

Having established that the cytoplasmic tail of TGN38 was sufficient to confer TGN localization on another protein, we attempted to further delineate the sequences and specific amino acid residues that were critical for this function. To this end, COOH-terminal truncated forms of T-T-G were constructed and transiently expressed in NRK cells (Fig. 4, Δ1–Δ3). Removal of the last four amino acids from the COOH-terminus resulted in a protein that was still localized to a TGN-like structure (Fig. 4, Δ1) and colocalized with endogenous TGN38 (not shown). This was in contrast to Δ2 and constructs with more extensive truncations, all of which showed prominent staining of the plasma membrane (Fig. 4, Δ2 and Δ3). These results implicated the sequence YQRL as containing information necessary for TGN localization. A series of internal deletion mutants of Δ1 (Fig. 4, *ID1–ID5*) were then made, to determine whether additional sequences were also essential for TGN localization. Interestingly, every internal deletion mutant displayed a characteristic TGN pattern of localization in transiently transfected NRK cells (Fig. 4, *ID1–ID5*), with only a minority of cells having distinct plasma membrane staining. In addition to the TGN, some cells showed punctate staining of the cytoplasm (not shown in the figure), which was more pronounced for some of the

Constructs:		IF Pattern
T-T-G	...KRKIIAFALEGKRSKVTRRPKASDYQRLNLKL*	TGN
Δ1	...KRKIIAFALEGKRSKVTRRPKASDYQRL*	TGN
Δ2	...KRKIIAFALEGKRSKVTRRPKASD*	PM
Δ3	...KRKIIAFALEGKRSKVTRRP*	PM
ID1	...KRKIIAFALEGKRSKVT-------YQRL*	TGN
ID2	...KRKIIAFALE-------RRPKASDYQRL*	TGN
ID3	...KRK-------GKRSKVTRRPKASDYQRL*	TGN
ID4	...KRKIIAFALE----------YQRL*	TGN
ID5	...KRK----------RRPKASDYQRL*	TGN

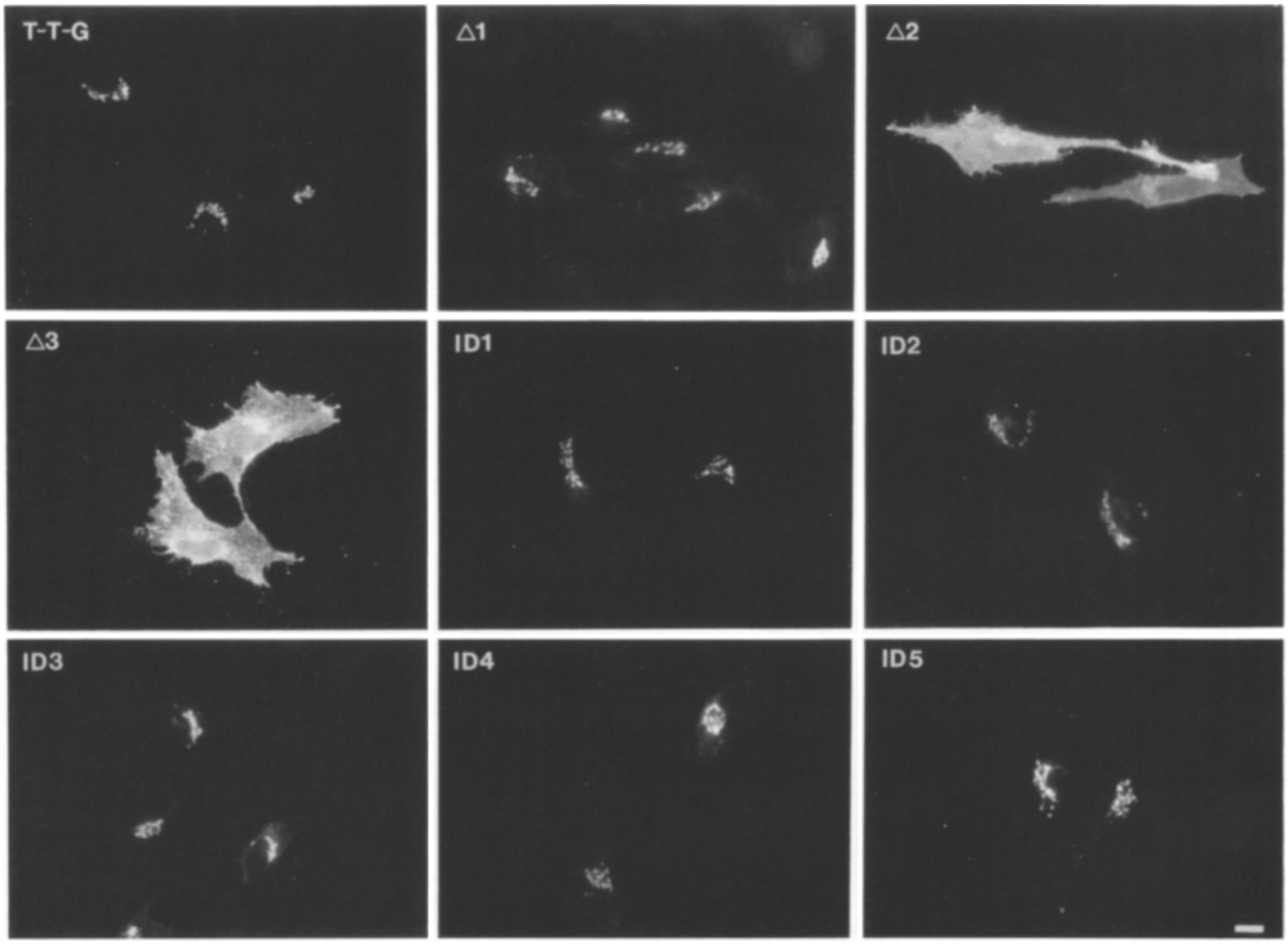


Figure 4. Delineation of cytoplasmic sequences involved in TGN localization. (*Top*) Summary of cytoplasmic COOH-terminal sequences and immunofluorescence (*IF*) patterns of the different constructs derived from T-T-G. Asterisks indicate the COOH termini of the different constructs. (*Bottom*) Immunofluorescence microscopy of transiently transfected NRK cells expressing the different chimeric constructs. Fixed, permeabilized cells were stained with a mouse monoclonal antibody to Tac (7G7) and rhodamine-conjugated antibodies to mouse IgG. Double labeling with antibodies against TGN38 revealed a perfect co-distribution of those chimeras showing TGN-like staining, with TGN38 (data not shown). Bar, 10 μm.

internal deletion constructs, such as ID1. The fact that YQRL was the only sequence, other than the perimembrane basic residues, common to all constructs showing TGN-like staining suggests that these four residues play a preeminent role in localization of the chimeric proteins to the TGN. These observations do not completely rule out the involvement of sequences located NH_2-terminal to YQRL in TGN localization, although no other strict requirement for specific residues was apparent from our deletion analysis.

To unequivocally establish the intracellular localization of a deletion mutant having the fewest residues of the TGN38 cytoplasmic tail, the construct ID5 (Fig. 4, *top*) was stably

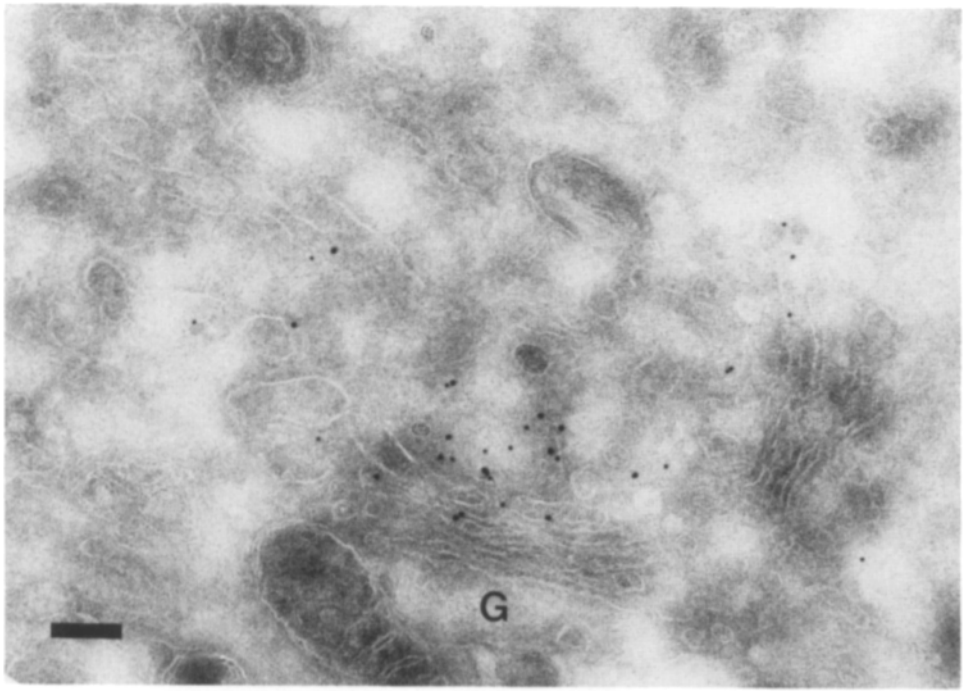
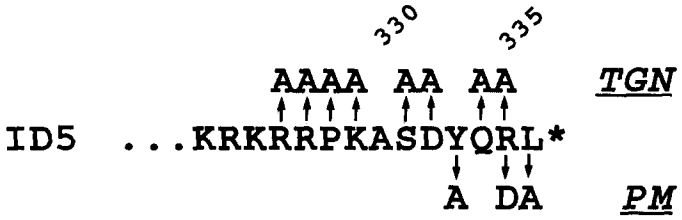


Figure 5. Immunoelectron microscopic co-localization of the ID5 chimeric protein and endogenous TGN38 in stably transfected NRK cells. Thin frozen sections of cells were sequentially immunostained with a rabbit antibody to Tac and 15-nm gold-conjugated protein A, followed by a rabbit antibody to endogenous TGN38 and 10-nm gold-conjugated protein A, as described in the Materials and Methods section. (*G*) Golgi complex. Bar, 0.2 μm.



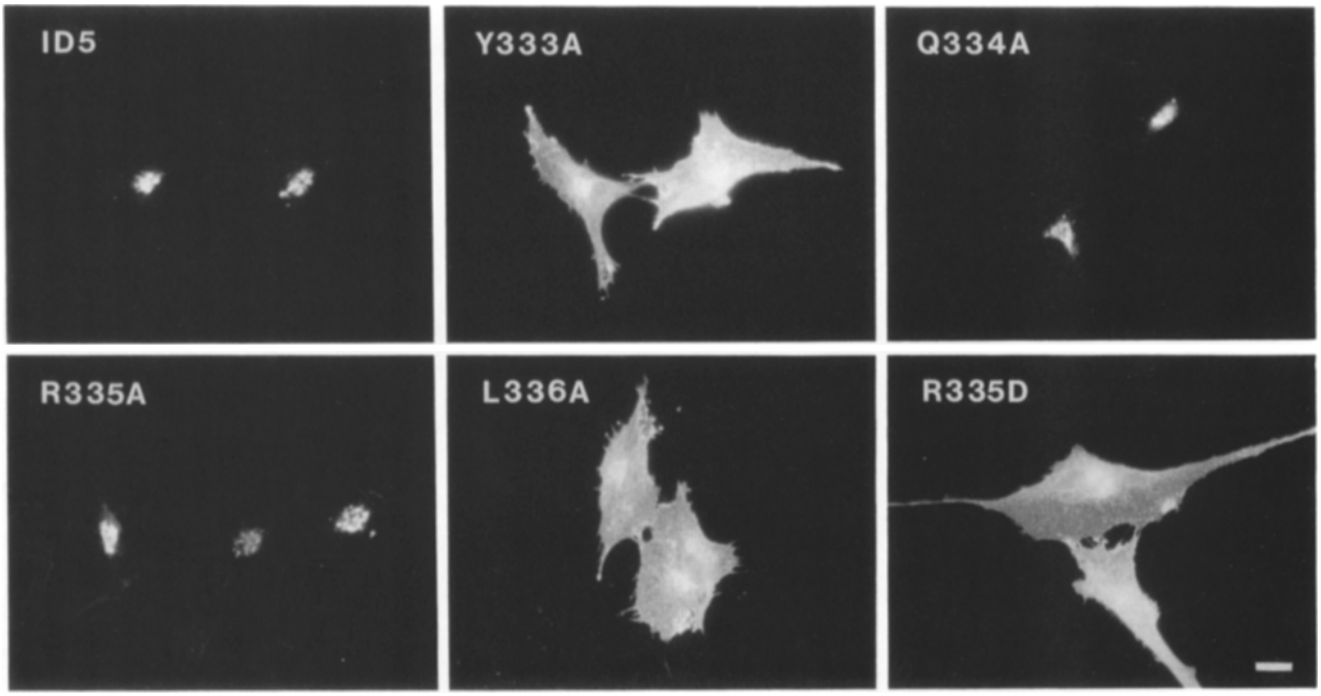


Figure 6. Identification of cytoplasmic residues critical for TGN localization. Single residues within the cytoplasmic tail of the ID5 construct (see Fig. 4) were substituted by alanine or aspartic acid residues, as indicated in the scheme. Numbering corresponds to the published sequence of TGN38 (Luzio et al., 1990). Mutants were expressed by transient transfection of NRK cells and localized by indirect immunofluorescence using a mouse mAb to Tac (7G7) and rhodamine-conjugated antibodies to mouse IgG. Constructs Y333A, L336A, and R335D were observed at the plasma membrane, whereas Q334A, R335A, and the rest of the constructs (not shown in the figure) showed a TGN localization similar to ID5. Bar, 10 μm.

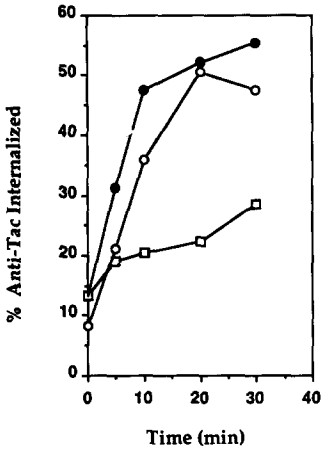


Figure 7. Internalization of ^{125}I-labeled anti-Tac in CV-1 cells expressing Tac (□), ID5 (●), or R335D (○). Internalization of ^{125}I-labeled anti-Tac pre-bound to transiently transfected CV-1 cells was measured as described in Materials and Methods. Each point represents the average value derived from two (Tac) or three (ID5 and R335D) independent experiments performed in duplicate. Duplicate determinations varied by <10%. No detectable binding or internalization was observed in untransfected CV-1 cells.

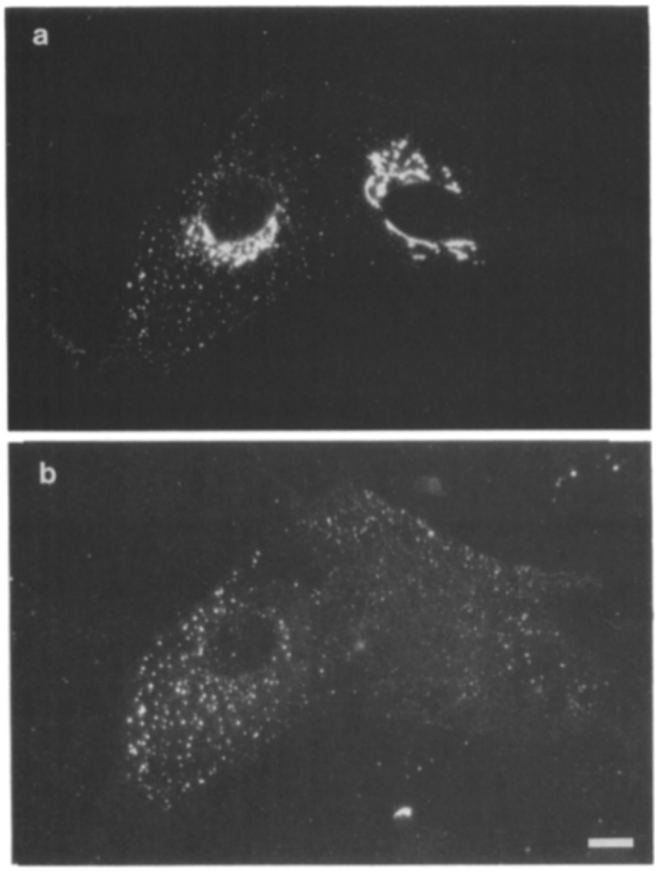


Figure 8. Comparison of the distributions of endocytosed transferrin and T-G-G. Stably transfected CV-1 cells expressing T-G-G were incubated for 2 h at 37°C with rhodamine-conjugated transferrin, fixed, permeabilized, and stained with an antibody to Tac (7G7) and fluorescein-conjugated antibodies to mouse IgG. (*a*) Fluorescein channel, T-G-G; (*b*) rhodamine channel, transferrin. Cells having some T-G-G in cytoplasmic vesicles were deliberately photographed to compare the distribution of the chimera with endocytosed transferrin. Notice the co-localization of endocytosed transferrin with T-G-G in cytoplasmic vesicles but not in the TGN. Bar, 10 μm.

expressed in NRK cells and its subcellular distribution studied by immunoelectron microscopy. Frozen sections of the stable transfectants were labeled sequentially for both the ID5 chimera and endogenous TGN38 using gold-conjugated protein A. As shown in Fig. 5, the chimeric protein (15-nm gold particles) was found in tubulo-vesicular structures adjacent to the Golgi stacks. These structures were identified as the TGN by the presence of endogenous TGN38 (10-nm gold particles). Both the chimeric protein and TGN38 were occasionally observed in vesicular organelles with the morphology of endosomes, but were only rarely detected at the plasma membrane (data not shown).

To identify specific amino acid residues critical for signaling localization to the TGN, 10 of the 11 COOH-terminal residues of the ID5 chimeric protein were individually mutated to alanine residues (Fig. 6, *top*). The immunofluorescence microscopy pattern of each mutant was determined in transiently transfected NRK cells. Substitution of an alanine residue for either Y333 or L336 resulted in loss of TGN localization and a concomitant increase in plasma membrane labeling (Fig. 6); the remaining alanine mutations had no effect on the TGN localization of the chimeric protein (Fig. 6, and data not shown). Interestingly, whereas replacement of R335 by an alanine residue did not affect the distribution of the protein, replacement by an aspartate residue caused decreased intensity of TGN staining and increased expression at the plasma membrane and in cytoplasmic vesicles (Fig. 6). Thus, these results indicated that Y333 and L336 are critical for TGN localization of the chimeric molecules. R335, on the other hand, can be replaced by an alanine but not an aspartic acid residue for efficient TGN localization.

Internalization of Proteins Having a TGN Localization Determinant

The sequence YQRL, identified by our mutagenesis studies as essential for TGN localization, resembles other sequences involved in internalization of cell surface receptors. The internalization signals of the transferrin receptor, mannose-6-phosphate receptors, asialoglycoprotein receptor, polymeric immunoglobulin receptor, and others are all tetrapeptides having an aromatic residue in the first position and a bulky hydrophobic residue in the fourth position of the sequence (reviewed by Trowbridge, 1991). These aromatic amino acid–based motifs have been proposed to form a tight turn structure that acts as a conformational signal for endocytosis (Collawn et al., 1990). Our analysis of the cytoplasmic sequence of TGN38 using the algorithm of Chou and Fasman (1978), predicted a high probability of a β-turn in the region around Y333. These considerations led us to test whether the sequence YQRL could also function as an internalization signal.

Transiently transfected CV-1 cells expressing Tac (no signal), the ID5 chimera (containing YQRL), or the R335D mutant (containing YQDL) were incubated for 30 min at 4°C with ^{125}I-labeled anti-Tac mAb (Uchiyama et al., 1981). Upon warming the cells to 37°C, Tac was found to be poorly internalized (Fig. 7), in agreement with the observations of Weissman et al. (1986). The ID5 chimera was internalized at a rate of 3.5% per minute (Fig. 7), significantly faster than normal Tac, but slower than other internalized receptors with tyrosine-based motifs (Weissman et al., 1986; Hunziker et al., 1991; Casanova et al., 1991). Interestingly, the R335D

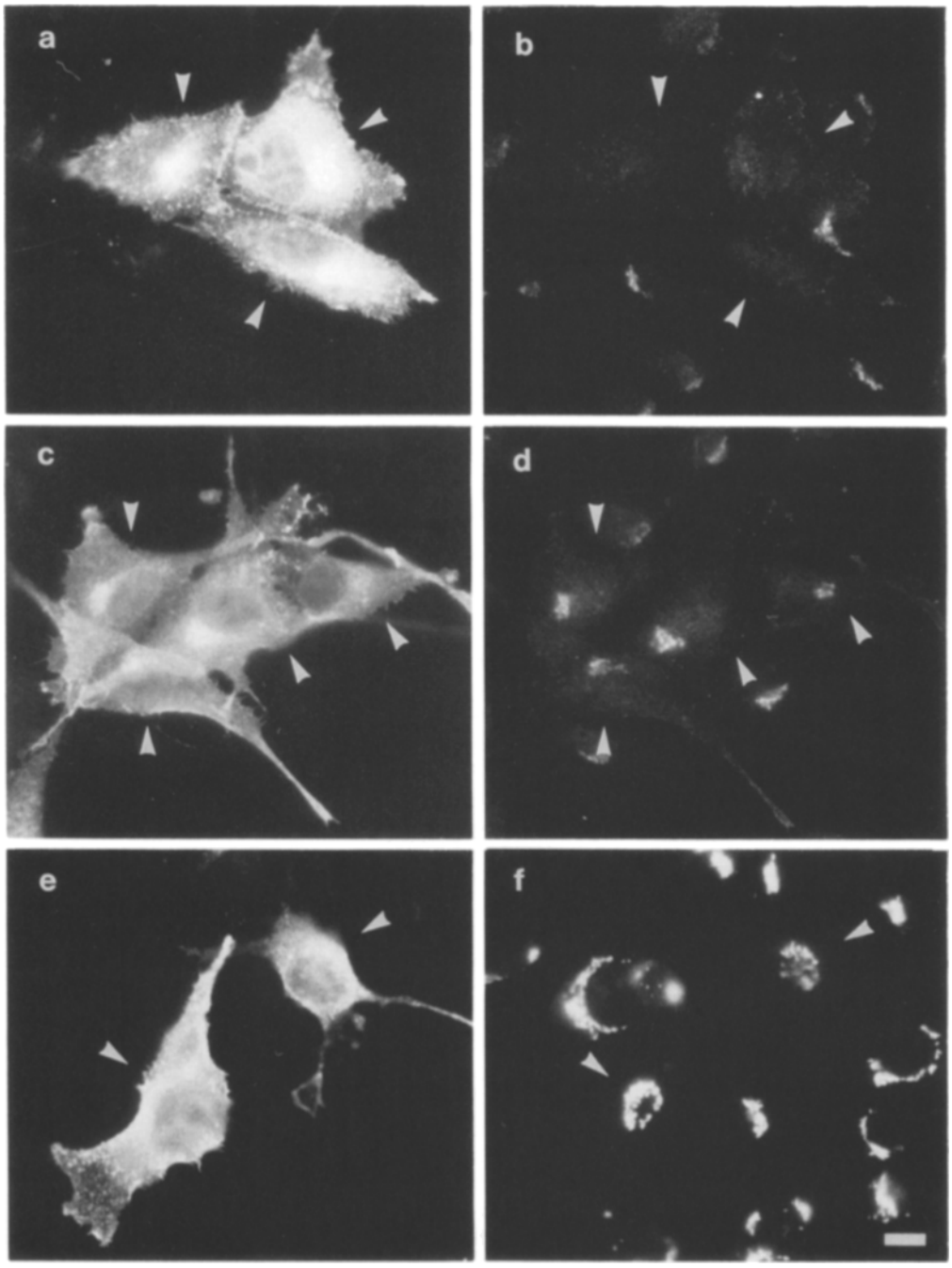


Figure 9. Dimming of endogenous TGN38 staining by overexpressed chimeric constructs. (*a* and *b*) MOP-8 cells overexpressing T-G-G were stained with a mouse antibody to Tac and a rabbit antibody to endogenous TGN38, followed by a fluorescein-conjugated donkey antibody to mouse IgG and a rhodamine-conjugated donkey antibody to rabbit IgG. (*a*) T-G-G, fluorescein channel; (*b*) TGN38, rhodamine channel. Notice the disappearance of perinuclear staining for TGN38 and the slight increase in surface staining for TGN38 in cells overexpressing T-G-G. Untransfected cells in the same field show normal staining of endogenous TGN38. (*c* and *d*) MOP-8 cells overexpressing normal Tac were stained with antibodies to Tac and TGN38 as above. (*c*) Tac, fluorescein channel; (*d*) TGN38, rhodamine channel. (*e* and *f*) MOP-8 cells overexpressing T-G-G were stained with a mouse antibody to Tac and a rabbit antibody to endogenous mannosidase II, followed by a fluorescein-conjugated donkey antibody to mouse IgG and a rhodamine-conjugated donkey antibody to rabbit IgG. (*e*) T-G-G, fluorescein channel; (*f*) mannosidase II, rhodamine channel. Arrowheads point to edges of the positive cells. Bar, 10 μm.

mutant, which did not contain a strong TGN localization determinant, was still internalized at 85% the rate of ID5 (Fig. 7). These experiments demonstrated that both YQRL- and YQDL-containing sequences can function as internalization determinants, even though only YQRL-containing sequences seem to be effective for TGN localization.

Different Steady State Distributions of Tac-TGN38 Chimeras and Endocytosed Receptors

Whereas the previous experiments suggested that TGN localization and endocytosis determinants were related but not identical, we were still intrigued by the possibility that the observed localization of the Tac-TGN38 chimeras could simply correspond to the distribution of any molecule internalized by virtue of a tyrosine-containing motif. This prompted us to compare the distribution of T-G-G with that of the transferrin receptor, which also carries a cytoplasmic tyrosine–containing internalization motif (YTRF). The recycling pool of transferrin receptors in CV-1 cells was marked by incubation of intact cells for 2 h at 37°C with rhodamine-conjugated transferrin. Under these conditions, intracellular transferrin would be expected to be bound to recycling receptors (Klausner et al., 1983; Dautry-Varsat et al., 1983), and to be localized mainly to early endosomes (Hopkins, 1983), with smaller amounts present in coated vesicles and tubules in the area of TGN (Willingham and Pastan, 1985; Fishman and Fine, 1987; Stoorvogel et al., 1988). Cells were then

fixed, permeabilized, and stained for T-G-G using an antibody to Tac and a fluorescein-conjugated second antibody (Fig. 8). No internalized transferrin could be detected in the central TGN structure containing T-G-G using this methodology; instead, transferrin was found within vesicles distributed throughout the cytoplasm (Fig. 8, *a* and *b*). As mentioned earlier, in some cells T-G-G was also found in cytoplasmic vesicles; the presence of internalized transferrin in the same vesicles identified them as early endosomal structures (Fig. 8, *a* and *b*). Similarly, the distribution of endogenous transferrin receptors detected with specific antibodies differed from that of T-G-G in the TGN of human RD4 cells (data not shown).

The results of these experiments suggest that the localization of Tac-TGN38 chimeras to the TGN is not solely a consequence of their being internalized from the cell surface by virtue of a tyrosine-based motif. Rather, YQRL-containing sequences appear to have additional information that specifies localization to the TGN.

Disappearance of Endogenous TGN38 Staining by Overexpression of Tac-TGN38 Chimeric Proteins

As mentioned above, a fraction of transiently transfected cells expressed the Tac-TGN38 chimeras at the plasma membrane. Almost invariably, this population of cells had visibly higher levels of fluorescence intensity as compared to cells displaying pure TGN staining, consistent with the previous observation that overexpression of TGN38 in CV-1 cells results in the appearance of the protein at the cell surface (Luzio et al., 1990). In a few of the most brightly stained NRK cells expressing T-G-G at the plasma membrane, no endogenous TGN38 staining was visible by immunofluorescence microscopy (data not shown). This phenomenon appeared to occur only in cells with very high levels of protein expression.

To evaluate this phenomenon further, T-G-G was expressed by transient transfection in mouse MOP-8 cells. MOP-8 cells are transformed with an originless polyoma virus and thus replicate to high copy number the expression vector used in these studies, pCDM8, which has the polyoma virus origin of replication (Muller et al., 1984). Consistent with the expected high levels of protein expression, immunofluorescent labeling of T-G-G in transfected MOP-8 cells showed surface staining in 63% of expressing cells (versus only ~10% in NRK cell transfections). Of MOP-8 cells expressing T-G-G at the plasma membrane, nearly all showed dimming or complete disappearance of endogenous TGN38 staining of the TGN as compared to untransfected cells in the same microscope field (Fig. 9, *a* and *b*). Weak staining of TGN38 at the plasma membrane was observed in some of these cells (Fig. 9 *b*). Disappearance of endogenous TGN38 staining was also observed in cells overexpressing other TGN-localized chimeras, such as T-T-G and ID5 (data not shown). However, neither disappearance nor dimming of endogenous TGN38 staining was seen in MOP-8 cells overexpressing Tac (Fig. 9, *c* and *d*), demonstrating the specificity of this phenomenon for overexpression of TGN-localized chimeras. Moreover, overexpression of T-G-G had no effect on the intensity and pattern of staining of a *cis*-medial Golgi marker, mannosidase II (Fig. 9, *e* and *f*). One possible explanation for these observations is that TGN-localized chimeric proteins compete with endogenous TGN38 for interaction with a specific, saturable localization mechanism. However, alternative explanations, such as complete disruption of the TGN or masking of the TGN38 epitope by overexpression of the chimeric proteins, have not been ruled out at this time. More definitive evidence for saturability of a TGN localization mechanism will first require a demonstration of the structural integrity of the TGN in overexpressing cells and a quantitative demonstration that endogenous TGN38 is displaced from the TGN to the cell surface or other intracellular compartments. In any case, this phenomenon demonstrates an additional specificity of the cytoplasmic determinant, which not only can confer TGN localization but also can affect the distribution of an endogenous TGN resident protein.

Discussion

Cytoplasmic Determinants of TGN Localization

The identification of transmembrane signals for protein retention in the Golgi stacks (reviewed by Machamer, 1991) represented a significant advance in the understanding of the organization of the Golgi complex and, in a more general sense, implicated a new mechanism for protein localization within the secretory pathway. Our observations that the cytoplasmic domain of TGN38 contains sufficient information for localization to the TGN demonstrates the existence of a second mechanism operating to localize proteins within the Golgi complex that depends on cytoplasmic rather than transmembrane determinants. The two mechanisms differ not only in the topological domain harboring the localization signal, but also in the characteristics of the localization process. The available evidence indicates that overexpression of proteins normally localized in the Golgi stacks results in their increased detection in the ER but not at the plasma membrane (Munro, 1991; Nilsson et al., 1991; Moremen and Robbins, 1991; Colley et al., 1992). This is in contrast to TGN-localized proteins which when overexpressed appear at the cell surface and in cytoplasmic vesicles (Luzio et al., 1990; this study). Although the generality of these observations will have to be assessed for other TGN proteins, the present data reinforce the idea that the Golgi stacks and the TGN are intrinsically different in structure, dynamics and function (Klausner et al., 1992; Mellman and Simons, 1992; Rothman and Orci, 1992).

The participation of a cytoplasmic sequence in localization of a protein to TGN-associated structures has also been suggested for the insulin-regulatable glucose transporter, GLUT-4 (Piper et al., 1992). Immunoelectron microscopy studies have localized GLUT-4 and chimeric proteins having the GLUT-4 NH_2-terminal cytoplasmic segment to tubulovesicular structures associated with the TGN (Slot et al., 1991; Piper et al., 1992). The intracellular distribution of these proteins, however, seemed to be less restricted to the TGN than in our studies. Although the identity of residues critical for this localization of GLUT-4 has not been reported, it is noteworthy that the NH_2-terminal sequence contains a potential aromatic amino acid-based tetrapeptide motif (FQQI; Piper et al., 1992).

In yeast cells, cytoplasmic sequences also play a role in localization to a late Golgi compartment that may be the functional equivalent of the mammalian TGN. The cytoplasmic

domain of dipeptidyl aminopeptidase A (DPAP A), a yeast Golgi peptidase, has been shown to be sufficient for retention of the protein in Golgi structures (Roberts et al., 1992). Similarly, deletion of the cytoplasmic tail of kex2p, a yeast Golgi pro-hormone processing peptidase, has been shown to result in mislocalization of the protein from a late Golgi compartment (Fuller et al., 1989). Preliminary observations from our laboratory have indicated that the cytoplasmic domain of a rat kex2p homologue, furin (Misumi et al., 1990), also has information sufficient for localization to the mammalian Golgi complex (J. S. Humphrey, J. Davidson, and J. S. Bonifacino, unpublished observations). Interestingly, several proteins localized to late compartments of the Golgi complex have potential aromatic amino acid–based motifs (Trowbridge, 1991) that may be involved in late Golgi or TGN localization. These proteins include DPAP A from *Saccharomyces cerevisiae* (Roberts et al., 1992); the kex2p homologues from *S. cerevisiae* (Fuller et al., 1989), *Kluyveromyces lactis* (Tanguy-Rougeau, 1988), *Drosophila melanogaster* (Hayflick et al., 1991), mouse (Hatsuzawa et al., 1990), rat (Misumi et al., 1990), and human (van den Ouweland et al., 1990); and kex1p from *S. cerevisiae* (Dmochowska et al., 1987). Additional mutagenesis studies will be needed to establish whether these cytoplasmic motifs are involved in late Golgi or TGN localization, as is the case for TGN38. If true, it will mean that cytoplasmic motifs are widely used as signals for localization to late Golgi compartments and will raise the possibility that an evolutionarily conserved mechanism exists to recognize such signals.

Characteristics of the TGN Localization Determinant and Relationship to Internalization Signals

Molecular dissection of the cytoplasmic tail of TGN38 identified an 11-amino acid sequence sufficient to confer TGN localization. Within this sequence, the tetrapeptide YQRL was found to contain the most critical elements for this function. Although mutations of other residues within the 11-amino acid sequence elicited minor or no effects, the subtle increase in the number of cytoplasmic vesicles observed for some of the deletion mutants raises the possibility that residues NH_2-terminal to YQRL may contribute to TGN localization. The requirements for specific residues at defined positions within this segment, however, would have to be much less stringent than for the YQRL motif.

The sequence YQRL has the structural features of an internalization signal (Trowbridge, 1991) and, as demonstrated by our experiments, can cause endocytosis of surface molecules. Moreover, the critical role of the Y and L residues in TGN localization is in line with the known sequence requirements for internalization signals (Trowbridge, 1991). Some of our results, however, argue that the function of YQRL in TGN localization is not limited to its ability to cause endocytosis from the cell surface. For instance, the sequence YQDL was considerably less efficient for TGN localization, even though it was almost as effective as YQRL for internalization. This agrees with previous observations that YQDL is the internalization signal of the asialoglycoprotein receptor H1 subunit (Spiess, 1990), and that replacement of the transferrin receptor internalization signal by YQDL results in a mutant transferrin receptor with an internalization rate virtually identical to that of the wild-type receptor (Collawn et al., 1991).

The disruption of TGN localization, but not internalization, by the mutation of R to D demonstrates an apparent fine specificity of the sequence YQRL for TGN localization which has precedent in studies of cytoplasmic signals for sorting to lysosomes and to the basolateral surface of polarized epithelial cells. For instance, most lysosomal integral membrane proteins have a cytoplasmic tyrosine residue that is critical for both lysosomal targeting and internalization (reviewed by Fukuda, 1991). For the lysosomal protein lgp120, a glycine immediately before the tyrosine residue is essential for targeting to lysosomes at low expression levels, but is dispensable for internalization (Harter and Mellman, 1992). Similarly, for lysosomal acid phosphatase, mutation of the critical cytoplasmic tyrosine residue to a phenylalanine abrogates basolateral targeting of the enzyme in polarized cells without affecting internalization (von Figura; cited in Mostov et al., 1992). Taken together, these observations suggest that cytoplasmic aromatic amino acid–containing motifs can be recognized at more than one point in the secretory and endocytic pathways, with overlapping but distinct specificities. The sequence YQRL probably belongs to a subset of internalization signals that have a specific capability to confer TGN localization.

Possible Mechanisms of TGN Localization

The ability of a tyrosine-containing cytoplasmic sequence to cause localization to the TGN can be explained by at least two different, although not mutually exclusive models. The first model assumes that the cytoplasmic determinant is directly recognized at the level of the TGN, resulting in retention or slow exit from this compartment. By analogy with tyrosine-based internalization signals (Pearse, 1985, 1988; Glickman et al., 1989; Beltzer and Spiess, 1991), the TGN localization determinant characterized here may bind to TGN-specific clathrin–associated adaptor molecules, such as γ-adaptin (Robinson, 1990). Interestingly, a large fraction of the yeast Golgi peptidase kex2p, whose cytoplasmic tail is necessary for Golgi localization, is missorted to the plasma membrane in yeast mutants deficient in clathrin heavy chains (Payne and Schekman, 1989; Seeger and Payne, 1992). Interaction with components of non-clathrin coats of the TGN is also possible. For any of these potential interactions to result in TGN retention, however, association of cytoplasmic sequences with coated structures would have to be a relatively stable event. The specificity and kinetics of the interaction would be dictated by amino acid residues in or around the tyrosine-based motif, such that single amino acid substitutions could perturb the interaction.

A second model entails recognition of the cytoplasmic determinant at a post-TGN location, such as the plasma membrane, with TGN localization being a consequence of retrieval from another compartment. According to this model, TGN38 can traffic normally from the TGN to the plasma membrane where the YQRL motif then functions as an internalization signal. From the endosomal compartment, the protein would be transported back into the TGN by default or by additional sorting information in the localization determinant. This explanation would be analogous to the view of the ER localization motif, KDEL, as a retrieval rather than retention signal (Pelham, 1991). Consistent with this retrieval model is our observation of a small amount of T-G-G at the cell surface of CV-1 stable transfectants, the ability of

the chimeras to internalize bound antibody and the detection of chimeras in early endosomes. In addition, recent observations have suggested the existence of a pool of TGN38 cycling between the TGN and the cell surface (Ladinsky, M. S., and K. E. Howell. 1992. *Mol. Biol. Cell.* 3:309). To what extent a retrieval mechanism contributes to the localization of TGN38 chimeras to the TGN, however, remains to be established. The failure of the endocytosed R335D mutant to localize to the TGN and the different distributions of transferrin receptor and TGN-localized Tac-TGN38 chimeras suggest that the property of internalization per se is insufficient to specify TGN localization.

In conclusion, the observations reported here demonstrate that a short, tyrosine-containing sequence within the cytoplasmic tail of TGN38 is sufficient to confer TGN-localization to a reporter protein, Tac, and reveal a novel function for a cytoplasmic tyrosine-based motif—the ability to mediate protein localization to the TGN. Study of other proteins, such as furin, will ascertain whether similar cytoplasmic signals mediate localization of additional type I integral membrane proteins to the Golgi complex. The conservation of TGN localization across species boundaries and the demonstrated importance of the cytoplasmic domain for localization of yeast proteins to the late Golgi suggest the existence of an evolutionarily conserved mechanism that relies on the specific recognition of cytoplasmic determinants. The identification of sequences that are sufficient for TGN localization should allow the development of biochemical and genetic approaches to define the mechanisms by which membrane proteins become localized to the TGN.

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