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TGN38 Recycles Basolaterally in Polarized Madin-Darby Canine Kidney Cells

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Sorting of newly synthesized plasma membrane proteins to the apical or basolateral surface domains of polarized cells is currently thought to take place within the trans-Golgi network (TGN). To explore the relationship between protein localization to the TGN and sorting to the plasma membrane in polarized epithelial cells, we have expressed constructs encoding the TGN marker, TGN38, in Madin-Darby canine kidney (MDCK) cells. We report that TGN38 is predominantly localized to the TGN of these cells and recycles via the basolateral membrane. Analyses of the distribution of Tac-TGN38 chimeric proteins in MDCK cells suggest that the cytoplasmic domain of TGN38 has information leading to both TGN localization and cycling through the basolateral surface. Mutations of the cytoplasmic domain that disrupt TGN localization also lead to nonpolarized delivery of the chimeric proteins to both surface domains. These results demonstrate an apparent equivalence of basolateral and TGN localization determinants and support an evolutionary relationship between TGN and plasma membrane sorting processes.

INTRODUCTION

The Golgi complex of higher eukaryotes is organized into three compartments with distinct biochemical and functional characteristics (Mellman and Simons, 1992; Rothman and Orci, 1992). Proteins leaving the endoplasmic reticulum (ER) enter the first of these compartments (known as the intermediate compartment, salvage compartment, or *cis*-Golgi network) where proteins are sorted into those that recycle back to the ER and those that are routed forward into the Golgi stack (Huttner and Tooze, 1989; Hsu *et al.*, 1991; Pelham, 1991; Mellman and Simons, 1992). After undergoing sequential processing of their carbohydrate side chains in the Golgi stack, proteins are transported into the *trans*-Golgi network (TGN). The TGN, a tubulo-reticular structure adjacent to the *trans*-most cisternae of the Golgi stack, is the major sorting organelle in the secretory pathway (Griffiths and Simons, 1986; Mellman and Simons, 1992). The TGN is the site where secretory and membrane proteins are sorted to the plasma membrane, lysosomes, endosomes, and regulated secretory granules. In polarized epithelial cells, the TGN is believed to be the site where plasma membrane proteins are sorted to apical or basolateral surface domains (Simons and Wandinger-Ness, 1990; Rodriguez-Boulan and Powell, 1992).

The mechanisms by which proteins are sorted at the level of the TGN are currently not well understood. In higher eukaryotes, transport along the secretory pathway appears to occur by bulk flow, such that proteins lacking any retention or sorting information are delivered to the cell surface or the extracellular space by default (Pfeffer and Rothman, 1987). Hence, protein

retention or sorting within organelles of the secretory pathway must be mediated by specific information encoded within the proteins' structure. This information can exist in the form of a discrete sequence or structural motif or of a particular physico-chemical property of the molecules, such as a tendency to aggregate in particular organellar environments (Pfeffer and Rothman, 1987; Klausner, 1989). The existence of retention or sorting signals has now been demonstrated for many proteins, including proteins that reside within the ER and the Golgi complex (Machamer, 1991; Pelham, 1991). Signals for sorting to the apical and basolateral surfaces of polarized epithelial cells have also been described (Rodriguez-Bouland and Powell, 1992).

Recent studies have begun to address the mechanisms by which some proteins become localized to the TGN. To date, the best characterized of these proteins is TGN38/41 (Luzio *et al.*, 1990; Reaves *et al.*, 1992). TGN38/41 is predominantly found within the TGN but is also expressed at some level at the plasma membrane. The surface expression of TGN38/41 is manifested by the ability to mediate uptake of antibodies directed to its lumenal domain (Ladinsky and Howell, 1992; Bos *et al.*, 1993; Reaves *et al.*, 1993). The internalized antibodies are eventually delivered to the TGN. These observations have suggested that TGN38/41 recycles between the TGN and the plasma membrane (Ladinsky and Howell, 1992; Bos *et al.*, 1993; Reaves *et al.*, 1993). The cytoplasmic domain of TGN38 has recently been shown to play a role in both TGN localization and internalization from the cell surface (Luzio *et al.*, 1990; Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993). Both functions are dependent on a tyrosine-containing sequence within the cytoplasmic domain (Humphrey *et al.*, 1993; Bos *et al.*, 1993; Wong and Hong, 1993). The properties of TGN38/41 are thus unique in that it can cycle through the cell surface even though its steady-state distribution shows predominant localization to the TGN.

In polarized epithelial cells, the differentiation of the plasma membrane into apical and basolateral domains raises the question of whether TGN38/41 recycles in a polarized fashion and, if so, which one is the domain preferred for recycling. To address these questions, we examined the intracellular localization and trafficking of an epitope-tagged form of TGN38 and of chimeric proteins having transmembrane and/or cytoplasmic sequences from TGN38. Our results indicate that TGN38 recycles preferentially through the basolateral domain of MDCK cells and that the cytoplasmic domain of TGN38 contains information not only for TGN localization but also for basolateral targeting.

MATERIALS AND METHODS

Cells and Reagents

Madin-Darby canine kidney (MDCK) II cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Laboratories, Grand Island, NY) supplemented with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT), glutamine, and nonessential amino acids (GIBCO Laboratories) in an air-5% CO_2 atmosphere at constant humidity. When grown on filters, 2×10^6 cells were seeded on 24.5-mm Transwell polycarbonate filters (Costar, Cambridge, MA) and cultured for 5–7 d with changes of medium every day. Protein A-Sepharose was purchased from Pharmacia (Uppsala, Sweden). Sulfo-N-hydroxy succinimido-biotin (S-NHS-biotin) and streptavidin-agarose beads were from Pierce (Rockford, IL). All other reagents were purchased from Sigma (St. Louis, MO).

Antibodies

Monoclonal antibodies (mAb) to myc epitope (9E10.2) (Evan *et al.*, 1985) and to Tac (7G7) (Rubin *et al.*, 1985) were prepared from hybridomas obtained from the American Type Culture Collection (Rockville, MD). A rat mAb to human Tac was obtained from AMAC (Westbrook, ME). An antibody to Golgi mannosidase II was kindly provided Dr. K. Moremen (University of Georgia, Athens, GA). A mAb to the γ-subunit of the HA1 adaptor was kindly provided by Dr. E. Ungewickell (Washington University, St. Louis, MO). The mouse mAb against the ectodomain of influenza hemagglutinin (HA) was obtained from Dr. W. Gerhardt (Wistar Institute, Philadelphia, PA). Affinity-purified antibodies (rabbit anti-mouse IgG) were purchased from Cappel (West Chester, PA). Fluorescently labeled secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

DNA Transfection

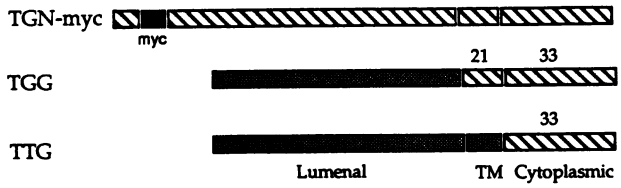
The procedures for the preparation of several Tac-TGN38 chimeras are described in Humphrey *et al.* (1993). A TGN38 construct with a myc epitope at the lumenal domain was generated by the polymerase chain reaction (to be published elsewhere). The sequence of all the recombinant constructs was confirmed by the dideoxy chain termination method. MDCK cells were plated at a density of 1×10^6 cells/100-mm culture dish and grown overnight at 37°C. Cells were trypsinized and incubated for 9–12 h with 20 μg plasmid DNA and 2 μg pMV6(neo) precipitated by calcium phosphate (Graham and Van der Eb, 1973). Cells were then washed with phosphate-buffered saline (PBS) and treated with 15% glycerol for 2 min, washed four times in PBS and then incubated in complete medium for 48 h before selection in 500 μg/ml active geneticin (G418, GIBCO BRL, Gaithersburg, MD) in complete medium. Stably transfected clones were identified by immunofluorescence as described below.

Immunofluorescence and Laser Scanning Confocal Microscopy

Transiently transfected cells were plated directly on glass coverslips and processed after 48 h for immunofluorescence as described (Rodriguez-Boulan, 1983). Bound IgG was visualized with either fluorescein- or Texas Red–conjugated secondary antibodies. A Nikon fluorescence microscope (Garden City, NY) coupled to a Phoibos 1000 laser scanning confocal microscope from Molecular Dynamics (Sunnyvale, CA) was used to scan the images. To detect simultaneously fluorescein- and Texas Red–labeled antigens, samples were excited at 514 nm, and the light emitted between 525 and 540 nm was recorded for fluorescein and above 630 nm for Texas Red. The cell monolayer was optically sectioned in horizontal (x-y) planes. Images were generated by an Iris Indigo workstation (Silicon Graphics, Mountain View, CA) and Image space software from Molecular Dynamics.

Biotinylation Assays

Biotinylation of monolayers on Transwells with S-NHS-biotin was carried out twice successively for 20 min each from either the apical or the basolateral compartment (Sargiacomo *et al.*, 1989). The cells



Construct	Cytoplasmic sequence	Localization
TTG	..KRKIIAFALEGKRSKVTRRPKASDYQRLNLKL*	TGN
D3	..KRKIIAFALEGKRSKVTRRP*	PM (Non polar)
ID1	..KRKIIAFALEGKRSKVT-------YQRL*	TGN
ID2	..KRKIIAFALE-------RRPKASDYQRL*	TGN
ID3	..KRK-------GKRSKVTRRPKASDYQRL*	TGN
ID5	..KRK----------RRPKASDYQRL*	TGN/Vesicles
Y333A	..KRK----------RRPKASDAQRL*	PM (Non polar)

Figure 1. Schematic representation of TGN-myc and Tac-TGN38 constructs and the cytoplasmic sequences of Tac-TGN38 chimeras used in this study.

were detergent extracted and immunoprecipitated using protein A-Sepharose beads coated with mAb 7G7, separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was blotted onto a nitrocellulose filter and visualized using [^{125}I]-labeled streptavidin as described (LeBivic *et al.*, 1989).

Antibody Internalization

To determine the recycling pattern of TGN38, filter-grown MDCK cells were incubated with anti-Tac rat mAb in complete medium added either to the apical or the basal compartment for 90 min at 37°C. The filters were washed with PBS, fixed and stained with Texas Red–conjugated goat anti-rat antibody, and visualized by immunofluorescence microscopy. Filter grown cells were also incubated with [^{125}I]-labeled anti-myc mAb at 1.3×10^8 cpm/ml as described (Weissman *et al.*, 1986) from either apical or basolateral side for 0.5, 1, and 2 h at 37°C. As control the same amount of [^{125}I]-labeled mouse mAb against the ectodomain of influenza HA was used. The labeled antibody was cleared of aggregates by centrifugation for 15 min before use. At the indicated times, the filters were removed and placed on ice, after which they were incubated with 500 μg/ml proteinase K in DMEM for 30 min at 4°C. The filters were then washed five times in cold DMEM, and the radioactivity was determined in a Packard Auto Gamma 5650 counter (Downers Grove, IL).

Analysis of Sialylation

Stably transfected MDCK cells expressing Tac or TGG were trypsinized and metabolically labeled in suspension for 1 h at 37°C with ^{35}S-methionine (0.5 mCi/ml) in methionine-free DMEM containing 2% dialyzed fetal bovine serum (labeling medium) and chased for 4 h at 37°C in regular culture medium. Cells were then incubated for 1 h at 37°C in serum-free DMEM in the absence or presence of 0.1 U of neuraminidase from *Vibrio cholerae* (Calbiochem, La Jolla, CA). The reaction was stopped by washing three times at 4°C in regular culture medium containing 0.1 mg/ml fetuin. Cells were collected by centrifugation and extracted with 1% Triton X-100, 0.3 M NaCl, 50 mM tris(hydroxymethyl)aminomethane HCl pH 7.4 (lysis buffer) containing 0.1 mg/ml fetuin. Lysates were cleared by centrifugation, and supernatants incubated for 2 h at 4°C with mAb 7G7 bound to protein A-Sepharose. The beads were washed five times with lysis buffer and one time with distilled water at 4°C. Samples were analyzed by two-dimensional (2-D) nonequilibrium pH gel electrophoresis (NEPHGE)/SDS-PAGE, according to the method of O'Farrell *et al.* (1975).

RESULTS

Localization of TGN38-myc and a Tac-TGN38 Chimera in MDCK Cells

Previous immunofluorescence and immunoelectron microscopic analyses revealed that in normal rat kidney (NRK) cells (a fibroblastoid cell line), TGN38 is predominantly localized to the TGN (Luzio *et al.*, 1990). The polyclonal antibody used in those experiments binds to TGN38 in rodents but not in MDCK cells. We therefore utilized a TGN38 construct that was tagged with the myc epitope in the ectodomain (Figure 1). Immunofluorescence microscopy of MDCK cells expressing the TGN38-myc construct showed localization of the protein to a perinuclear structure characteristic of the Golgi complex (Figure 2B). We also examined the localization in the same cells of a chimeric protein generated by fusion of the ectodomain of the Tac antigen (interleukin 2-receptor α-chain) (Leonard *et al.*, 1984) to the transmembrane and cytoplasmic domains of TGN38 (TGG) (Humphrey *et al.*, 1993). This chimeric protein was found to colocalize perfectly with TGN38-myc (Figure 2A). That this structure corresponded to the Golgi complex was demonstrated by showing colocalization of TGG with γ-adaptin (Figure 2, C and D) and mannosidase II (Figure 2, E and F). These observations indicated that the epitope-tagged TGN38 construct was accurately targeted to the Golgi complex in MDCK cells and that a segment comprising the transmembrane and cytoplasmic domains of TGN38 was sufficient for localization to this compartment. Interestingly, we found that the organization of TGN in MDCK cells grown on glass coverslips and Transwells was very different. Whereas on glass coverslips the TGN displayed a typical Golgi-like staining appearing like a juxtanuclear cap (Figure 3B, see also Figure 2), on Transwells the TGN appeared fragmented and distributed more randomly in the cytoplasm (Figure 3A). Similar distributions of the Golgi complex of Transwell-grown MDCK (van Meer and van't Hof, 1993) and Caco-2 (van't Hof and van Meer, 1990) cells have been reported.

Internalization and Recycling of TGN38 in Polarized MDCK Cells

Because TGN38 recycles to the cell surface in nonpolarized cells (Ladinsky and Howell, 1992; Bos *et al.*,

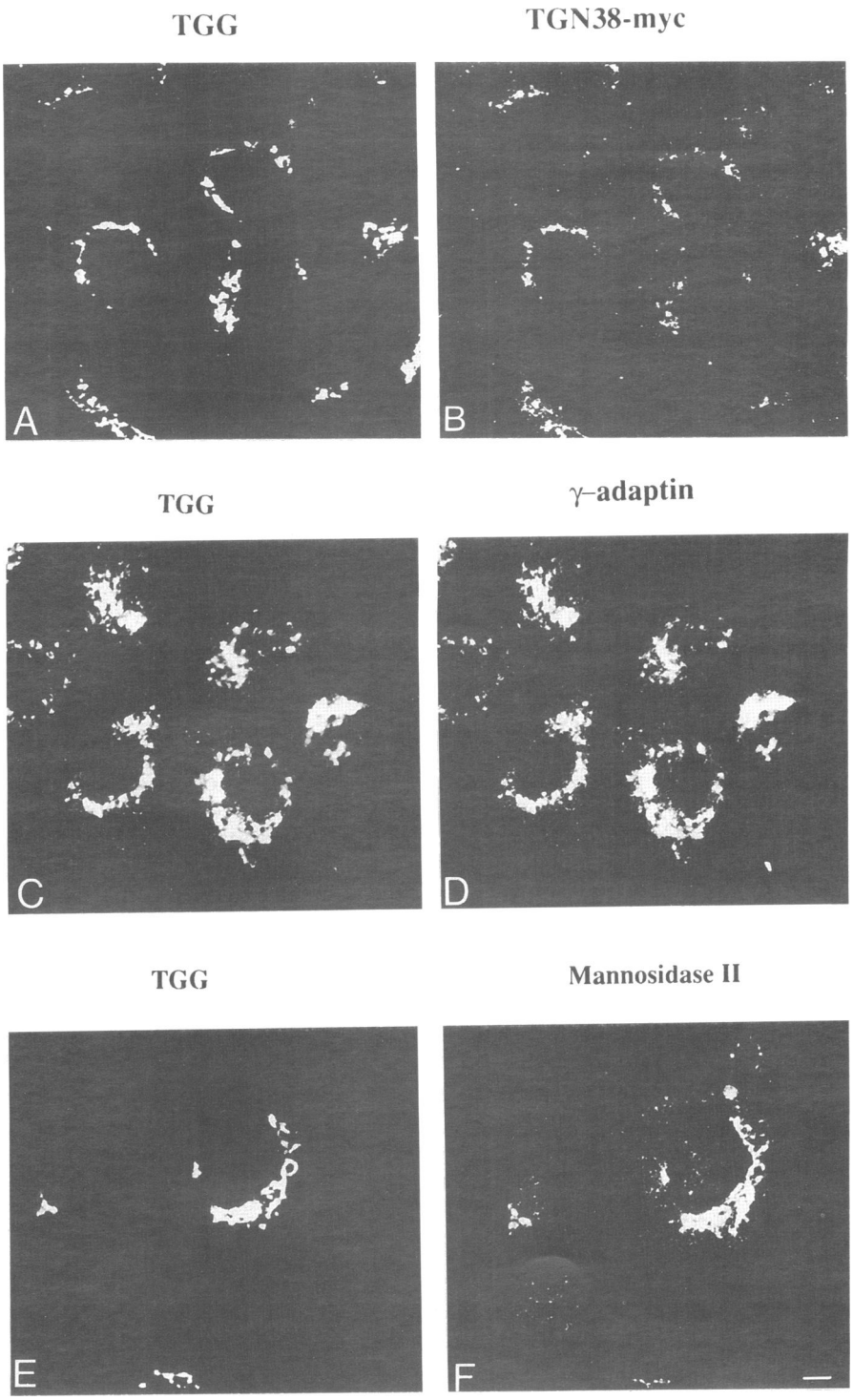


Figure 2. Immunofluorescence localization of TGN38-myc and TGG by laser scanning confocal microscopy. (A and B) MDCK cells stably expressing TGG were transiently transfected with a plasmid encoding TGN38-myc. (C–F) MDCK stably transfected with a plasmid encoding TGG. Cells were fixed, permeabilized, and stained with a rat mAb to Tac. TGG was visualized using a Texas Red–conjugated donkey antibody to rat IgG (A, C, and E). A and B show colocalization of TGG with TGN-myc visualized using a myc-epitope specific mouse mAb and fluorescein-conjugated rabbit anti-mouse IgG. C and D show colocalization of TGG with γ-adaptin stained with a specific mAb and fluorescein-conjugated rabbit anti-mouse IgG. E and F show colocalization of TGG with mannosidase II visualized with a rabbit polyclonal antibody to mannosidase II and a fluorescein-conjugated goat anti-rabbit IgG. Bar, 5 μm.

1993), it is likely that the TGN localization of TGN38 results from a dynamic equilibration of the molecule between the cell surface and the TGN. To determine whether the TGN38-myc construct was expressed at either surface of polarized MDCK cells, filter-grown MDCK cells expressing TGN38-myc were incubated at 37°C with an iodinated antibody to the myc epitope added to the apical or basolateral surfaces. As shown

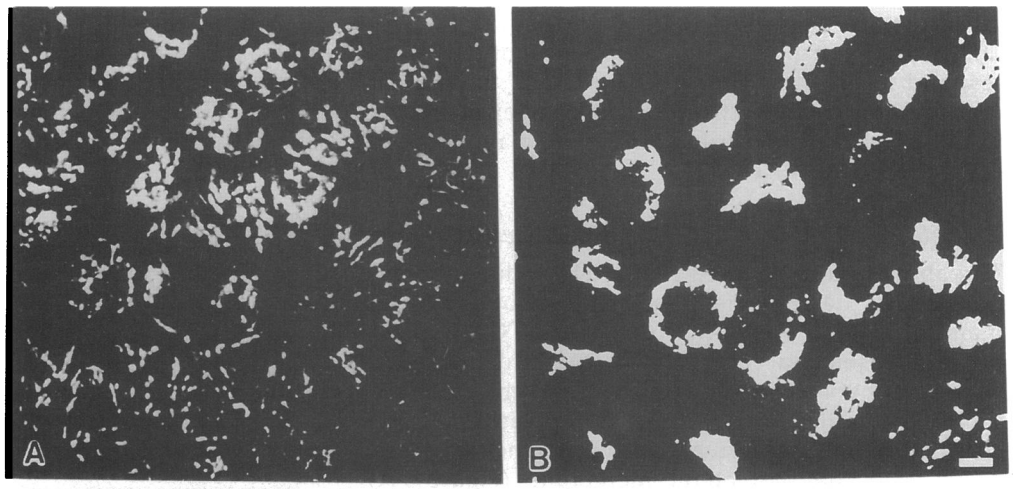


Figure 3. Comparison of the structure of TGN in MDCK cells grown on transwells and glass coverslips. MDCK cells were grown on transwells (A) for 5 d and on glass coverslips (B) for 3 d. Cells were then fixed, permeabilized, and stained for TGG using the rat mAb to Tac. TGG was visualized using a Texas Red–conjugated donkey antibody to rat IgG and visualized by laser scanning confocal microscopy. Bar, 5 μm.

in Figure 4, the antibody was preferentially taken up from the basolateral side by approximately three- to fourfold. Uptake of the anti-myc antibody from the apical side was similar in level to the uptake of a control anti-HA antibody; furthermore uptake of anti-HA antibody was approximately similar from the apical and basal sides (Figure 4). These results suggest that basolateral uptake of anti-myc antibody was facilitated by basolateral recycling of TGN38-myc.

To confirm the results of the antibody uptake experiments and to determine the intracellular destination of the internalized antibody, MDCK cells stably transfected with TGG were grown on filters and incubated with a rat mAb to Tac from either the apical or the basolateral domain for 2 h at 37°C. The cells were then fixed, permeabilized, and visualized by immunofluorescence microscopy (Figure 5). Incubation with antibodies added to the basolateral side (Figure 5B) resulted in the staining of structures remeniscent of the TGN observed on filter-grown cells (see Figure 3). On the other hand antibody incubation from the apical side did not show any specific staining (Figure 5A). To confirm that the structures stained by internalized antibody are TGN, the filters were double labeled with an antibody against a TGN marker, γ-adaptin. As shown in Figure 5, C and D the structures stained by the internalized antibody completely colocalized with γ-adaptin. Because the Tac antigen is not normally internalized (Weissman *et al.*, 1986), these results are consistent with the transmembrane and cytoplasmic domains of TGN38 mediating internalization and retrieval to the TGN. No uptake of influenza HA antibody from either apical or basolateral sides of the MDCK cells stably transfected with TGG was observed (Figure 5, E and F). The staining patterns of TGG antibodies uptaken by MDCK cells were different in cells grown on filter or glass coverslips (Figure 5, G and H), reflecting the different TGN structures under both conditions (Figure 3).

If TGN38 recycles from the cell surface, it may be detectable in endosomal compartments. To address this issue, MDCK cells expressing TGG or TGN38-myc were incubated with fluorescein-conjugated dog transferrin at 37°C for 1 h, fixed, and stained with mAb to Tac or myc. As expected, transferrin, TGG, and TGN38 were all found in intracellular vesicles throughout the cytoplasm. In addition, transferrin was also detected in a compartment that roughly codistributed with TGN, identified by the TGG and TGN38-myc antibodies. Codistribution, however, was not pre-

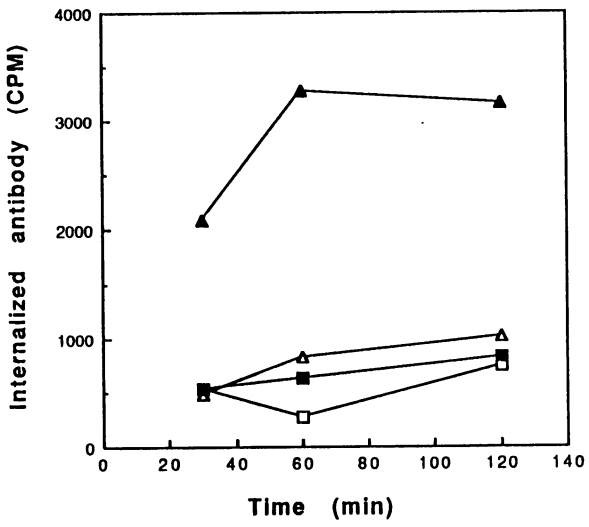


Figure 4. Internalization of [^{125}I]-labeled anti-myc mAb (9E10) by MDCK cells expressing TGN38-myc. MDCK cells grown on filters were fed with [^{125}I]-labeled anti-myc or influenza HA antibodies from apical or basolateral sides for 30 min, 1 h, and 2 h at 37°C, and the amount of internalized antibody was determined after proteinase K treatment and subsequent washing as described in MATERIALS AND METHODS. (△ TGN38-myc, apical; ▲ TGN38-myc, basolateral; □ HA, apical; ■ HA, basolateral).

cise (compare Figure 6A with B and Figure 6C with D); the transferrin Golgi-like compartment might correspond to the "recycling compartment", a deep endosomal compartment from which transferrin recycles to the cell surface, described by Maxfield and coworkers (McGraw *et al.*, 1993).

Distribution of Other Tac-TGN38 Chimeric Proteins

The above results showed that a 54 residue sequence comprising the transmembrane and cytoplasmic domains of TGN38 was sufficient to confer TGN localization and basolateral recycling. To further delineate the sequences involved in these processes, several other Tac-TGN38 constructs were expressed in polarized MDCK cells (Figure 7). The normal Tac antigen showed a clear staining of the basolateral membrane (Figure 7A), whereas TGG was found inside the cells in a structure typical of the Golgi complex (Figure 7B). The construct TTG, generated by fusion of the lumenal and transmembrane domains of Tac with the cytoplasmic tail of TGN38, also displayed Golgi localization (Figure 7C). Deletion of 12 residues from the carboxy terminus of the TTG chimera (D3, see Figure 1) resulted in loss of Golgi localization and nonpolar distribution of the chimera. (Figure 7D). Internal deletions upstream from residue 333 (ID1, ID2, ID3) had no effect in the localization pattern of the chimeras (Figure 7, E–G). These observations indicated that information critical for Golgi complex localization in polarized cells was contained within residues 333–336 of the protein (YQRL), in agreement with previous observations in nonpolarized cells (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993).

A more extensive internal deletion of 14 residues (ID5, see Figure 1), which still preserved the critical YQRL sequence, resulted in a protein that showed a more diffuse staining of cytoplasmic vesicles (Figure 7H). This was in contrast to the pattern observed in the nonpolarized fibroblasts cell lines NRK and CV-1 and even in the polarized Fischer rat thyroid epithelial cell line (unpublished results), in which the protein was clearly localized to the Golgi complex. This observation emphasizes the importance of the context in which residues critical for TGN localization are placed, as well as the fact that different cell types may recognize contextual features with varying degrees of stringency. Mutation of the critical tyrosine residue within ID5 (Y333A) resulted in loss of intracellular localization and nonpolar distribution of the chimera with predominant expression at the apical plasma membrane (Figure 7I).

Polarity of Tac-TGN38 Constructs in MDCK Cells

The immunofluorescence data discussed above suggests that cytoplasmic sequences of TGN38 might play a role in its polarized delivery to the plasma membrane. For instance, removal of residues 328–340 from the cytoplasmic tail of TTG (D3, Figure 7D) or mutation of the critical tyrosine residue within the ID5 construct (Y333A, Figure 7I) resulted in nonpolarized transport to the plasma membrane, with predominant expression on the

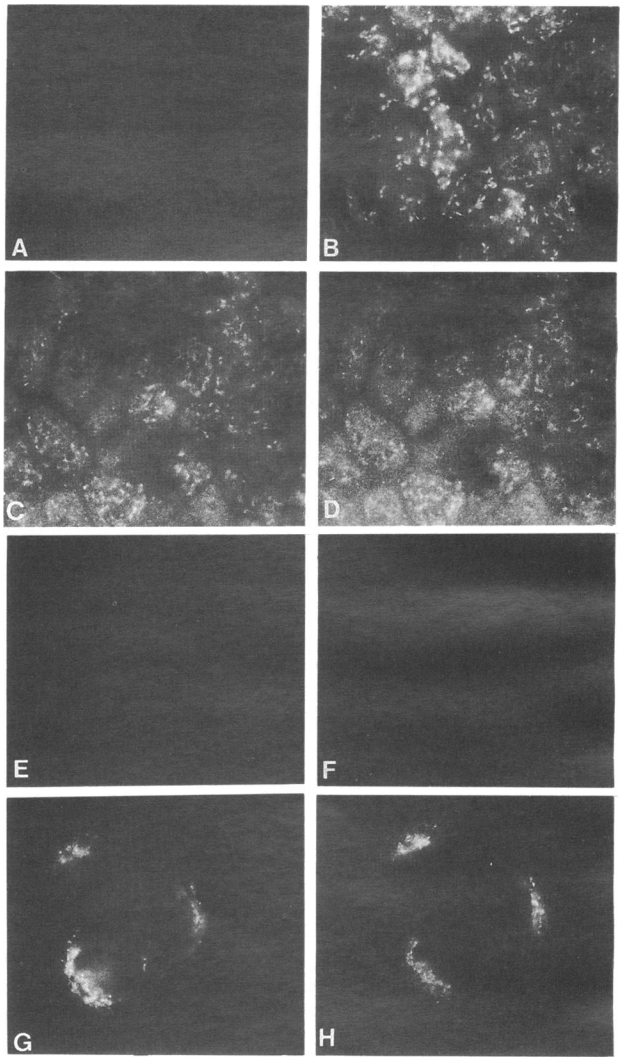


Figure 5. Uptake of antibodies to Tac (rat mAb) by MDCK cells. MDCK cells transfected with TGG were grown on filters and incubated with anti-Tac (A–D), or control (E and F) antibodies from either the apical (A and E) or basolateral (B, D, and F) sides for 2 h. Cells were also grown on glass coverslips (G and H) and were incubated with anti-tac antibody for 30 min. The cells were then fixed, permeabilized, and stained with Texas Red–conjugated donkey anti-rat IgG secondary antibodies. Also, cells were stained for γ-adaptin using a mouse mAb and fluorescein isothiocyanate-conjugated anti mouse secondary antibodies (D and H). Samples were viewed by immunofluorescence microscopy. Antibody incubation from the basolateral side showed TGN staining (B). No uptake of an equivalent concentration of control antibody was observed (E and F). Note the difference in the structure of the TGN in cells grown on glass coverslips and filters. Magnification 2000×.

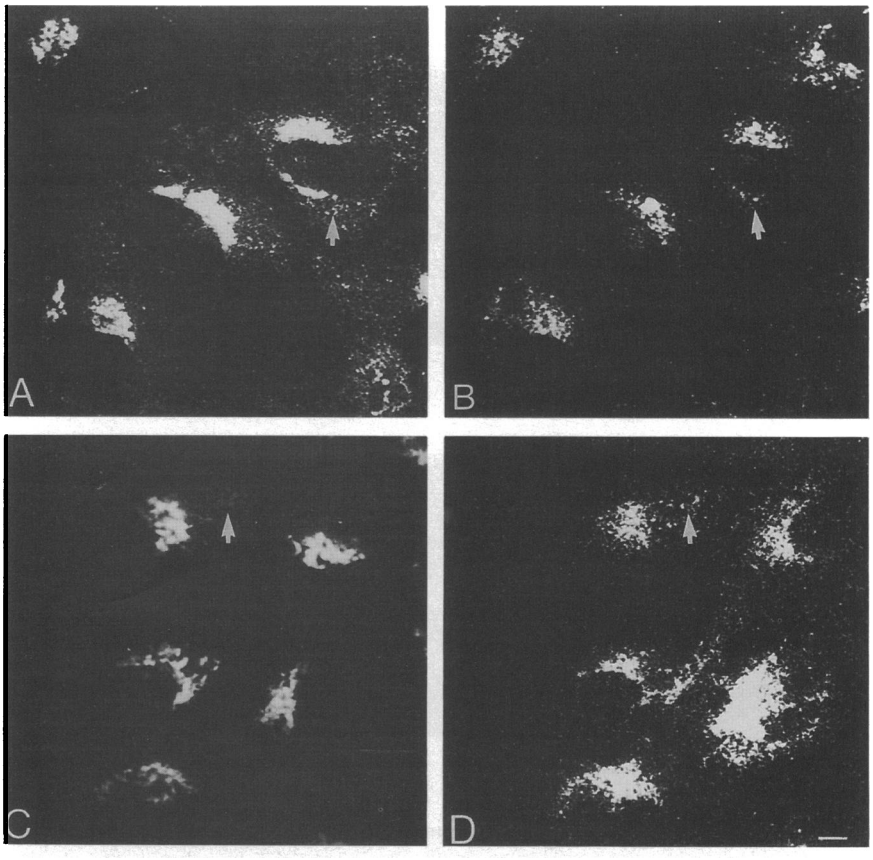


Figure 6. Distribution of endocytosed transferrin and TGG in MDCK cells. MDCK cells expressing TGG and TGN38-myc were incubated with fluorescein-conjugated transferrin for 1 h at 37°C, fixed, permeabilized, and stained with a rat mAb to Tac and Texas Red–conjugated antibodies to rat IgG. Cells were visualized by laser scanning confocal microscopy. (A and C) Texas-Red channel, TGG and TGN38-myc, respectively, (B and D) fluorescein channel, transferrin. Bar, 5 μm. Arrows indicate vesicles containing TGG and internalized transferrin.

apical side. To further investigate this point, we compared the distribution of Tac, TGG, ID5, and Y333A by cell surface biotinylation (Figure 8A). Although TGG was undetectable at the cell surface by immunofluorescence microscopy, the sensitive biotinylation assay detected this fusion protein at the basolateral membrane. Quantitation of the steady-state cell surface expression of these chimeras by biotinylation analysis showed that 75% of TGG was expressed basolaterally (Figure 8B). Sixty percent Tac displayed basolateral distribution. The cell surface expression of the construct ID5 was found predominantly (95%) at the basolateral membrane. Interestingly, mutation of the critical tyrosine residue to an alanine, resulted in a protein (Y333A) that showed increased expression at the apical surface (58%). Furthermore, we determined the total population of Tac or TGG expressed on the cell surface by assessing their neuraminidase sensitivity in intact cells (Figure 9). In this assay metabolically labeled Tac or TGG were treated with neuraminidase and were immunoprecipitated using mAb 7G7 and analysed by 2-D nonequilibrium pH gel electrophoresis. This analysis revealed that 76% of Tac and 6% of TGG gained access to externally applied neuraminidase. In other words only a small population (6%) of the TGG is recycled through the cell surface at any given time. Our results indicate that TGN38 cytoplasmic sequences direct both basolateral recycling and TGN localization. The function of TGN38 cytoplasmic sequences in polarized delivery is dependent, however, on the same tyrosine residue that is critical for intracellular sequestration of the protein.

DISCUSSION

The results of this study indicate that proteins carrying the TGN38 cytoplasmic tail are predominantly localized to the TGN in MDCK cells. Although we failed to observe staining of the plasma membrane by immunofluorescence microscopy, a sensitive biotinylation assay detected a small amount of the TGG chimera at the cell surface, predominantly on the basolateral surface. Because TGN38 has been shown to recycle to the cell surface in nonpolarized cells (Ladisnky and Howell, 1992; Bos *et al.*, 1993; Reaves *et al.*, 1993), this observation suggests that in epithelial cells surface recycling might involve the basolateral domain of the plasma membrane. To address this issue, we determined the polarity of uptake of specific ectodomain antibodies by cells transfected with TGN38-myc or TGG. Immunofluorescence micros-

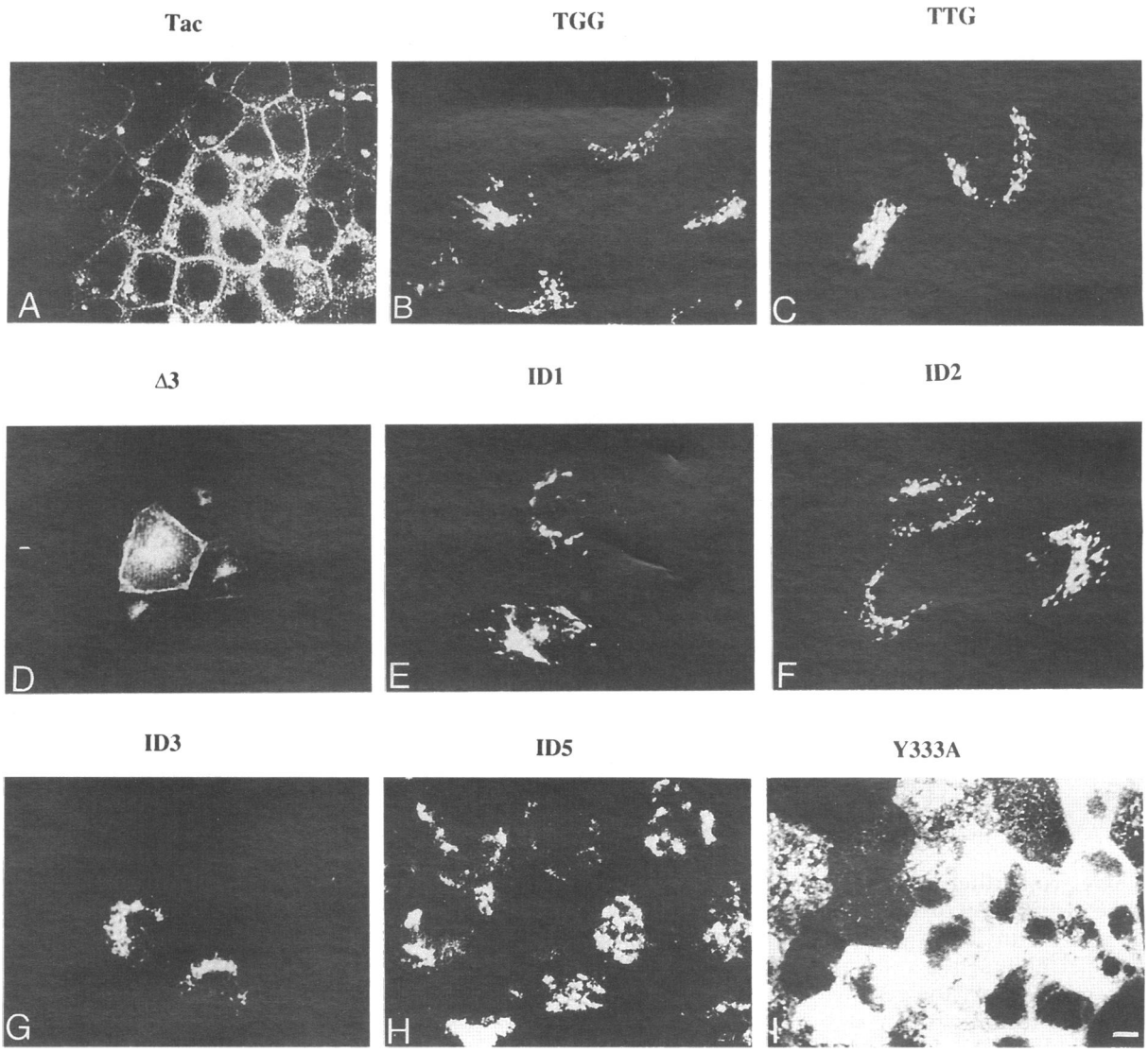


Figure 7. Immunofluorescence localization of Tac-TGN38 chimeric proteins in transfected MDCK cells by laser scanning confocal microscopy. MDCK cells were either stably transfected with Tac (A), TGG (B), ID5 (H), or Y333A (I) or transiently transfected with TTG (C), D3 (D), ID1 (E), ID2 (F), ID3 (G), or ID3 (I). Cells were stained with anti-Tac rat mAb and Texas Red-conjugated donkey antibody to rat IgG. Bar, 5 μm.

copy analysis of these cells upon staining with fluorescent second antibodies revealed that uptake indeed occurred from the basolateral surface. Experiments with radiolabeled antibodies demonstrated a three- to four-fold higher antibody uptake from the basolateral medium. This must reflect the basolateral recycling of TGN38 because nonspecific antibody uptakes from apical and basolateral surfaces were similar, probably reflecting the similar apical and basolateral surface areas of MDCK II cells grown on polycarbonate filters (Butor and Davoust, 1992) and the similar fluid phase endocytic ability of these membranes (Bomsel *et al.*, 1989). These results strongly suggest that TGN38-myc and TGG recycle through the basolateral surface of MDCK cells. At steady state only 6% of the total population of the TGG was expressed on the cell surface as determined by its neuraminidase sensitivity. Analysis of the intracellular localization of several Tac-TGN38 chimeric proteins indicated that determinants for TGN localization are similarly recognized in nonpolarized and polarized cells. In nonpolarized cells, information that is sufficient for TGN localization is mainly contained within a tyrosine-containing cytoplasmic sequence of TGN38 (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993). Using chimeras of Tac and TGN38,

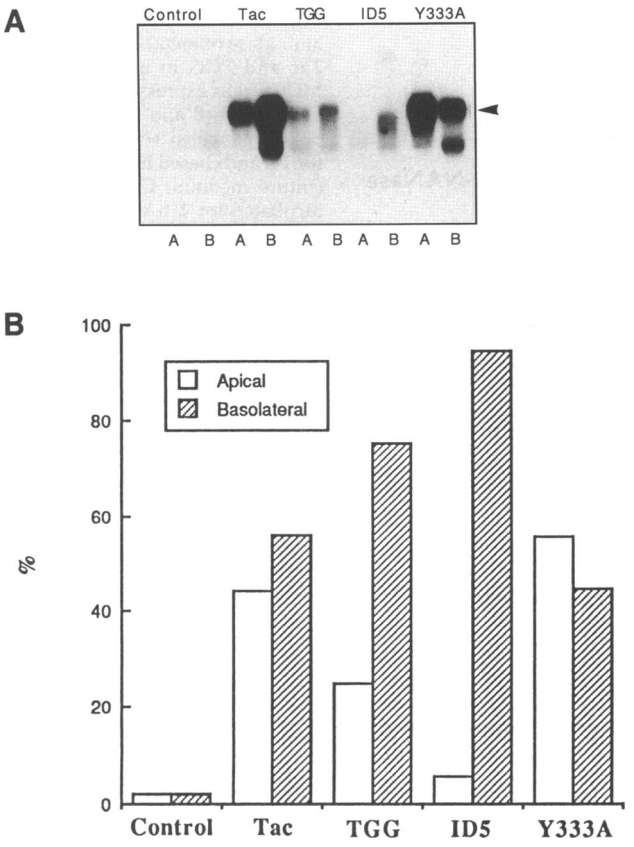


Figure 8. Analysis of the polarity of Tac-TGN chimeras in MDCK cells by cell surface biotinylation. (A) Cells grown on filters were biotinylated from the apical (A) or basolateral (B) sides and Tac proteins immunoprecipitated from detergent-solubilized cells. Biotinylated proteins were revealed by [^{125}I]-streptavidin blotting after 10% SDS-PAGE and transfer to nitrocellulose. (B) Fluorograms from two (Tac) or three (control, TGG, ID5, and Y333A) independent gels were quantitated, and the results were expressed as percentage apical and percentage basolateral surface expression of Tac-TGN38 chimeras in MDCK cells. Arrowhead indicates the band used in the densitometric quantification.

we show here that similar tail mutations to those that result in relocation to the cell surface in nonpolarized cells result in the redistribution to the plasma membrane in MDCK cells. Interestingly, the expression of these mutant proteins at the cell surface was either nonpolarized or concentrated in the apical domain.

The sequence that is critical for TGN localization of the chimeric proteins has a tyrosine-containing motif that bears similarity to signals involved in other processes, such as rapid endocytosis via clathrin-coated vesicles (Trowbridge, 1991), targeting to lysosomes (Kornfeld and Mellman, 1989; Peters *et al.*, 1990; Johnson and Kornfeld, 1992; Mathews *et al.*, 1992), and targeting to the basolateral surface of the epithelial cells (Brewer and Roth, 1991; Casanova *et al.*, 1991; Hunziker *et al.*, 1991; Le Bivic *et al.*, 1991; Matter *et al.*, 1992). Although similar in appearance, there are subtle distinctive aspects of these different types of signals that may be responsible for their different function. For example, individual mutation to alanine of all 27 amino acids in the cytoplasmic tail of lysosomal acid phosphatase, reveals slight differences between the requirements for basolateral targeting and targeting to lysosomes (Prill *et al.*, 1993).

The similar, yet nonidentical, sequence requirements for lysosomal, basolateral, and TGN targeting suggest that a family of different "adaptor" proteins may be regulating these different processes. To date, only clathrin-associated adaptor molecules have been implicated in sorting, with AP1 (HA1, containing γ-adaptin) involved in TGN sorting of hydrolases and AP2 (HA2, containing α-adaptin) participating in endocytic processes. Coatomer proteins, although showing homology to clathrin adaptors, are thought to participate only in bulk membrane flow processes (Rothman and Orci, 1992). The similarity of the sorting signals used for different cellular localizations suggests that all of these mechanisms developed during evolution from a similar ancestral mechanism, or that one of the three mechanisms preceded all the others. Because evolutionary theories about the cell predict that the plasma membrane is the oldest organelle (Sabatini *et al.*, 1982), it is likely that the endocytic mechanism is the oldest one, and the TGN and lysosome localization mechanisms derived from the endocytic mechanism as cells became more complex and developed the endomembrane system. In fact, the evolutionary relationship between the TGN and the plasma membrane is supported by three different sets of observations: 1) the effects of the fungal metabolite Brefeldin A, which increases recycling of TGN markers to the cell surface whereas Golgi markers are delivered to the ER (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989, 1990; Ulmer and Palade, 1989), effectively generating two separate endomembrane systems; 2) the location of Golgi localization signals in the transmembrane domain (reviewed by Machamer, 1991) in contrast with the importance of cytoplasmic determinants in TGN localization; 3) the ER "leakage" of overexpressed Golgi proteins (Munro, 1991; Colley *et al.*, 1992), in contrast to the plasma membrane localization of overexpressed TGN markers (Luzio *et al.*, 1990; Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993).

It cannot be excluded that the basolateral recycling of the TGN may have some functional significance. It has been speculated that the heavily glycosylated luminal domain of TGN38 might serve to bind nonspecifically luminal TGN proteins leaked into secretory vesicles to recycle them back to the TGN (Bos *et al.*, 1993). Extending this speculation, it may be suggested that the basolateral recycling of the TGN might partic-

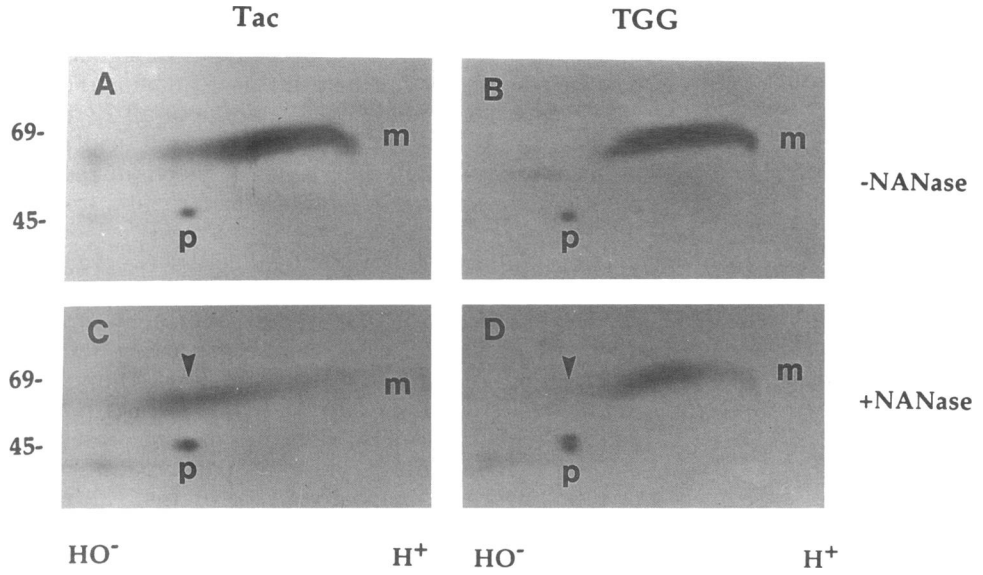


Figure 9. Sensitivity to neuraminidase of metabolically labeled Tac and TGG in intact cells and MDCK cells expressing Tac (A and C) or TGG (B and D) were metabolically labeled with methionine for 1 h and chased for 4 h in regular culture medium. Cells were then incubated for 1 h without (A and B, −NANase) or with (C and D, +NANase) neuraminidase. Tac and TGG were isolated from metabolically labeled cells using the mAb 7G7 and analyzed by 2-D NEPHGE/SDS-PAGE. The acidic and basic sides of the gels are indicated by the H^+ and HO^- symbols, respectively. The positions of M_r standards (expressed as $10^{-3} \times M_r$) are indicated on the left. Arrowheads point to the position of the desialylated species. *p*, the precursor; *m*, the mature species of Tac and TGG. Densitometric analyses of autoradiograms revealed that 76% of Tac and 6% of TGG were shifted to the basic side of the NEPHGE upon treatment with neuraminidase. Densitometry was performed using a Phosphor Imager (Molecular Dynamics).

ipate in the retrieval of missorted apical proteins for a second round of sorting in the TGN, an editing process that would greatly enhance the fidelity of plasma membrane sorting in the TGN.

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