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Targeting of Glycoprotein I (gE) of Varicella-Zoster Virus to the *trans*-Golgi Network by an AYRV Sequence and an Acidic Amino Acid-Rich Patch in the Cytosolic Domain of the Molecule

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Previous studies suggested that varicella-zoster virus (VZV) envelope glycoproteins (gps) are selectively transported to the *trans*-Golgi network (TGN) and that the cytosolic domain of gpI (gE) targets it to the TGN. To identify targeting signals in the gpI cytosolic domain, intracellular protein trafficking was studied in transfected cells expressing chimeric proteins in which a full-length or mutated gpI cytosolic domain was fused to the gpI transmembrane domain and interleukin-2 receptor (tac) ectodomain. Expressed protein was visualized with antibodies to tac. A targeting sequence (AYRV) and a second, acidic amino acid-rich region of the gpI cytosolic domain (putative signal patch) were each sufficient to cause expressed protein to colocalize with TGN markers. This targeting was lost when the tyrosine of the AYRV sequence was replaced with glycine or lysine, when arginine was replaced with glutamic acid, or when valine was substituted with lysine. In contrast, tyrosine could be replaced by phenylalanine and valine could be substituted with leucine. Mutation of alanine to aspartic acid or deletion of alanine abolished TGN targeting. Exposure of transfected cells to antibodies to the tac ectodomain revealed that the TGN targeting of expressed tac-gpI chimeric proteins occurred as a result of selective retrieval from the plasmalemma. These data suggest that the AYRV sequence and a second signaling patch in the cytosolic domain of gpI are responsible for its targeting to the TGN. The observations also support the hypothesis that the TGN plays a critical role in the envelopment of VZV.

The intracellular route of maturation and assembly of herpesviruses has been controversial. While it is clear that nucleocapsids are assembled in the nucleus and that they acquire an envelope during budding from the inner nuclear membrane (21), the nature of that envelope is uncertain. One idea, primarily supported by observations made on the processing of herpes simplex virus, is that the envelope derived from the inner nuclear membrane is essentially the final viral envelope (4, 16, 21). Although the sugars of envelope glycoproteins (gps) may be posttranslationally modified during transport through the cisternal space, tegument and the core proteins of the viral envelope are thought to all be present in the particles as they emerge from the nucleus. A second idea, developed from observations made on pseudorabies (3) and varicella-zoster virus (VZV) (6), is that the envelope acquired from the inner nuclear membrane is a temporary one that enables enveloped particles to fuse with the rough endoplasmic reticulum (RER), delivering free nucleocapsids to the cytosol. The nucleocapsids are then believed to be reenveloped in the *trans*-Golgi network (TGN) by a process that causes tegument to be trapped between the nucleocapsids and the viral envelope. This secondary envelope is thought to be the final one that is found on infectious particles. Conceivably, the same mechanism may not be responsible for the envelopment of all alphaherpesviruses.

One VZV gp, gpI, has been investigated as a model to determine whether a TGN-targeting sequence is present in its primary structure (30). These studies have indicated that the cytosolic domain of gpI contains all of the information needed for targeting that molecule to the TGN. In cells transfected with cDNA encoding wild-type gpI (gpI_{wt}), the protein concentrates in the TGN; however, when cells are transfected with cDNA encoding a truncated gpI, which lacks cytosolic and transmembrane domains, the protein does not concentrate in the TGN and is either secreted or degraded in the RER. When cells are transfected with a cDNA encoding a chimeric protein in which the ectodomain of a plasmalemmal marker, the interleukin-2 receptor (tac), is fused to the transmembrane and cytosolic domains of gpI_{wt}, the chimeric protein concentrates in the TGN. In contrast, in cells transfected with cDNA encoding the ectodomain of tac fused only to the transmembrane domain of gpI, the resultant protein concentrates in endosomes and not the TGN. The full-length tac itself is targeted to the plasma membrane of transfected cells. Transfection of cells with cDNA encoding mutant forms of gpI in which one, two, or all three potential glycosylation sites have been deleted does not affect the targeting of the resultant proteins. These observations support the idea that the cytosolic domain of at least one VZV gp contains targeting information that causes the protein to accumulate in the TGN.

This study was carried out to identify the sequence(s) of the cytosolic domain of gpI that is responsible for its targeting to the TGN. In addition, since the prototypic TGN protein, TGN38 (1), has been demonstrated to concentrate in the TGN by selective retrieval from the plasma membrane (13, 14, 19), we tested the hypothesis that gpI is similarly routed to the TGN via the plasmalemma. The observation that a chimeric protein, in which the transmembrane domain of gpI was fused to the ectodomain of tac, is targeted to endosomes is compatible with the possibility that the retrieval of gps from the plasma membrane to endosomes is a default pathway; retention in the plasma membrane, as in tac, or concentration in the TGN, as in gpI_{wt}, may require targeting signals in the cytosolic domains of the proteins. To study potential gpI-targeting se-

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TABLE 1. PCR amplimers used to prepare tac-gpI constructs (with internal primers)

Construct	3′ primer	Upstream internal primer	Downstream internal primer
R1-7 (gpI_{wt})	5′CCCTCTAGATGCCCCGGTTCGGTGATCA3′	5′GGGGGTAATTTCTGTTGTAAATATGGACGT3′	5′TTTACAACAGAAATTACCCCCGTAAAC3′
R1/R4-6	5′CCCTCTAGATGCCCCGGTTCGGTGATCA3′	5′GTAATACATGGCTTTAACCCTCATTCG3′	5′GTTAAAGCCATGTATTACGCTGGCCTT3′
R1/R5-6	5′CCCTCTAGATGCCCCGGTTCGGTGATCA3′	5′GAAATCGTCGGCTTTAACCCTCATTCG3′	5′GTTAAAGCCGACGATTTCGAGGACTCG3′
R1-2/R4-6	5′CCCTCTAGATGCCCCGGTTCGGTGATCA3′	5′GTAATACATTACCCTATAGGCTTTAAC3′	5′TATAGGGTAATGTATTACGCTGGCCTT3′
R1-2/R5-6	5′CCCTCTAGATGCCCCGGTTCGGTGATCA3′	5′GAAATCGTCTACCCTATAGGCTTTAAC3′	5′TATAGGGTAGACGATTTCGAGGACTCG3′

TABLE 2. PCR amplimers used to prepare tac-gpI constructs (without internal primers)

Construct	3′ primer
R1	5′CCCTCTAGAGGCTTTAACCCTCATTCGTTTAGCC3′
R1-2	5′CCCTCTAGATACCCTATAGGCTTTAACC3′
R1-3	5′CCCTCTAGAGCTTTGGTTATACGGGGAC3′
R1-4	5′CCCTCTAGACACTGGAAGGCCAGCGTAA3′
R1-5	5′CCCTCTAGACGTAGATTCCGAGTCCTCG3′
R1-6	5′CCCTCTAGACGCGTTACCAAACTCTTCT3′
$R1_{\delta A}$-R2	5′CCCTCTAGATACCCTATATTTAACCCTCATTCG3′
$R1_{A\rightarrow D}$-R2	5′CCCTCTAGATACCCTATAGTCTTTAAC-CCTCATTCG3′
R1-$R2_{Y\rightarrow F}$	5′CCCTCTAGATACCCTAAAGGCTTTAACC3′
R1-$R2_{Y\rightarrow G}$	5′CCCTCTAGATACCCTACCGGCTTTAACC3′
R1-$R2_{Y\rightarrow K}$	5′CCCTCTAGATACCCTTTTGGCTTTAACC3′
R1-$R2_{R\rightarrow E}$	5′CCCTCTAGATACCTCATAGGCTTTAACC3′
R1-$R2_{V\rightarrow K}$	5′CCCTCTAGATTTCCTATAGGCTTTAAC3′
R1-$R2_{V\rightarrow L}$	5′CCCTCTAGATAGCCTATAGGCTTTAACC3′

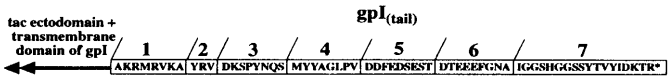
quences, cells were transfected with cDNA encoding chimeric proteins in which the ectodomain of tac was fused to the transmembrane and cytosolic domains of gpI from which specific sequences were deleted or mutated. To investigate the possibility that the targeting of gpI to the TGN involves selective retrieval from the plasma membrane, cells transfected with cDNA constructs encoding tac-gpI fusion proteins were incubated with antibodies to the ectodomain of tac. Since the tac ectodomain of the resultant fusion proteins would be exposed to the extracellular medium, retrieval of tac-gpI chimeric proteins from the plasma membrane would be expected to cause these antibodies to become concentrated in the TGN. Similarly, deletion or mutation of the responsible targeting signal in the cytosolic domain of gpI would be expected to eliminate the concentration in the TGN of antibodies to tac. Our observations support the idea that the cytosolic domain of gpI contains a TGN-targeting signal with the sequence AYRV. Additional portions of the cytosolic domain of gpI may contribute to a second TGN-targeting patch. Targeting of gpI to the TGN occurs as a result of selective retrieval from the plasma membrane.

MATERIALS AND METHODS

PCR cloning. cDNA encoding gpI_{wt} was cloned from VZV genomic DNA, using the amplimers 5′-CCCGGTACCGAGGGTCGCCTGTAATAT-3′ (VZGP1L5) and 5′-CCCTCTAGATGCCCCGGTTCGGTGATCA-3′ (VZGP1L3) as previously described (30). Briefly, reaction mixtures were initially incubated for 3 min at 94°C and then subjected to 35 cycles of 1 min at 94°C, 1 min at 58°C, and 3 min at 72°C. The PCR-amplified DNA was digested with *Asp*718 and *Xba*I and gel purified, and the resultant fragments were cloned into the multiple cloning sites of the eucaryotic expression vector pSVK3 (Pharmacia, Piscataway, N.J.). Transformed *Escherichia coli* DH5α clones were selected, and plasmid DNA from selected subclones was amplified and purified by CsCl gradient centrifugation. All constructs were sequenced to verify their authenticity. Authenticity of cloned cDNA was also verified by in vitro translation, which was carried out with a transcription-translation coupling kit (rabbit reticulocyte lysate system; Promega Biotec).

Preparation of chimeric proteins. We prepared cDNA constructs encoding chimeric proteins in which selected domains of gpI were fused to the extracellular domain of tac. Methods used were essentially those described previously (30). In the first step, two overlapping DNA fragments were amplified by the terminal primers and overlapping internal primers encompassing the desired mutant. Gel-purified products of this first amplification were used for a second PCR amplification in which only the terminal primers were used. For preparation of fusion proteins, the terminal 5′ tac primer was 5′-CCCGGTACCAAG GGTCAGGAAGATGGA-3′, which was paired with VZGP1L3. Primers (other than the 5′ primer) are shown in Tables 1 and 2.

Transfection of cells. The targeting of tac-gpI fusion proteins was investigated in transfected cells. Cos-7 cells were cultured at 37°C in Dulbecco's modified Eagle's medium containing heat-inactivated 10% fetal bovine serum, 100 U of penicillin per ml, and 100 U of streptomycin per ml in an atmosphere containing



Construct	Sequence	Localization	
		TGN	endosomes
R1-7 (gpI_{wt})	AKRMRVKAYRVDKSPYNQSMYYAGLPVDDFEDSESTDTEEEFGNAIGGSHGGSSYTVYIDKTR*	+++	0
R1-6	AKRMRVKAYRVDKSPYNQSMYYAGLPVDDFEDSESTDTEEEFGNA*	+++	0
R1-5	AKRMRVKAYRVDKSPYNQSMYYAGLPVDDFEDSEST*	+++	0
R1-4	AKRMRVKAYRVDKSPYNQSMYYAGLPV*	+++	±
R1-3	AKRMRVKAYRVDKSPYNQS*	++	+
R1-2	AKRMRVKAYRV*	++	+
R1	AKRMRVKA*	0	+++

FIG. 1. Identification of TGN-targeting sequences of the cytosolic domain of gpI. *, COOH terminus.

5% CO_2. Electroporation for used to transfect cells. For this purpose, 10^7 cells were released from dishes by trypsinization (0.5 ml), which was terminated by adding 0.5 ml of cold electroporation buffer (Dulbecco's modified Eagle's medium, 10% bovine calf serum, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]). The suspended cells (0.8 ml) were transferred into a 0.4-cm gap cuvette (Bio-Rad Laboratories, Richmond, Calif.), 40 μg of DNA was added, and the preparations were incubated on ice for 5 min. The cells were then placed in the chamber of a Gene Pulser electroporation unit (300 V, 960 μF; Bio-Rad). After electroporation, the cells were again incubated on ice for 5 min prior to transfer to a 10-cm-diameter dish containing growth medium. Media were changed after 12 h, and cells were incubated for a further period of 48 h.

Subcellular markers. Transfected cells were grown on glass coverslips in a six-well tissue culture dish and fixed for 2 h at room temperature with 2% formaldehyde (freshly prepared from paraformaldehyde). When endosomes were to be specifically identified, cells were incubated for 60 min prior to fixation with α_2-macroglobulin labeled with the green fluorescent dye fluorescein isothiocyanate (FITC). For immunocytochemistry, fixed cells were washed with phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100 in PBS. The cells were then exposed for 2 h at room temperature to mouse monoclonal antibodies to tac (1:100 diluted with PBS–2 mg of bovine serum albumin [BSA] per ml; Amersham Corp., Arlington Heights, Ill.), rinsed with PBS, and exposed to FITC-labeled goat anti-mouse immunoglobulin G (1:80 diluted with PBS–2 mg of BSA per ml; (Kirkegaard & Perry, Gaithersburg, Md.). Coverslips were washed in PBS and mounted in the presence of β-nitrophenol to minimize fading. The lipid *N*-(ε-7-nitrobenz-2-oxa-1,3-diazo-4-yl-aminocaproyl)-D-*erythro*-sphingosine (C_6-NBD-Cer), which selectively concentrates in the TGN (17), was used as a TGN marker. Immunoreactivity and the fluorescence of C_6-NBD-Cer was visualized by vertical fluorescence microscopy using a narrow-band dichroic mirror-filter combination (exciting filter BP 450-490; dichroic mirror RKP 510, barrier filter BP 525/20).

RESULTS

A targeting sequence in its cytosolic domain is sufficient to target gpI to the TGN. Constructs encoding chimeric proteins in which elements of the transmembrane and cytosolic domains of gpI were fused to the ectodomain of tac (Tables 1 and 2; Fig. 1) were transfected into Cos-7 cells, and tac immunoreactivity was localized in order to analyze the targeting of the resultant protein (30). In vitro translation was used to verify all constructs (Fig. 2). The TGN was identified with C_6-NBD-Cer, and endosomes were identified by endocytosis of Cy3-labeled α_2-macroglobulin. As noted in the earlier investigation (30), tac immunoreactivity was colocalized with TGN markers when cells were transfected with cDNA encoding tac fused to the full-length transmembrane and cytosolic domains of gpI_{wt} (Fig. 1) and with endosomal markers when cells were transfected with cDNA encoding tac fused only to the transmembrane domain of gpI. To delineate the sequences of the cytosolic domain of gpI that might contribute to the TGN targeting of the expressed fusion proteins, the cytosolic domain of gpI was divided into seven regions (R1 to R7) which were systematically deleted. Constructs encoding the ectodomain of tac fused to the transmembrane domain and the remaining segments of the cytosolic domain of gpI were then prepared and expressed in Cos-7 cells. A YRV sequence is found in the cytosolic tail of gpI (Fig. 1), and a TGN targeting signal of the TGN resident protein, TGN38, contains a YQRL sequence (9, 29). Although the YRV sequence of gpI is not identical to the YQRL sequence of TGN38, Q has been shown to be unnecessary for the targeting of TGN38 to the TGN, and the properties of L and V are not dissimilar; moreover, YRV also contains a tyrosine motif, which is essential for the sorting of proteins to the TGN (1). The regions into which the cytosolic domain of gpI were divided were thus designed to test the hypothesis that the YRV sequence of gpI (R2) is part of a targeting sequence that targets gpI to the TGN. In addition, since regions R5 and R6 of the cytosolic domain of gpI are unusually rich in the acidic amino acids E and D, we also tested the possibility that these acidic domains contribute to the targeting of the protein to the TGN.

The localization of expressed proteins in the TGN or endosomes of transfected cells was scored on an arbitrary scale of + to +++ by three independent observers. TGN localization was considered that which coincided with C_6-NBD-Cer. An endosome localization was considered that found for α_2-macroglobulin coupled to Cy3, which was evenly distributed in small cytosolic vesicles. Deletion of neither R6 nor R7 greatly affected the localization of tac immunoreactivity in transfected cells (Fig. 1). As was true of tac-gpI_{wt} (Fig. 1 and 3A and B), both tac-R1-6 (Fig. 1 and 3C) and tac-R1-5 were concentrated in the TGN. Although deletions of R6 and R7 did cause some tac immunoreactivity to appear in endosomes, endosomal localization was minimal until further deletions of regions from the C-terminal end of the cytosolic domain were made. These resulted in the localization of significant amounts of expressed protein in endosomes (Fig. 1 and 3E and F); however, the

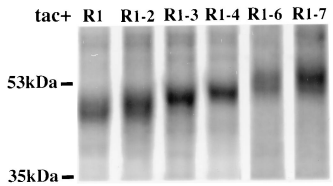


FIG. 2. In vitro translation. Constructs encoding tac-gpI chimeric proteins were translated in vitro by using a rabbit reticular cell lysate system. The constructs included the ectodomain of tac fused to the transmembrane domain and regions of the cytoplasmic domain of gpI. The positions of the regions of the gpI cytosolic domain are indicated in Table 1. The full-length gpI_{wt} encompasses R1 to R7. Note that a single product is translated from each construct and that the sizes of these products decrease appropriately as elements of the cytosolic domain of gpI are deleted.

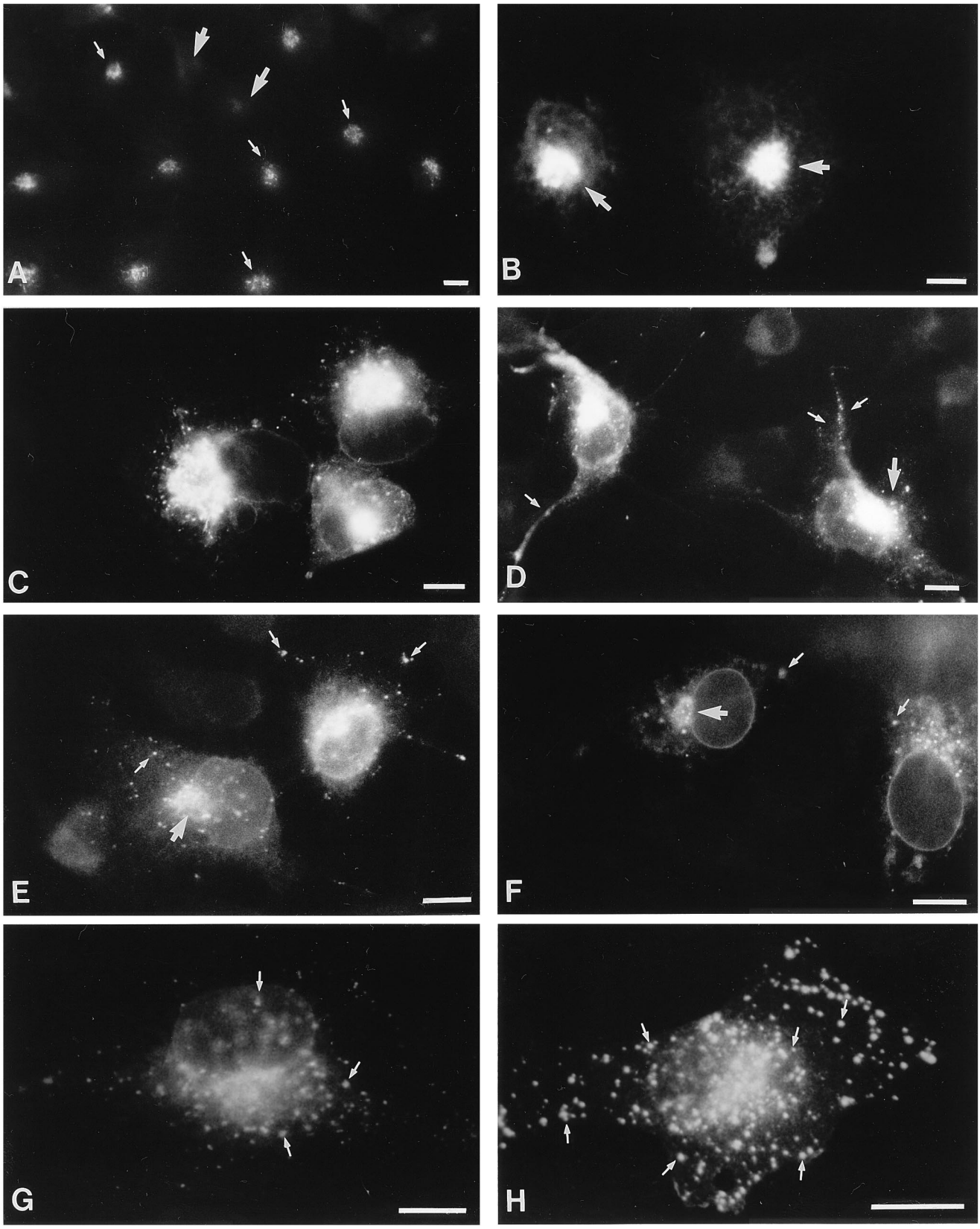

A
B
C
D
E
F
G
H

FIG. 3. Progressive deletion of regions of the cytosolic domain of gpI in tac-gpI constructs reveals the existence of a TGN targeting sequence in R2. Cos-7 cells were transfected with a construct encoding the tac ectodomain fused to the transmembrane domain of gpI and regions of the gpI cytosolic domain. The expressed protein was detected with antibodies to tac. The location of the TGN was assessed with C_6-NBD-Cer (not illustrated), and the location of endosomes was determined by demonstrating the uptake of Cy3-labeled α_2-macroglobulin (not illustrated). The markers represent 10 μm. (A) Control (construct R1-7, containing the full-length gpI_{wt} cytosolic domain). Note that at low magnification there is little or no background and that specific immunofluorescence (thin arrows) is seen only in the TGN of transfected cells. Cells that were not transfected (thick arrows) are not fluorescent. (B) Control (R1-7). At higher magnification, the limitation of specific immunofluorescence to the TGN (arrow) is more evident, and the absence of immunofluorescence in endosomes, ER, plasma membrane, or other organelles should be noted. (C) R1-6. Localization of specific immunofluorescence is similar to that of the control, primarily in the TGN. Very few small endosomes are evident. (D) R1-4. The TGN (thick arrow) is still labeled; however, the processes of the cells can also be seen because specific immunofluorescence is present in endosomes (thin arrows), which are diffusely distributed in the cytoplasm. In addition, the plasma membrane is immunostained, which adds apparent background and outlining to the transfected cells. (E) R1-3. The TGN (thick arrow) is still immunostained, and endosomes (thin arrows) are evident. No immunostaining of the plasma membrane is apparent; however, the expressed protein can be detected by a faint but specific immunofluorescence in the perinuclear cisterna which outlines the nonfluorescent nucleus. (F) R1-2. The TGN (thick arrow) is brightly immunostained, but there is also some specific immunofluorescence in endosomes (thin arrows) and the perinuclear cisterna. No immunostaining of the plasma membrane is apparent; however, the expressed protein can be detected by a faint but specific immunofluorescence in the perinuclear cisterna which outlines the nonfluorescent nucleus. (G and H) R1. There is almost no specific immunofluorescence remaining in the TGN, although the centrosomal regions of transfected cells are thicker than the periphery and show more autofluorescence. As a result, there is a diffuse nonspecific brightness in this region that is different from the more intense and sharply localized specific fluorescence found in immunostained organelles. Expressed protein is found mainly in endosomes; however, faint immunofluorescence can also be detected in the perinuclear cisterna (G). The plasma membrane is not immunostained.

fusion proteins continued to be concentrated primarily in the TGN until R2 was deleted (Fig. 1 and 3G and H). When R2 was deleted from the chimeric constructs, none of the resultant fusion protein concentrated in the TGN; instead, the expressed protein was located entirely in endosomes, a pattern identical to that observed in cells transfected with cDNA encoding the ectodomain of tac fused only to the transmembrane domain of gpI. These observations are consistent with the hypothesis that the YRV sequence (R2) of the cytosolic domain of gpI is essential for targeting the protein to the TGN. Certainly, the observation that the addition of only the YRV sequence to R1 is sufficient to confer TGN targeting on the resultant chimeric protein suggests that this sequence is a TGN-targeting signal, although it may not be the only one. The presence of the YRV sequence alone, however, did not prevent the appearance of some of the expressed protein in endosomes. Endosomes, as well as the TGN, became labeled when cells were transfected with many of the constructs encoding chimeric proteins with deletions from the C-terminal region of the cytosolic domain of gpI. This observation suggests that sequences other than YRV may also contribute to gpI targeting, at least to restrict retention in endosomes.

A second targeting patch in its cytosolic domain contributes to the targeting of gpI to the TGN. To determine whether regions of the cytosolic domain of gpI, in addition to the YRV of R2, contribute to TGN targeting, Cos-7 cells were transfected with constructs encoding tac-gpI chimeric proteins in which R2 was deleted while other regions of the gpI cytosolic domain closer to the C terminus were retained. Since the initial studies (Fig. 1) had indicated that the localization of fusion proteins that contained R1 to R3 (Fig. 3E) was similar to that of proteins that contained only R1 and R2 (Fig. 3F), these studies focused on R4 to R6, which included the acidic regions (R5 and R6) of the molecule. When cells were transfected with cDNA encoding chimeric proteins that lacked the YRV domain but contained R4 to R6, the expressed protein concentrated in the TGN but was also found in endosomes (Table 3; Fig. 4A). The further deletion (individually) of R4 (Table 3; Fig. 4B), R5 (Table 3), or R6 (Table 3; Fig. 4C), may have reduced but did not eliminate targeting to the TGN. There seemed to be a reciprocal relationship between targeting of constructs with deletions to the TGN and concentration of tac immunoreactivity in endosomes. Deletion of both R5 and R6 greatly increased the concentration of expressed protein in endosomes (Table 3; Fig. 4D). These observations are consistent with the idea that R4 to R6 encompass a targeting patch that contains TGN-targeting information.

Combining the targeting sequence with the targeting patch in its cytosolic domain potentiates the targeting of gpI to the TGN. Expressed proteins containing R2 and those containing R4 to R6 are each targeted to the TGN but also to endosomes. The presence of expressed protein in endosomes thus distinguishes the trafficking of these chimeric proteins from that of the fusion protein containing the full-length cytosolic domain of gpI_{wt}. To test whether the combination of these regions would be targeted like tac-gpI_{wt}, Cos-7 cells were transfected with a construct that encoded a chimeric protein that contained R2 and R4 to R6. The corresponding expressed protein was essentially confined to the TGN in transfected cells, and almost no labeling of endosomes was detected (Table 4; Fig. 5A). The appearance of tac immunoreactivity in cells transfected with the fusion protein containing R2 and R4 to R6 could not be distinguished from that of tac-gpI_{wt} (Fig. 5B). In contrast, expressed protein was found in endosomes when cells were transfected with constructs encoding proteins that contained R2 and only R4 (Table 4; Fig. 5C) or R4 and R5 (Table 4; Fig. 5D). These observations suggest that all of the targeting

TABLE 3. Identification of a TGN-targeting patch in the cytosolic domain of gpI

Construct[a]	Sequence[b]	Localization	
		TGN	Endosomes
R1/R4-6	AKRMRVKA_____MYYAGLPVDDFEDSESTDTEEEFGNA	++	+
R1/R5-6	AKRMRVKA_____DDFEDSESTDTEEEFGNA	+	++
R1/R4-5	AKRMRVKA_____MYYAGLPVDDFEDSEST	+	++
R1/R4	AKRMRVKA_____MYYAGLPV	±	+++[c]
R1-2/R4-6	AKRMRVKAYRV_____MYYAGLPVDDFEDSESTDTEEEFGNA	+++	0

[a] See Fig. 1.
[b] COOH termini are on the right.
[c] Also locates strongly in plasma membrane.

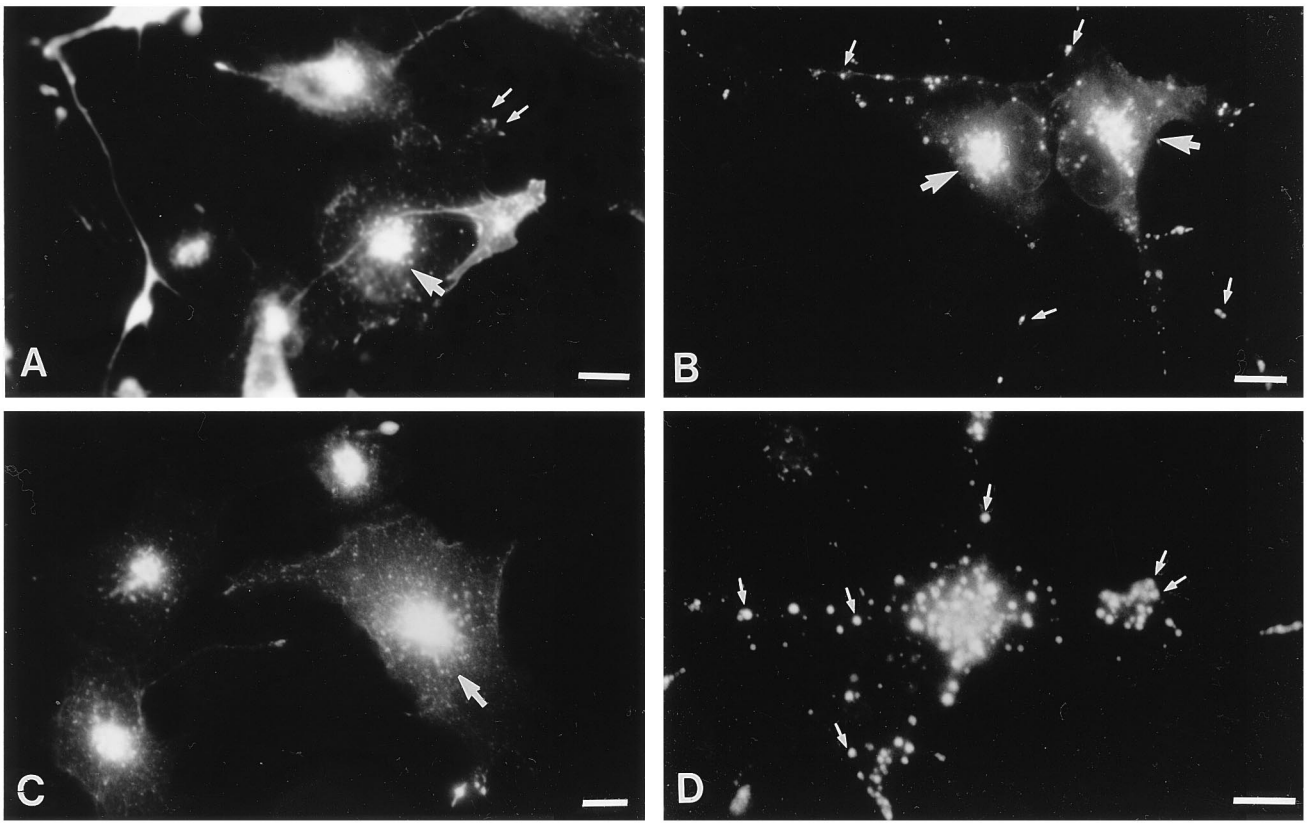


FIG. 4. Deletion of the region (R2) that includes the TGN-targeting sequence enables a second targeting patch to be detected. Cos-7 cells were transfected with a construct encoding the tac ectodomain fused to the transmembrane domain of gpI and regions of the gpI cytosolic domain from which R2 was deleted. The expressed protein was detected as for Fig. 3. The markers represent 10 μm. (A) R1/R4-6. Specific immunofluorescence can be detected in the TGN (thick arrow) and in endosomes (thin arrows). In many cells the plasma membrane is also very immunofluorescent, which obscures the visualization of intracellular structures. (B) R1/R5-6. Specific immunofluorescence is evident in the TGN (thick arrow) and in endosomes (thin arrows). There is less immunostaining of the plasma membrane than when R4 is present. (C) R1/R4-5. The TGN (thick arrow) and endosomes are immunostained. (D) R1/R4. Specific immunofluorescence is primarily located in endosomes (thin arrows); the TGN is not labeled.

information in the cytosolic domain of gpI is found in R2 and R4 to R6.

Mutations of individual amino acids of the targeting sequence identify those which are critical for TGN targeting. As controls, Cos-7 cells were transfected with cDNA encoding chimeric proteins in which the ectodomain of tac was fused to the transmembrane and R1 and R2 of the cytosolic domains of gpI (Table 5). The targeting of expressed protein was then compared with that found in cells transfected with tac-R1-2 constructs in which individual amino acids of R2 or the last amino acid of R1 were mutated. Targeting to the TGN was almost entirely lost when the tyrosine of the R2 sequence was replaced with glycine or lysine (R1-$R2_{Y\rightarrow G}$ or R1-$R2_{Y\rightarrow K}$ [Table 5]). The expressed R1-$R2_{Y\rightarrow G}$ protein appeared to be mainly retained in the endoplasmic reticulum (Fig. 6A). Intracellular expressed protein in cells transfected with the construct R1-$R2_{Y\rightarrow K}$ was restricted to endosomes (Fig. 6B). In contrast, tyrosine could be replaced by phenylalanine (R1-$R2_{Y\rightarrow F}$ [Table 5; Fig. 6C]) without altering the targeting of the expressed protein. These observations suggest that an aromatic amino acid rather than tyrosine itself is critical in position 569. Targeting to the TGN was also lost when the arginine of R2 was replaced with glutamic acid (R1-$R2_{R\rightarrow E}$ [Table 5; Fig. 6D]) or the valine of R2 was replaced with lysine (R1-$R2_{V\rightarrow K}$ [Table 5; Fig. 6E]). In both cases, intracellular tac immunoreactivity was again found only in endosomes. Although TGN targeting thus was abolished when valine was mutated to a basic amino acid, it could be replaced by another hydrophobic amino acid, leucine, without altering the targeting of expressed protein to the TGN or increasing localization in endosomes

TABLE 4. Combination of the putative TGN-targeting signal and patch in the cytosolic domain of gpI

Construct[a]	Sequence[b]	Localization	
		TGN	Endosomes
R1-2/R4-6	AKRMRVKAYRV_____MYYAGLPVDDFEDSESTDTEEEFGNA	+++	0
R1-2/R4-5	AKRMRVKAYRV_____MYYAGLPVDDFEDSEST	++	+
R1-2/R4	AKRMRVKAYRV_____MYYAGLPV	++	+

[a] See Fig. 1.
[b] COOH termini are on the right.

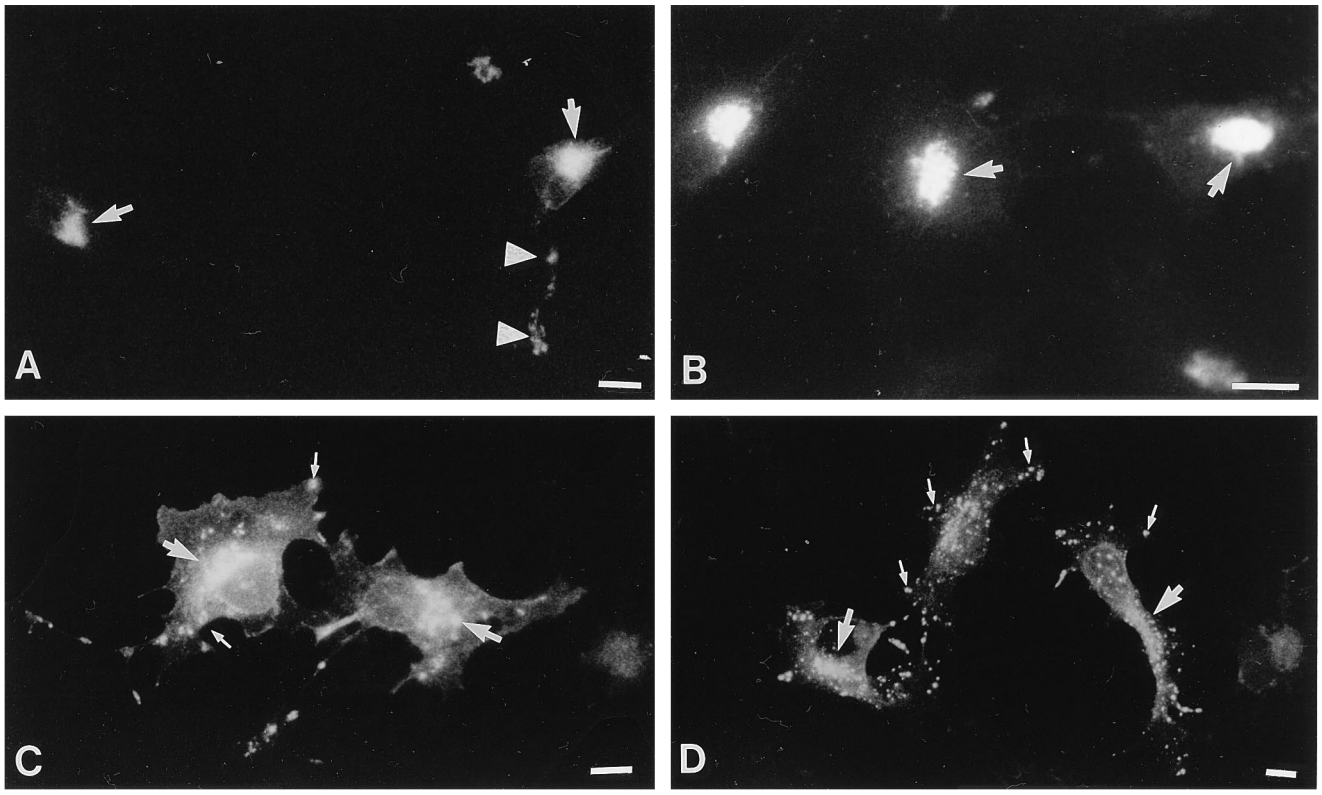


FIG. 5. Inclusion of regions of the gpI cytosolic domain that contain both the targeting sequence (R2) and the second signal patch (R4 to R6) improves targeting of expressed protein to the TGN. Cos-7 cells were transfected with a construct encoding the tac ectodomain fused to the transmembrane domain of gpI and regions of the gpI cytosolic domain from which R3 and R7 were deleted. The expressed protein was detected as in Fig. 3. The markers represent 10 μm. (A) R1-2/R4-6. The localization of tac immunoreactivity is limited to the TGN (thick arrows) of transfected cells. The long fluorescent structure below the transfected (arrowheads) is noncellular debris. (B) R1-7 (the full-length gpI_{wt} cytosolic domain). Immunofluorescence is confined to the TGN (thick arrows). The pattern of specific labeling is essentially the same as that found with R1-2/R4-6 (A). (C) R1-2/R4. Specific immunofluorescence is found in the TGN (thick arrow); however, the plasma membrane and endosomes (thin arrows) are also labeled. (D) R1-2/R4-5. Again, specific immunofluorescence is found in the TGN (thick arrow). Endosomes (thin arrows) are also labeled, but there is very little specific immunostaining of the plasma membrane.

($R1\text{-}R2_{V\rightarrow L}$ [Table 5; Fig. 6F]). Since the TGN targeting sequence of TGN38 includes four amino acids (9, 19, 20, 29), we evaluated the role of the alanine in position 568 of gpI, in R1, immediately preceding the YRV sequence. Mutation of alanine to aspartic acid ($R1_{A\rightarrow D}$-R2 [Table 5; Fig. 6G]) or deletion of alanine ($R1_{\Delta A}$-R2 [Table 5; Fig. 6H]) abolished TGN targeting and resulted in the concentration of all of the expressed protein in endosomes. These observations suggest that the alanine in position 568 is critical and that the TGN targeting signal of gpI is thus AYRV.

TABLE 5. Identification of critical amino acids in the TGN signal sequence

Construct[a]	Sequence[b]	Localization	
		TGN	Endosomes
R1-2 (control)	AKRMRVKAYRV	++	+
$R1\text{-}R2_{Y\rightarrow G}$	AKRMRVKAGRV	0	+[c]
$R1\text{-}R2_{Y\rightarrow K}$	AKRMRVKAKRV	0	+++
$R1\text{-}R2_{Y\rightarrow F}$	AKRMRVKAFRV	++	+
$R1\text{-}R2_{R\rightarrow E}$	AKRMRVKAYEV	0	+++
$R1\text{-}R2_{V\rightarrow K}$	AKRMRVKAYRK	0	+++
$R1\text{-}R2_{V\rightarrow L}$	AKRMRVKAYRL	++	+
$R1_{A\rightarrow D}$-R2	AKRMRVKDYRV	0	+++
$R1_{\delta A}$-R2	AKRMRVK_YRV	0	+++

[a] See Fig. 1.
[b] COOH termini are on the right.
[c] Retained in the ER (see Fig. 6A).

The targeting of gpI to the TGN involves the selective retrieval of the protein from the plasmalemma. To test the hypothesis that the trafficking of gpI to the TGN involves a route that takes the protein to the plasma membrane, living cells that were transfected with cDNA constructs encoding tac-gpI chimeric proteins were exposed to monoclonal antibodies to the ectodomain of tac. The cells were then fixed, permeabilized, and probed with FITC-labeled secondary antibodies to mouse immunoglobulin G. Two methods were used to expose cells to antibodies to the tac ectodomain. In one set of experiments, cultures of transfected Cos-7 cells were cooled and exposed to the antibodies for 2 h at 4°C. These cells were then rewarmed to 37°C and incubated for an additional 60 min. In the other set of experiments, cultures were not cooled but were incubated with the antibodies to tac for 2 h at 37°C. The location of tac immunoreactivity within transfected cells was the same regardless of how the cells were exposed to the antibodies.

Cos-7 cells that were transfected with cDNA encoding a chimeric protein in which the tac ectodomain was fused to the transmembrane and full-length cytosolic domains of gpI_{wt} took up antibodies to tac (Table 6; Fig. 7A). Nontransfected cells in the same cultures did not take up significant amounts of antibody. Within the transfected cells, the antibodies to tac were found to have become strikingly localized in the TGN (codis-

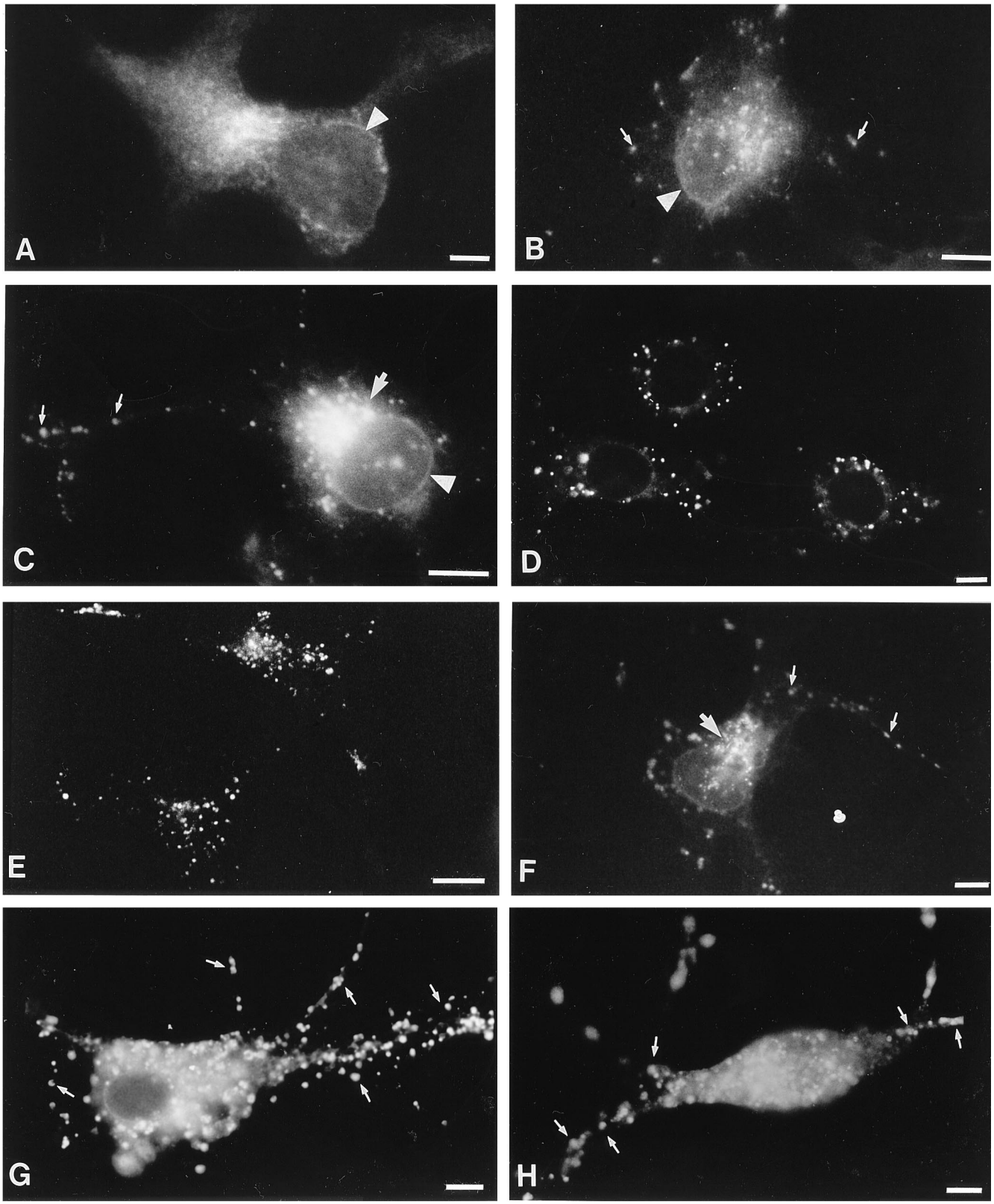


FIG. 6. Point mutations made in the targeting sequence enable amino acids critical for the targeting of expressed proteins to the TGN to be identified. Cos-7 cells were transfected with a construct encoding the tac ectodomain fused to the transmembrane domain of gpI and R1 and R2 of the cytosolic domain of gpI. Individual amino acids were mutated or deleted. The expressed protein was detected as for Fig. 2. The markers represent 10 μm. (A) R1-R2$_{Y\rightarrow G}$. tac immunoreactivity is primarily seen in an ER pattern. The perinuclear cisterna is immunofluorescent (arrowhead). The centrosomal regions of the transfected cells are brighter than the periphery, but there is little or no specific immunofluorescence in the TGN. (B) R1-R2$_{Y\rightarrow K}$. Immunofluorescence appears in endosomes (thin arrows) and the perinuclear cisterna (arrowhead). There is also a faint ER pattern of immunostaining in the background; however, there is no specific immunofluorescence in the TGN. (C) R1-R2$_{Y\rightarrow F}$.

TABLE 6. Intracellular targeting of antibodies to the tac ectodomain in cells transfected with cDNA encoding tac-gpI chimeric proteins

Construct	Localization		
	TGN	Endosomes	Plasma membrane
gpI_{wt}	+++	0	0
R1-6	+++	0	0
R1-5	++	+	0
R1-4	++	+	++
R1-3	++	+	0
R1-2	++	+	0
R1	0	++	+++
R1/R4-6	++	+	0
R1/R5-6	++	+	0
R1/R4-5	+	+	+
R1/R4	0	+	+++
R1-2/R4	++	+	+
R1-2/R5-6	++	0	0
R1-2/R4-6	+++	0	0

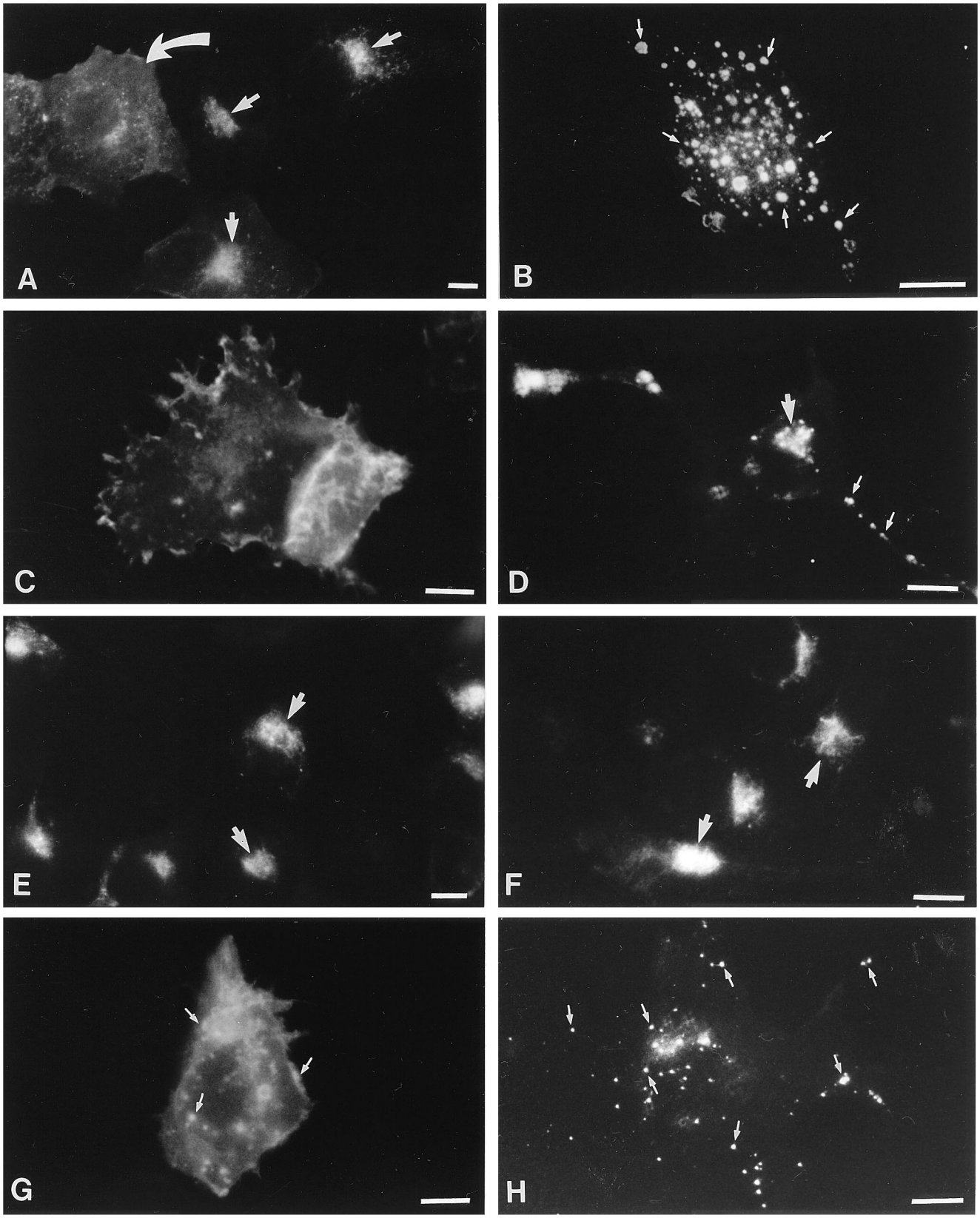
tributed with C_6-NBD-Cer). Almost none of the antibody was found in endosomes (located with α_2-macroglobulin), and very little appeared to be adherent to the plasma membrane. Cos-7 cells that were transfected with cDNA encoding a protein in which the tac ectodomain was fused to the transmembrane and only the R1 region of the cytosolic domain of gpI also took up antibodies to tac; however, in contrast to tac-gpI_{wt}, almost none of the intracellular anti-tac was found in the TGN (Table 6; Fig. 7B). Instead, intracellular antibodies to tac were found in endosomes and in some large vacuoles with a bizarre appearance, and a great deal remained on cell surfaces. In many of the transfected cells, the anti-tac appeared to be restricted to the plasma membrane and was hardly internalized at all (Fig. 7C). In sharp contrast, when Cos-7 cells were transfected with cDNA encoding a chimeric protein that contained both R1 and R2 of the cytosolic domain of gpI, the cells internalized antibodies to tac and targeted the internalized antibodies to the TGN (Fig. 7D). These observations suggest that antibodies bound at the plasma membrane to the tac ectodomain of tac-gpI chimeric proteins are internalized by transfected cells and that their subsequent targeting is determined by the gpI cytosolic domain of the chimeric protein. The striking difference in intracellular targeting between chimeric proteins that differ in the cytosolic domain only by the YRV sequence implies that this targeting sequence is responsible for targeting the internalized chimeric proteins to the TGN.

Additional constructs were prepared to determine whether the intracellular trafficking of antibody-labeled tac-gpI chimeric proteins internalized from the plasma membrane in transfected cells would mimic the targeting of the proteins themselves (Table 6). Studies of the distribution of these constructs suggested that both the targeting sequence (R2) and the signal patch (R4 to R6) play roles in the routing to the TGN of internalized chimeric proteins tagged with antibodies to tac, just as they do the routing of the similar proteins expressed in transfected Cos-7 cells. The targeting of antibody-labeled internalized chimeric protein to the TGN was maximal in cells transfected with cDNA encoding proteins that contained either the full-length cytosolic domain of tac-gpI_{wt}, R1 to R6, or R1 and R2 plus R4 to R6 (Table 6; Fig. 7E and F). The deletion of one of the regions, R4 to R6, that comprises the signal patch found previously, did not abolish targeting of internalized chimeric protein to the TGN, but it increased the distribution of the internalized protein in endosomes at the expense of that which is concentrated in the TGN (Table 6). Curiously, when the cytosolic domain of the tac-gpI construct ended at R4 (construct R1-4 or R1/R4 [Table 6; Fig. 7G]), internalization was reduced and a great deal of the bound antibodies to tac remained at the plasma membrane. When cells were transfected with constructs that lacked R2 and contained only R4, very little concentration of internalized protein was found in the TGN; most of the bound tac internalized in those transfected cells was found in endosomes (Fig. 7H). Both the previously identified targeting sequence, R2, and the signal patch, R4 to R6, therefore, were sufficient by themselves to direct internalized antibody-labeled chimeric proteins to the TGN. The TGN thus became labeled as long as constructs contained either the targeting signal (R2) or the targeting patch (R4 to R6) (Table 6). The combination of the targeting sequence with the signal patch sharpened TGN targeting, in the sense that the amount of internalized protein found in endosomes decreased. In contrast to the two critical regions of the cytosolic domain of gpI, other regions, such as R1, R3, and R7, appeared not to affect the relative targeting of antibodies to the TGN or endosomes.

DISCUSSION

Previous experiments have suggested that envelopment may occur twice during the transport and maturation of VZV. When particles emerge from the nuclei of infected cells, the virions are in the perinuclear cisterna and RER (6). At this stage, the particles appear to lack tegument and gpI (equivalent to gE of herpes simplex virus [7]) immunoreactivity (6). If the envelope acquired from the inner nuclear membrane were to contain gps, then these proteins would have to move within the plane of the membrane through nuclear pore complexes to reach the inner nuclear membrane. No such movement, however, could be detected in pulse-chase experiments with [^{3}H]mannose (6). Instead, [^{3}H]mannose-labeled gps were transported, independently of nucleocapsids, to the Golgi complex and the TGN. Apparent fusion of the envelope of intralumenal particles with RER membranes delivering nucleocapsids to the cytosol has been visualized. TGN sacs become curvilinear in infected cells. The concave TGN face contains viral gps and has tegument-like material attached to its cytosolic surface. The convex face lacks viral gps but contains the cation-independent mannose 6-phosphate receptor (MPR^{ci}). Nucleocapsids adhere to the accumulations of tegument-like material on the concave face and appear to become wrapped by the TGN sac so that the viral gp-rich concave membrane, still displaying TGN markers, becomes the viral envelope, while the convex membrane becomes a transport vesicle. Subsequent routing of vesicles carrying the enveloped VZV evidently follows the itin-

Specific immunofluorescence is found in the TGN (thick arrow) and in some endosomes (thin arrows). The perinuclear cisterna is also immunofluorescent (arrowhead). (D) R1-$R2_{R\rightarrow E}$. Specific immunofluorescence is confined to endosomes. The TGN is not immunofluorescent. (E) R1-$R2_{V\rightarrow K}$. Specific immunofluorescence is again confined to endosomes. The TGN is not immunofluorescent. (F) R1-$R2_{V\rightarrow L}$. Specific immunofluorescence is found in the TGN (thick arrow) and in some endosomes (thin arrows). (G) $R1_{A\rightarrow D}$-R2. Specific immunofluorescence is confined to endosomes (thin arrows). The TGN is not immunofluorescent. (H) $R1_{\delta A}$-R2. Specific immunofluorescence is abundant in endosomes (thin arrows), and there is considerable background from the apparent immunostaining of the ER; however, there is no labeling of the TGN.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erary of the MPR^{ci} to deliver virions to acidic prelysosomes (late endosomes). Inactivation of virions in these structures may account for the cell-associated nature of VZV in vitro. These observations suggest that the targeting of viral gps to the TGN might be critical for the final envelopment of VZV.

Since earlier studies indicated that the cytosolic domain of VZV gpI contains TGN-targeting information (30), the current investigation was carried out to identify the responsible regions of this portion of the molecule. The targeting of expressed protein in Cos-7 cells transfected with cDNA encoding the ectodomain of tac fused to the transmembrane and cytosolic domains of gpI was studied. To determine the role played in targeting by specific regions of the cytosolic domain of gpI, segments or individual amino acids of this domain were deleted or mutated. The selective localization of C_6-NBD-Cer served as a TGN marker (17, 30), and the localization of α_2-macroglobulin, taken up by endocytosis, was used as marker for endosomes (30).

Two regions of the cytosolic domain of gpI were found to be critical for the targeting of expressed chimeric proteins to the TGN. One was a short stretch of four amino acids with the sequence AYRV, and the other was a much longer sequence nearer the C-terminal end of gpI. Both of these regions of the gpI cytosolic domain contained information which by itself was sufficient to target chimeric proteins expressed in transfected cells to the TGN. We postulate that the longer acidic sequence constitutes a targeting patch, the effectiveness of which depends not on a sequence of contiguous amino acids but on the tertiary structure of the molecule. The individual amino acids of the shorter targeting sequence were each found to be important in signaling. In contrast, relatively large portions of the more distal patch could be deleted without abolishing its TGN-targeting function. While such deletions reduced the proportion of the expressed protein that was targeted to the TGN and increased the relative amount in endosomes, they did not prevent some of the expressed protein from concentrating in the TGN. Combining the shorter targeting sequence with the longer putative signal patch enhanced the targeting of the resultant expressed protein to the TGN and virtually eliminated its appearance in endosomes. It is of interest that TGN38, the prototypic TGN resident protein, also contains both a targeting sequence of amino acids and another signal patch, each of which contributes to the concentration of this molecule in the TGN (18).

The role in TGN targeting played by individual amino acids of the AYRV-targeting sequence was studied by evaluating the effects of point mutations made in constructs encoding chimeric proteins that lacked the targeting patch. The targeting of expressed proteins in cells transfected with these constructs was thus determined solely by the effectiveness of the targeting sequence and was not subject to a confounding effect of the more distal patch. Although the tyrosine of the AYRV sequence could not be replaced with glycine or lysine, it could be replaced by phenylalanine; therefore, an aromatic amino acid, rather than tyrosine itself, is probably required in this position. Phosphorylation on this tyrosine residue is thus not likely to play a role in TGN targeting. Like gpI, TGN38, contains a four-amino-acid sequence, YQRL, that is critical for its localization in the TGN (9, 19, 20, 29). The tyrosine of the YQRL sequence is thought to be essential; however, its substitution with phenylalanine has not been investigated. Since the arginine of AYRV could not be replaced with glutamic acid, a basic amino acid may be needed at this residue. Although targeting to the TGN was not disrupted when valine was substituted with leucine, targeting was lost when valine was substituted with lysine, suggesting that a hydrophobic amino acid and not a basic amino acid is critical at this position. Since alanine could not be deleted (which has the effect of converting AYRV to KYRV) or substituted with aspartic acid, targeting is evidently lost if an acidic or basic amino precedes the aromatic amino acid in the targeting sequence. Deletion of the entire region (R3) between the AYRV sequence and the more distal putative signal patch did not alter the targeting of expressed chimeric protein to the TGN, suggesting that valine is the last amino acid of the AYRV-targeting sequence. Another TGN resident protein, furin, is directed to the TGN by signalling regions in its cytosolic domain (2, 26). Furin also cycles between the plasma membrane and the TGN and concentrates in the TGN as a result of selective retrieval (24). As is true of gpI and TGN38, two nonoverlapping regions participate in signalling (22, 26). One of these is a YKGL motif that is likely to be functionally similar to the shorter targeting sequence of gpI, and the other is a longer region, the sequence of which is acidic (26) and resembles that found in R4 to R6 of the cytosolic domain of gpI. In fact, the S residues that have been demonstrated in these regions of furin to be critical for TGN targeting (24) are also present in analogous location in gpI. In addition to TGN38 and furin, the cytosolic domains of other proteins of interest contain sequences of amino acids that resemble the AYRV of gpI. One of these is the cation-dependent mannose 6-phosphate receptor (MPR^{cd}), which cycles between the plasma membrane and the TGN (11). The cytosolic domain of the MPR^{cd} contains both the YQRL that is found in TGN38 and an AYRGV sequence that resembles gpI. For it to be similar to AYRV, one would have to suppose that the additional glycine in the MPR^{cd} is a small linker that does not affect the signaling function. This supposition has not been tested, and the roles played by YQRL and AYRGV in the intracellular targeting of the MPR^{cd} have not been studied. The AYRGV sequence, however, has been found to be critical for internalization of the MPR^{cd} (10). The itinerary of the MPR^{cd}, moreover, includes stops at both the plasma membrane and the TGN (10, 11, 15, 27). Another protein with a similar sequence in a probable internal cytosolic loop is the putative

FIG. 7. Incubation of living transfected cells with antibodies to tac reveals that targeting of expressed protein to the TGN involves selective retrieval from the plasma membrane. Cos-7 cells were transfected with a construct encoding the tac ectodomain fused to the transmembrane and cytosolic domains of gpI. The cells were incubated with mouse monoclonal antibodies to tac prior to fixation. The cells were subsequently permeabilized and probed with a goat anti-mouse reagent to locate internalized antibodies to tac. Specific regions of the cytosolic domain of gpI were deleted. The markers represent 10 μm. (A) R1-7 (the full-length gpI_{wt} cytosolic domain). Specific immunofluorescence of anti-tac is largely confined to the TGN of transfected cells (thick arrows). In some cells (curved arrow), fluorescence of the plasma membrane can also be detected. No internalization of anti-tac can be seen in cells that are not transfected; although these cells, which are not fluorescent, cannot be visualized, they actually are more numerous than transfected cells. Since the cultures are confluent, the nontransfected cells fill the black areas of the field. (B) R1. Anti-tac immunofluorescence has been internalized; however, it is confined to large endosomes (thin arrows). There is no fluorescence in the TGN. (C) R1. Anti-tac immunofluorescence has not been internalized but remains bound to the plasma membrane of a transfected cell. (D) R1-2. Specific immunofluorescence of anti-tac is most intense in the TGN of transfected cells (thick arrows). The immunofluorescence of endosomes is also apparent (thin arrows). (E) R1-6. Specific immunofluorescence of anti-tac is largely confined to the TGN of transfected cells (thick arrows). (F) R1-2/R4-6. Specific immunofluorescence of anti-tac is again essentially confined to the TGN of transfected cells (arrows). The pattern is the same as that of R1-7 (A) and R1-6 (F). (G) R1/R4. A great deal of anti-tac remains bound to the plasma membrane. Most of the internalized anti-tac is found in endosomes (thin arrows). (H) R1/R4. In this cell, the anti-tac has been internalized; however, it is restricted to endosomes (thin arrows). Almost no anti-tac is seen in the TGN.

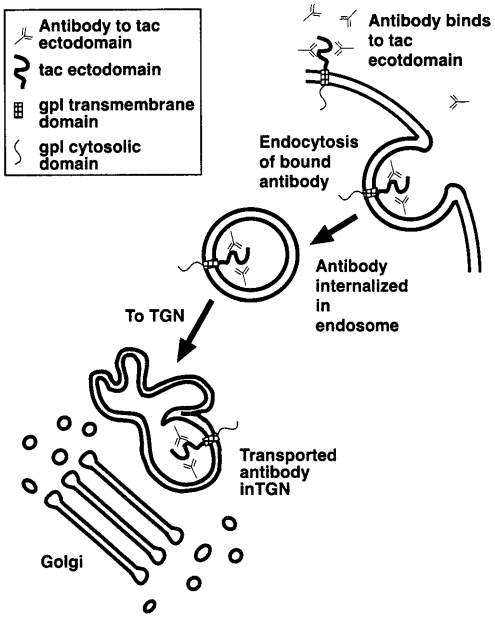

FIG. 8. Antibodies to the tac ectodomain are internalized and selectively concentrated in the TGN of cells transfected with gpI-tac constructs. The schematic diagram (not drawn to scale) shows how selective retrieval of expressed protein carrying bound antibodies can cause these antibodies to become concentrated in the TGN of transfected cells. The original forward transport of expressed protein to the plasma membrane is presumed to occur through the constitutive pathway and is not depicted. The antibodies to the tac ectodomain are postulated to bind to the tac ectodomain, which is exposed on the plasma membrane. The antibodies are then concentrated in the lumen of endosomes, which is topologically equivalent to extracellular space. The endosomes then presumably fuse with membranes of the TGN. Antibodies are again intralumenal, as the directionality of membranes is maintained. Since a change in the sequence of the gpI cytosolic domain of the chimeric proteins disrupts retrieval in the same manner as it disrupts targeting to the TGN, it is likely that targeting of expressed chimeric proteins occurs as a result of selective retrieval from the plasma membrane directed by the cytosolic domain of gpI.

Cu^{2+}-transporting ATPase that is defective in Menke's disease (12, 25). Although this sequence of the Cu^{2+}-transporting ATPase, LYRV, contains a leucine instead of an alanine, the two amino acids are likely to be functionally similar. The intracellular distribution of the Cu^{2+}-transporting ATPase has not yet been reported.

Since the targeting of TGN38 to the TGN has been shown to depend on selective retrieval of the protein from the plasma membrane, the possibility that a similar mechanism accounts for the targeting of gpI was investigated. Living cells that had been transfected with tac-gpI constructs were incubated with antibodies to the ectodomain of tac (Fig. 8). To minimize the possibility that antibodies to tac might enter cells nonspecifically by fluid-phase endocytosis, cells were initially exposed to the antibodies at 4°C. Subsequent warming after the washout of residual free antibody would enable bound antibodies to be internalized together with the proteins that they labeled. In practice, cooling the cells during exposure to antibodies proved to be unnecessary; transfected cells rapidly internalized antibodies to the tac ectodomain, regardless of whether the cells were exposed to these antibodies at 4 or at 37°C. In contrast, nontransfected cells did not take up the antibodies to tac in detectable amounts, even when they were incubated with the antibodies for 2 h at 37°C. Since expressed protein remains entirely at the plasma membrane when cells are transfected with the full-length tac (30), the internalization of the chimeric tac-gpI proteins suggests that an internalization signal is present in the cytosolic and/or transmembrane domains of gpI. The observation that antibodies to tac were internalized in all transfected cells, even in those transfected with constructs that contained only the transmembrane and R1 of the gpI cytosolic domain, suggests that the internalization signal of the gpI molecule is present either in its transmembrane region or in R1 of the cytosolic domain. Whether internalized chimeric proteins only reached endosomes or selectively concentrated in the TGN, however, was found to depend on signals in other regions of the cytosolic domain of gpI. These trafficking signals, determining the routing of protein retrieved from the plasma membrane, were the same domains of the gpI cytosolic domain, which were found to direct the targeting of expressed tac-gpI constructs. Again, both the AYRV-targeting sequence and the larger putative signal patch were found to play important roles. Either could direct some of the internalized protein to the TGN, and when both were present in the expressed protein, essentially all of the retrieved antibody-labeled protein was found in the TGN. These observations suggest that selective retrieval from the plasma membrane is the mechanism that is responsible for the targeting of gpI to the TGN (Fig. 8).

The conclusion that selective retrieval from the plasma membrane is responsible for the targeting of gpI to the TGN implies that the concentration of gpI in the TGN is likely to be an active process involving membrane recycling, rather than a stable process involving the anchoring of gpI in the TGN. To reach the plasma membrane, gpI would have to be transported from the RER through the *cis*-Golgi, Golgi stack, TGN, and transport vesicles. To carry bound antibodies to tac back to the TGN from the plasma membrane, the protein would have to be retrieved by endocytosis, which would deliver it to endosomes (Fig. 8). Endosomes are organelles that sort a great deal of membrane traffic (28). For example, both MPRs cycle between the plasma membrane and the TGN via endosomes (23). Endosomes also direct receptors and other membrane proteins to specialized postendosomal vesicles, including the transcytotic vesicles of epithelial cells, synaptic vesicles of neurons, and GLUT4-containing vesicles of adipocytes (28). The routing function of the targeting sequence and patch of the cytosolic domain of gpI, therefore, evidently interacts with a sorting mechanism that directs the traffic of membrane from endosomes. An equilibrium, in which vesicles containing gpI recycle between the TGN and the plasma membrane, is probably shifted by the presence of the targeting signal and patch in the cytosolic domain of gpI toward the TGN. Deletion of either the shorter signal or the longer patch may shift the equilibrium toward endosomes, while the loss of both signaling regions results in the failure of endosomes to transport gpI back to the TGN.

The observation that gpI contains targeting signals that cause it to concentrate in the TGN is compatible with the hypothesis that the TGN plays a critical role in viral envelopment. Neither the shorter targeting sequence nor the longer targeting patch of the gpI cytosolic domain is present in the cytosolic domains of VZV gpII to gpV (5). In fact, cytosolic tails of significant length are probably present only in gpI, gpII, gpIV, and gpVI; moreover, there is relatively little sequence identity in the putative cytosolic regions of any of the gps, although an AYRYV sequence is present in gpII. It is thus not yet clear what is responsible for routing gps other than gpI to the TGN. Conceivably, different targeting sequences in the other gps may be responsible. Alternatively, other gps may not be independently targeted to the TGN, but instead be routed to this organelle because they form a complex with gpI. The resulting multimeric complex could then be directed to the site of viral envelopment by the targeting signal in the cytosolic

domain of gpI. If so, then gpI would serve as a navigator gp, a role that is consistent with the fact that gpI is the most abundant protein synthesized by cells infected with VZV (8). Although the mechanism by which the TGN envelops newly synthesized virions in VZV-infected cells has not yet been made clear, the current observations support the idea that this organelle is the site where the process occurs and suggest that gpI, which contains TGN-targeting signals, may be critical for viral envelopment.

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