The EMBO Journal vol.15 no.22 pp.6096–6110, 1996

A tyrosine-based motif and a casein kinase II phosphorylation site regulate the intracellular trafficking of the varicella-zoster virus glycoprotein I, a protein localized in the trans-Golgi network

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We have studied the intracellular trafficking of the envelope glycoprotein I (gpI) of the varicella-zoster virus, a human herpes virus whose assembly is believed to occur in the trans-Golgi network (TGN) and/or in endocytic compartments. When expressed in HeLa cells in the absence of additional virally encoded factors, this type-I membrane protein localizes to the TGN and cycles between this compartment and the cell surface. The expression of gpI promotes the recruitment of the AP-1 Golgi-specific assembly proteins onto TGN membranes, strongly suggesting that gpI, like the mannose 6-phosphate receptors, can leave the TGN in clathrin-coated vesicles for subsequent transport to endosomes. Its return from the cell surface to the TGN also occurs through endosomes. The transfer of the gpI cytoplasmic domain onto a reporter molecule shows that this domain is sufficient to confer TGN localization. Mutational analysis of this domain indicates that proper subcellular localization and cycling of gpI depend on two different determinants, a tyrosine-containing tetrapeptide related to endocytosis sorting signals and a cluster of acidic amino acids containing casein kinase II phosphorylatable residues. Thus, the VZV gpI and the mannose 6-phosphate receptors, albeit localized in different intracellular compartments at steady-state, follow similar trafficking pathways and share similar sorting mechanisms.

Keywords: assembly proteins/recycling/sorting signals/ trans-Golgi network/varicella-zoster virus

Introduction

During the past years, the trans-Golgi network (TGN) has been characterized as a multi-functional compartment, involved in protein sorting and transport processes within the secretory pathway (Griffiths and Simons, 1986; Mellman and Simons, 1992). The late post-translational modifications that newly synthesized molecules undergo in the TGN include sialylation (Roth *et al.*, 1985), tyrosine sulfation (Huttner and Baeuerle, 1988) and, in some cases, proteolytic processing (Sossin *et al.*, 1990), and rely on a set of TGN resident proteins, such as the β-galactoside α2,6-sialyltransferase (STase) (Roth *et al.*, 1985), a type-II membrane protein related to other carbohydrate-processing Golgi enzymes, and furin, a type-I membrane protein that belongs to a family of subtilisin-related mammalian endoproteases (Barr *et al.*, 1991). Other TGN-resident proteins include TGN38 and TGN41, two isoforms of a highly glycosylated type-I membrane protein of an as yet uncharacterized function (Luzio *et al.*, 1990; Reaves *et al.*, 1992). Although it is clear that furin and TGN38 cycle between the TGN and the plasma membrane (Bos *et al.*, 1993; Molloy *et al.*, 1994; Voorhoes *et al.*, 1995), the molecular mechanisms responsible for retaining the bulk of these molecules in the TGN at steady-state remain poorly understood (Luzio and Banting, 1993). Intracellular traffic of furin relies on a tyrosine-based motif and an acidic stretch (Jones *et al.*, 1995; Schäfer *et al.*, 1995; Takahashi *et al.*, 1995; Voorhoes *et al.*, 1995), whereas that of TGN38 relies on a tyrosine-based motif and the transmembrane region (Ponnambalam *et al.*, 1994; Wong and Hong, 1993; Bos *et al.*, 1993).

The TGN also constitutes the major sorting station where proteins and lipids that must be targeted to different post-Golgi locations are packaged into distinct carrier vesicles. For example, the mannose 6-phosphate receptors (MPRs) and their bound lysosomal enzymes are packaged into clathrin-coated vesicles for delivery to endosomes (Kornfeld and Mellman, 1989), while other molecules destined for the plasma membrane are incorporated into other transport vesicles (Yoshimori *et al.*, 1996). In cells that show regulated secretion, the TGN is the place where concentration of proteins destined for export into secretory granules occurs (Bauernfeind and Huttner, 1993). In the case of those vesicles involved in lysosomal enzyme transport, it is now well established that they are generated by the successive recruitment of two cytosolic protein complexes onto the TGN membrane: the AP-1 Golgi-specific assembly proteins and clathrin triskelions (Kornfeld and Mellman, 1989; Pearse and Robinson, 1990; Schmid and Damke, 1995). The first of these complexes (AP-1) is a heterotetramer whose binding to the TGN membrane is regulated by the ADP-ribosylation factor ARF-1, a small GTP-binding protein (Stammes and Rothman, 1993; Traub *et al.*, 1993) and requires the presence of transmembrane proteins, in particular that of the MPRs that are sorted along the AP-1-dependent pathway (Le Borgne *et al.*, 1993, 1996). The translocation of AP-1 is a prerequisite for the subsequent binding of the clathrin triskelions, whose self-assembly properties are thought to generate the driving force for the formation of clathrin-coated vesicles (CCVs). The delivery of plasma membrane receptors to early endosomes also occurs by means of similar CCVs (Schmid, 1992). Both types of CCV can be distinguished on the basis of the underlying assembly proteins, since AP-1 and its plasma membrane counterpart AP-2 are made up of different, but related subunits (Pearse and Robinson, 1990). The intracellular

traffic of the MPRs requires sorting signals in their cytoplasmic tails, namely tyrosine-based and dileucine-based motifs and acidic stretches with casein kinase II phosphorylation sites (Kornfeld, 1992; Trowbridge *et al.*, 1993).

In addition to the MPRs, the envelope glycoproteins of the varicella-zoster virus (VZV) could provide an interesting model system to study sorting events occurring in the TGN. The VZV, an alpha herpes virus, is the causative agent of chickenpox and shingles (Weller, 1983a,b). It has been shown recently that during VZV biogenesis, its nucleocapsids and envelope glycoproteins are transported separately throughout the cell (Gershon *et al.*, 1994). The nucleocapsids, which are assembled in the nucleus, acquire a transient envelope as they translocate through the inner nuclear membrane into the periplasmic space. This envelope is lost as the virion fuses with the membrane of the endoplasmic reticulum, the nucleocapsids thus being released into the cytoplasm (Gershon *et al.*, 1994). On the other hand, the genome of the VZV codes for at least six membrane glycoproteins (gpI, gpII, gpIII, gpIV, gpV and gpVI) (Davidson and Scott, 1986), also known as gE, gB, gH, gI, gC and gL (Davidson *et al.*, 1986), which are all type-I membrane proteins. These molecules are synthesized in polyribosomes and transported along the secretory pathway until they reach the TGN, as evidenced by the presence in their polypeptide chains of both sialylated complex-type oligosaccharides, as well as other modifications known to occur within the TGN (Grose, 1990). The actual series of events leading to the final envelopment of the nucleocapsids remains largely unknown. When propagated in cultured cells, mature viruses are secreted into the medium in a non-infective state (Cook and Stevens, 1968; Gershon *et al.*, 1973; Gabel *et al.*, 1989), most likely due to their degradation in hydrolase-rich, pre-lysosomal compartments where the virus can be found (Gabel *et al.*, 1989; Gershon *et al.*, 1994). One model to explain the biogenesis of the VZV argues that budding of the nucleocapsids and enveloping occurs in the TGN, with the mature viruses being released in the TGN lumen (Gershon *et al.*, 1994). It has been proposed further that these viruses could bind to the MPRs through mannose 6-phosphate residues present on some oligosaccharides of the envelope glycoproteins, and subsequently be transported to endosomes in a manner similar to the lysosomal enzymes (Gershon *et al.*, 1994). An alternative, but not mutually exclusive, hypothesis is that the envelope glycoproteins, like the MPRs, are transported into CCVs to endocytic compartments where viral budding could occur.

We describe here the intracellular trafficking of gpI (or gE), the most abundant envelope glycoprotein of the VZV, which contains in its cytoplasmic tail signals similar to those found in the MPRs. Our data indicate that gpI is present at steady-state in the TGN and cycles between this compartment, endosomes and the cell surface. We also show that gpI is able to promote AP-1 recruitment onto Golgi membranes, probably reflecting its exit from the TGN via CCVs. In addition, specific signals within the gpI tail are responsible for this cyclic movement. These results may not only facilitate a better understanding of the biogenesis of the VZV, but also could provide additional support for the important role of cargo molecules in promoting the initial stages of coat formation. They also provide evidence for a novel TGN marker that can be used in future studies to address the mechanisms involved in TGN localization.

Results

In order to study the intracellular traffic of gpI, the complete open reading frame of the VZV gpI was inserted in the eukaryotic expression vector pSFFV6 (Chen *et al.*, 1993) and transiently expressed in HeLa cells. These cells were chosen for this study because TGN38 and STase have already been characterized as bona fide TGN markers in this cell type (Ponnambalam *et al.*, 1994; Rabouille *et al.*, 1995).

GpI localizes to the TGN at steady-state

GpI contains in its cytoplasmic tail motifs reminiscent of those found in the tails of furin and the MPRs, namely a tyrosine-based motif and casein kinase II phosphorylation sites. For this reason, we initially compared the intracellular distributions of gpI with those of furin, which is localized in the TGN (Bosshart *et al.*, 1994; Molloy *et al.*, 1994; Schäfer *et al.*, 1995; Takahashi *et al.*, 1995) and of the cation-independent MPR (CI-MPR) which, depending on the cell type, distributes to different extents between the TGN and late endosomes (Griffiths *et al.*, 1990; Klumperman *et al.*, 1993). Due to the poor reactivity of some antibodies against endogenous TGN markers, we co-expressed gpI together with VSV-G-tagged STase (Rabouille *et al.*, 1995), TGN38 and furin. As shown in Figure 1, confocal immunofluorescence microscopy of double-transfected HeLa cells revealed that gpI expression was restricted to the perinuclear region (Figure 1a, c, e and g), in a compartment that largely co-localized with the TGN, as judged by its simultaneous labelling with anti-VSV-G (Figure 1b), anti-TGN38 (Figure 1d) and anti-furin (Figure 1f) antibodies. Similar co-localization patterns could be observed in ~90% of the cells. In the remaining 10% of the cells, gpI was detected mainly in the cell surface, correlating in every case with the simultaneous surface expression of furin and TGN38 (not shown). These cells are probably expressing the transfected genes above physiological levels and were not investigated further. HeLa cells were then transfected with gpI and double labelled with anti-gpI antibodies and antibodies against the CI-MPR (Griffiths *et al.*, 1988). Conventional immunofluorescence microscopy revealed that the bulk of cellular CI-MPR was found in the perinuclear region, almost indistinguishable from the gpI-positive compartment (data not shown). However, visualization of the same samples by laser confocal microscopy revealed that the apparent co-localization of these two markers was, in general, less evident although both were clearly in the same region of the cell (Figure 1g and h). In addition, the anti-CI-MPR antibodies labelled numerous vesicles located close to the nucleus that were never labelled with the anti-gpI antibody (Figure 1g and h).

The poor reactivity of anti-gpI antibodies at the electron microscope level prevented a detailed localization. An alternative approach to distinguish closely apposed membrane compartments relies on the use of drugs that affect the morphology of the compartments under study. For this purpose, the drug brefeldin A (BFA) was used. This drug

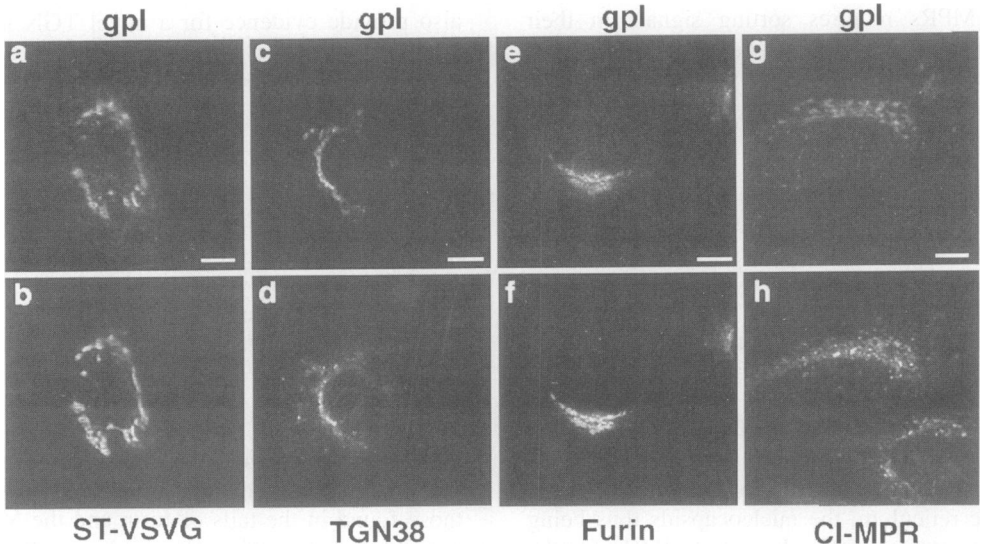

Fig. 1. Localization of gpI, TGN38, furin, sialyltransferase and the mannose 6-phosphate receptor by confocal microscopy. HeLa cells were double transfected with the plasmids pSFFV-gpI and pSRα-STVSVG (**a** and **b**), pSFFV-gpI and pCMV-TGN38 (**c** and **d**), pSFFV-gpI and pSG5-furin (**e** and **f**) or only pSFFV-gpI, fixed and processed for immunofluorescence, using in each case the SG1 anti-gpI monoclonal and polyclonal antibodies against the VSV-G epitope or TGN38 or furin or the cation-independent mannose 6-phosphate receptor. GpI was detected with fluorescein-conjugated anti-mouse IgG antibodies (a, c, e and g), whereas rhodamine-conjugated anti-rabbit IgG antibodies were used to detect VSV-G-tagged sialyltransferase (b), TGN38 (d), furin (f) and the CI-MPR (h). The percentage of transfected cells that showed co-expression of the two transfected markers was almost 100%.

is known to impair AP-1 binding (Robinson and Kreis, 1992) and to induce a rapid tubularization of the TGN as well as of the endosomes (Lippincott-Schwartz *et al.*, 1991; Reaves and Banting, 1992). Double-transfected HeLa cells were treated for 5 min with 10 μg/ml BFA, fixed and stained with antibodies against gpI, furin or TGN38. The formation of tubules simultaneously labelled with the anti-gpI, anti-furin and anti-TGN38 antibodies (Figure 2c–f) was observed after addition of BFA. However, this gpI-positive, tubular compartment remained poorly labelled with anti-CI-MPR antibodies, clearly segregating the bulk of gpI from the structures enriched for this MPR (Figure 2a and b). In addition, treatment of equally transfected cells with the microtubule-depolymerizing agent nocodazole also induced a segregation of the CI-MPR and gpI-positive compartment into distinct vesicle populations (not shown). Collectively, these data argue that gpI, furin, TGN 38 and STase are present in the same compartment, presumably the TGN, while the CI-MPR in HeLa cells is localized mostly in a different, although closely apposed compartment, most probably the late endosomes.

GpI cycles between the TGN and the cell surface

Furin and TGN38 cycle between the TGN and the cell surface (Bos *et al.*, 1993; Molloy *et al.*, 1994; Voorhoes *et al.*, 1995). To investigate whether gpI behaves in a similar manner, antibody uptake experiments were performed on gpI-expressing HeLa cells. When the cells were allowed to internalize a polyclonal anti-gpI antibody for 1 h, a strong perinuclear signal, together with some vesicular pattern scattered throughout the cytosol, was observed (Figure 3A,a). This perinuclear labelling overlapped with the bulk of intracellular gpI, detected with a monoclonal anti-gpI antibody (Figure 3A,b). The internalized antibody could not be detected without permeabilization of the cells (Figure 3A, c and d). When the polyclonal anti-gpI antibody was internalized for short time periods (5 min), most of the labelling was associated with peripheral structures (Figure 3B, a) that co-localized with the transferrin receptor (Figure 3B, b). Altogether, these results strongly suggest that gpI has access to the cell surface and recycles back to the TGN via an early endosomal compartment containing a fraction of the transferrin receptor.

GpI recruits AP-1 onto TGN membranes

We have shown that the overexpression of the MPRs promotes AP-1 recruitment onto Golgi membranes (Le Borgne *et al.*, 1993, 1996). Because gpI and the MPRs share similar protein motifs in their cytoplasmic domains, we investigated the possibility that gpI could be sorted in a similar way. For this purpose, either the wild-type gpI or two different truncation mutants that lack either most of the cytoplasmic tail (Δ1) or the carboxy-terminal domain containing an acidic cluster (Δ2) (see Figure 8) were expressed in HeLa cells using a T7 RNA polymerase recombinant vaccinia virus. These cells were labelled with a polyclonal anti-gpI antibody, to discriminate transfected from non-transfected cells, and with a monoclonal antibody against γ-adaptin, as a marker of the AP-1 complex. As shown in Figure 4, overexpression of either the wild-type gpI or the Δ2 mutant correlates with an increase of γ-adaptin staining in the perinuclear region (Figure 4b and d) that overlaps with the bulk of gpI (Figure 4a and c). In contrast, the tail-less mutant (Δ1), that was also found concentrated in the perinuclear region due to its overexpression, remains without any effect (Figure 4e and f). When the cells were treated for 5 min with 10 μg/ml BFA prior to fixation, no apparent γ-adaptin could be detected in the TGN (data not shown), indicating that the changes in AP-1 distribution observed in gpI-expressing cells

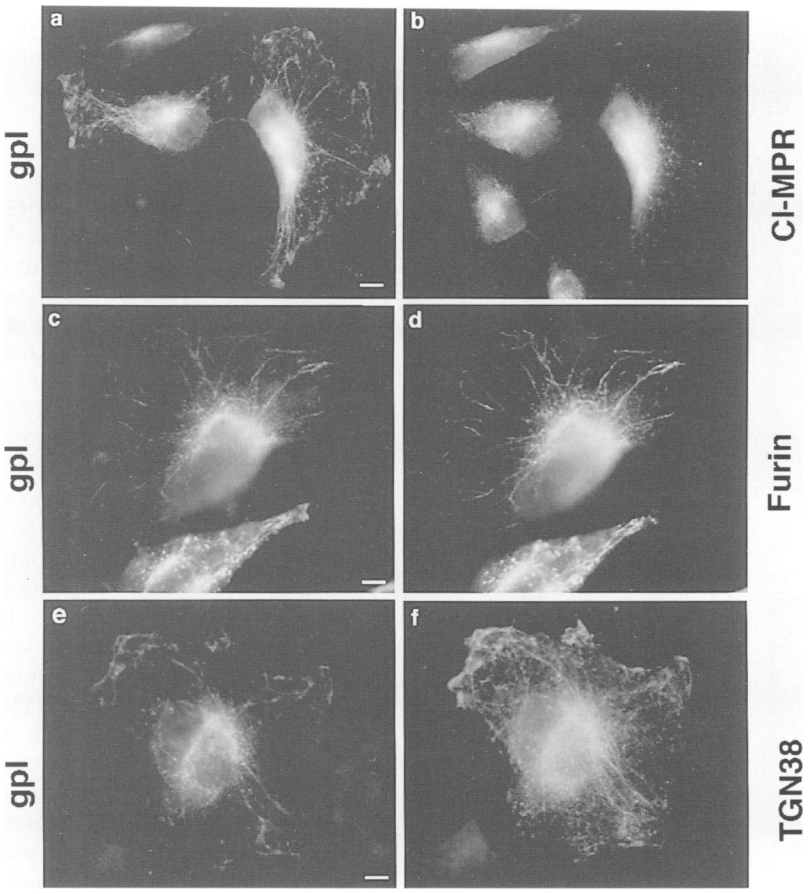

Fig. 2. Intracellular distribution of gpI, CI-MPR, furin and TGN38 in BFA-treated cells. HeLa cells transiently expressing gpI alone (**a** and **b**) or gpI and furin (**c** and **d**) or gpI and TGN38 (**e** and **f**) were treated with 10 μg/ml BFA for 5 min and then prepared for immunofluorescence microscopy. The cells were labelled with anti-gpI and anti-CI-MPR antibodies (a and b), with anti-gpI and anti-furin antibodies (c and d) or anti-gpI and anti-TGN38 antibodies (e and f). Bars: 20 μm.

reflect recruitment of AP-1 onto its physiological target compartment. The quantitation of the fluorescence associated with the Golgi region indicates that overexpression of the wild-type gpI or of the Δ2 mutant induces a 2-fold increase in the amount of bound AP-1 (Figure 5), whereas expression of the Δ1 mutant does not alter this value significantly (Figure 5).

The expression levels obtained with the vaccinia expression system are much higher than those normally observed for endogenous proteins. Therefore, we also took advantage of the MPR-negative fibroblast cell line that we have generated previously (Ludwig *et al.*, 1994). These cells exhibit a low capacity for recruiting AP-1 that can be restored by re-expressing physiological levels of the MPRs (Le Borgne *et al.*, 1996). Thus, wild-type gpI and the Δ1 mutant were stably re-expressed in these cells, and AP-1 staining was examined by indirect immunofluorescence in co-culture of each of these clones with the parental cell line. Because this cell type has a low content of lysosomal enzymes (Ludwig *et al.*, 1994), a large amount of gpI and immunoreactive breakdown products was also detected in cytoplasmic granules (Figure 6a and c) also positive for Lamp-1 (not shown). The same observation was made when TGN38 and furin were expressed in this cell line but not in the corresponding MPR-positive cells (not shown). As shown in Figure 6, cells expressing wild-type gpI exhibit a stronger γ-adaptin staining than non-transfected cells (Figure 6a and b). In contrast, when a mixture of cells expressing the Δ1 mutant and non-transfected cells was processed in an identical way, no apparent difference in the γ-adaptin staining could be observed between both cell types (Figure 6c and d). Thus, the expression of gpI in two different systems argues that this TGN marker is able to recruit AP-1.

The gpI cytoplasmic domain confers TGN localization

In an attempt to identify the domain of gpI that contains sorting information, we constructed two chimeric molecules in which the cytosolic domains of influenza haemagglutinin (HA; a protein normally expressed on the cell surface) (Gething and Sambrook, 1981) and that of gpI were exchanged (Figure 7A). These two molecules, and the intact HA and gpI, were expressed in HeLa cells, together with the VSV-G epitope-tagged STase and were processed further for double indirect immunofluorescence using anti-gpI or anti-HA and anti-VSV-G epitope antibodies. As expected, HA and gpI were found exclusively at the plasma membrane (Figure 7B, a and b) and in the TGN (Figure 7B, c and d), respectively. The HHE chimera in which the cytosolic domain of gpI was fused to the luminal and transmembrane domain of HA (Figure 7A) showed a perinuclear labelling that fully co-localized with the STase (Figure 7B, e and f). In contrast, the EEH chimeric molecule, made of luminal and transmembrane domain of gpI fused to the HA cytoplasmic tail, accumu-

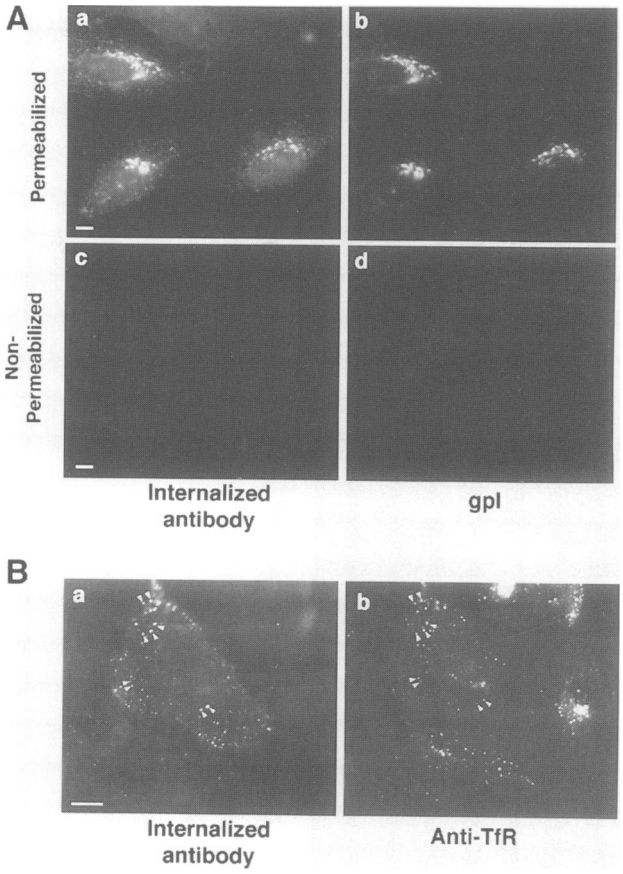

Fig. 3. Recycling of gpI and traffic through endosomes. (**A**) HeLa cells expressing gpI were incubated at 0°C with a human polyclonal anti-gpI serum for 1 h and then returned to 37°C for an additional hour. The cells were fixed and permeabilized (a and b) or left unpermeabilized (c and d). The internalized antibody was detected with FITC-conjugated anti-human IgG antibody (a and c), whereas gpI was detected with the SG1 monoclonal and rhodamine-conjugated anti-mouse IgG antibody (b and d). (**B**) HeLa cells as in (A) that had internalized anti-gpI antibody for 5 min were fixed, permeabilized and then stained with a fluorescein-conjugated anti-human IgG antibody (a) or with a monoclonal aganist the human transferrin receptor followed by rhodamine-conjugated anti-mouse IgG antibody (b). Arrowheads indicate vesicles that can be labelled with both antibodies. Bars: 20 μm.

lated at the cell surface (Figure 7B, g and h). These results support the view that the gpI cytoplasmic tail is sufficient to confer TGN localization and that the luminal and transmembrane region of this protein are devoid of any sorting information.

A tyrosine-based motif and a casein kinase II phosphorylation site regulate the intracellular distribution of gpI

In order to decipher the sorting determinants in the gpI tail, four progressive truncations in its cytoplasmic domain were introduced (Figure 8) and the steady-state distributions of the resulting mutants were analysed by immunofluorescence after transient expression. As shown in Figure 9, the truncation of the carboxy-terminal region removing either the last nine (Δ4, Figure 8) or 21 amino acids (Δ3, Figure 8) of the gpI tail did not affect the steady-state distribution of gpI, since these mutants still co-distributed with TGN38 (Figure 9c–f). However, the removal of the last 36 amino acids (Δ2), which included the acidic stretch containing the casein kinase II phosphorylation sites (Figure 8), resulted in a mixed distribution between the TGN (as shown by partial co-localization with TGN38) and the plasma membrane (Figure 9g and h). The most drastic effect was observed with the largest truncation mutant lacking a putative tyrosine-based motif (Δ1, Figure 8). This mutant was mislocalized completely to the cell surface and no longer detected in the TGN (Figure 9i and j). These results clearly point to the existence of two different signals within the tail of gpI that are necessary for proper localization of this molecule to the TGN, an acid cluster containing two potential casein kinase II phosphorylation sites (DDFEDSESTDTEEE) and a putative tyrosine-based motif (YAGL) related to known endocytosis signals.

Using site-directed mutagenesis, we then investigated in more detail the role of the acidic cluster in gpI localization. This sequence contains four hydroxylated residues, two threonines at positions 596 and 598 and two serines at positions 593 and 595. Both threonines fall into consensus sequences for casein kinase II phosphorylation. We constructed two single-point mutants in which Thr596 or Thr598 were mutated to alanine (SSTA and SSAT), one double mutant in which both threonines were replaced by alanine (SSAA) and one quadruple mutant in which the two threonines and both Ser593 and Ser595 were changed to alanine (AAAA) (Figure 8). The steady-state distribution of these molecules was determined by double indirect immunofluorescence using TGN38 as a TGN marker. As shown in Figure 10, the mutation of a single threonine (mutants SSTA, Figure 10c or SSAT, not shown) did not affect the intracellular distribution of gpI, since this protein largely co-localized with TGN38 (Figure 10d). However, the simultaneous replacement of the two threonine residues (SSAA) led to an increase in the amount of gpI detected at the cell surface (Figure 10e and f). The strongest effect was obtained with the mutation of the four hydroxylated residues (AAAA) which, as for the Δ2 mutant, resulted in a strong redistribution of gpI to the cell surface (Figure 10g and h) although a small amount of gpI was still detected together with TGN38. These results clearly show that the casein kinase II phosphorylation site is an important determinant for the TGN localization of gpI.

The expression of gpI truncation mutants indicates that the first 25 amino acids of the tail are also important for TGN localization. This region contains a canonical tyrosine-based motif, similar to those found to be critical for the localization of other TGN transmembrane proteins (Luzio and Banting, 1993). Therefore, we asked whether this tyrosine-containing tetrapeptide in the gpI tail could play a similar role. Therefore, a mutant (Y582A), in which the Tyr582 was replaced by alanine, was expressed. The indirect immunofluorescence shows that the mutated gpI was detected mainly on the cell surface, although some residual, but significant staining could still be observed over the TGN (Figure 11c), as judged by co-localization with TGN38 (Figure 11d). Moreover, a Y582A-AAAA mutant was constructed (Figure 8) in which the Tyr582 and the four hydroxylated residues within the acidic stretch were mutated to alanine. The combined mutations of the two different motifs resulted in a stronger effect than any

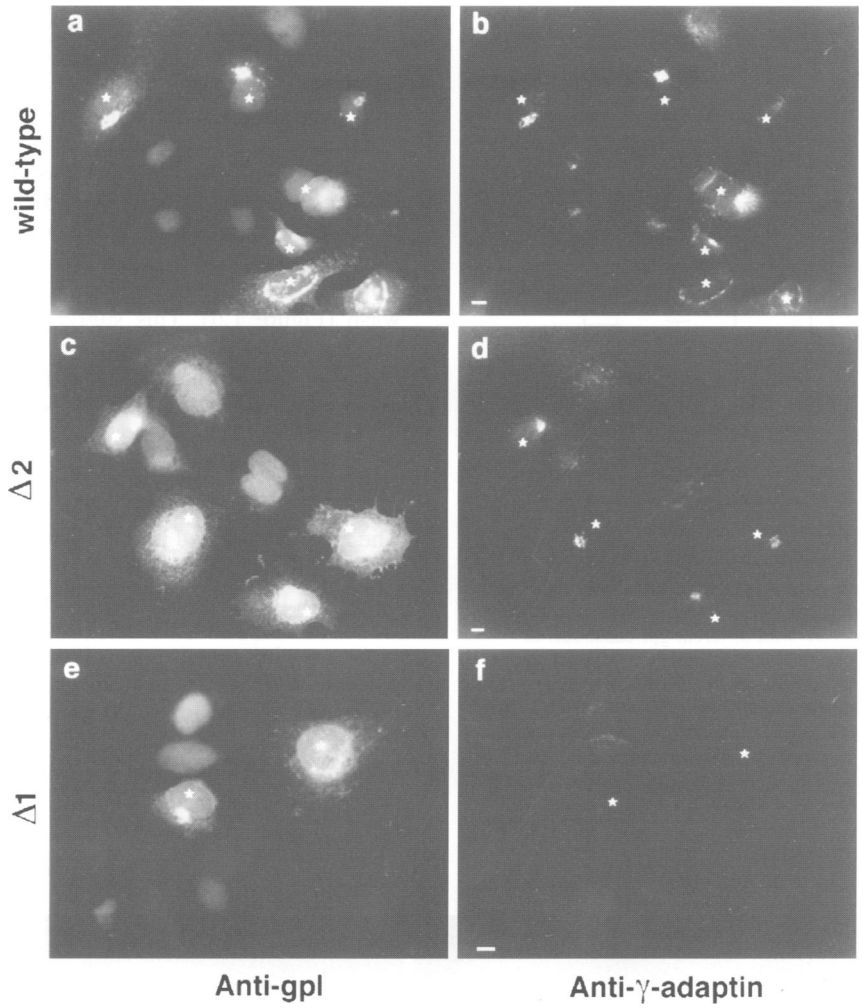

Fig. 4. Intracellular distribution of γ-adaptin in HeLa cells expressing different gpI constructs. HeLa cells were infected with a recombinant T7 RNA polymerase vaccinia virus, and transfected with the wild-type gpI (**a** and **b**), with the Δ2 mutant that lacks the acidic stretch (**c** and **d**) or with the Δ1 mutant that lacks most of the cytoplasmic tail (**e** and **f**) using the DOTAP reagent. After 2 h of expression, cells were fixed and labelled with a human convalescent zoster serum or with the monoclonal 100.3 against γ-adaptin and the corresponding FITC and rhodamine secondary antibodies. gpI is shown in (a), (c) and (e) and γ-adaptin in (b), (d) and (f). The stars indicate cells expressing gpI. Non-transfected cells can be also detected due to the nuclear background of the anti-gpI antibody. Bars: 20 μm.

of the individual mutations. At steady-state, this mutant was found mainly at the cell surface (Figure 11e and f). However, the effect was less drastic than the complete truncation (Δ1 mutant) since some weak, residual intracellular labelling could still be detected over the TGN (Figure 11e and f).

Altogether, these results clearly indicate the existence of two different, dominant determinants within the gpI cytoplasmic domain that confer TGN localization: one related to casein kinase II phosphorylation sites within an acidic cluster, and the other relying on a tyrosine residue. However, removal of both signals does not induce a complete missorting of the protein to the cell surface, as was seen with the tailless mutant. This finding most likely reflects the existence of additional sorting motifs within the gpI tail.

Characterization of the signals responsible for gpI internalization

Since gpI cycles between the TGN and the plasma membrane, its proper steady-state localization must also rely on its efficient retrieval from the cell surface. Efficient transport from the cell surface back to the TGN can be estimated in a qualitative manner by monitoring the internalization of exogenously added anti-gpI antibodies. Thus, HeLa cells transiently expressing wild-type gpI or gpI mutants were incubated with an anti-gpI antibody. After a 1 h internalization, the cells were either directly fixed or washed at low pH to remove the surface-bound antibody. The result of these experiments is shown in Figure 12. The antibodies internalized by cells expressing the wild-type protein could reach an acid-resistant, perinuclear location, consistent with an efficient recycling to the TGN (Figure 12a and b). Conversely, the Δ1 mutant, exclusively located at the cell surface at steady-state (Figure 9), failed to internalize the exogeneously added antibody since it could be removed efficiently by an acidic wash (Figure 12c and d). In contrast, only discrete vesicular structures within the cytoplasm can be detected in cells expressing the Δ2, the AAAA or the Y582A mutants after removal of the surface-bound antibody (Figure 12e–j). The vesicular structures were identified as early endosomes

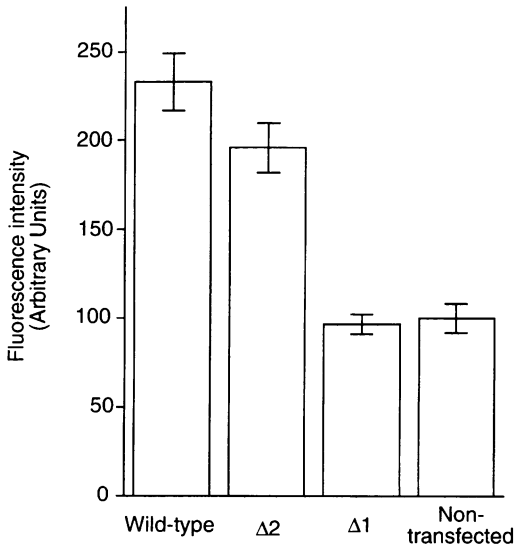

Fig. 5. Quantitation of the amount of γ-adaptin associated with the TGN in cells expressing wild-type gpI or truncated forms of it. HeLa cells were transiently transfected with different gpI constructs as described in the legend to Figure 4 and identically processed for double immunofluorescence. The intensity of the fluorescence signal in the rhodamine channel was quantitated for 30–50 transfected and for a similar number of non-transfected cells and the results processed as described in Materials and methods. Values correspond to mean ± SEM. The confidence limits of the sample populations were found to be in every case >99%, based on a Student's *t* test.

since they could also be labelled with anti-transferrin receptor antibodies (not shown). Thus, the tyrosine-based signal and the casein kinase II phosphorylation site are both necessary for the proper return of gpI to the TGN, although a single mutation still allows the protein to be internalized to some extent. However, the combination of these two mutations (mutant Y582A-AAAA) drastically affected the endocytosis of gpI. Most of the exogeneously added antibody was found attached to the cell surface (Figure 12k) and was removed completely by the low pH wash (Figure 12l). This suggests that both the tyrosine and the acidic cluster cooperate for rapid internalization from the cell surface, and that no additional signals are apparently required for this process.

Discussion

The results presented in this work illustrate several aspects of the intracellular traffic of the VZV gpI. Like TGN38 and furin, this protein accumulates in the TGN but cycles between this compartment and the cell surface. GpI promotes the recruitment of AP-1 onto the Golgi and probably leaves this compartment in CCVs for subsequent transport to endosomes. Its recycling pathway from the cell surface back to the TGN also occurs via endosomes. A tyrosine-based motif and a casein kinase II phosphorylation site in its cytoplasmic domain regulate its intracellular traffic, i.e. its TGN localization.

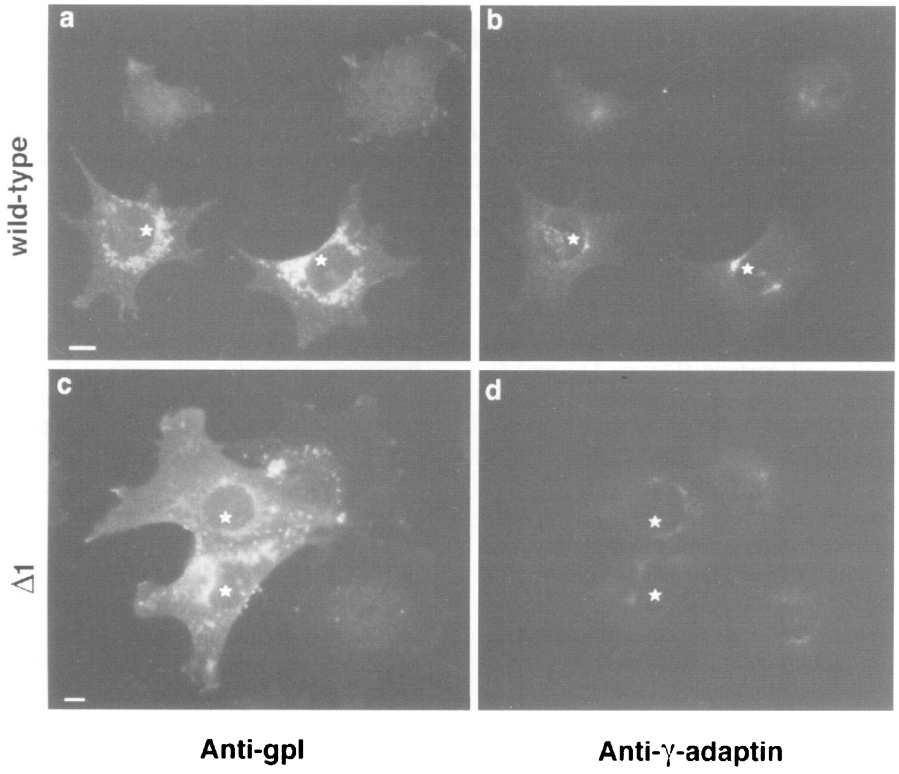

Fig. 6. Intracellular distribution of γ-adaptin in MPR-deficient mouse fibroblasts stably expressing different gpI constructs. MPR-deficient mouse fibroblasts were stably transfected with either wild-type gpI or with the tailless mutant (Δ1). Mixtures of the parental cell line with cells expressing the wild-type protein (**a** and **b**) or the Δ1 mutant (**c** and **d**) were plated on coverslips, fixed, permeabilized and stained with the SG1 monoclonal and with an antibody against the mouse γ-adaptin hinge region followed by fluorescein-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG antibodies. GpI is shown in (a) and (c) (fluorescein channel) and γ-adaptin in (b) and (d) (rhodamine channel). The stars indicate cells expressing gpI. Bars: 20 μm.

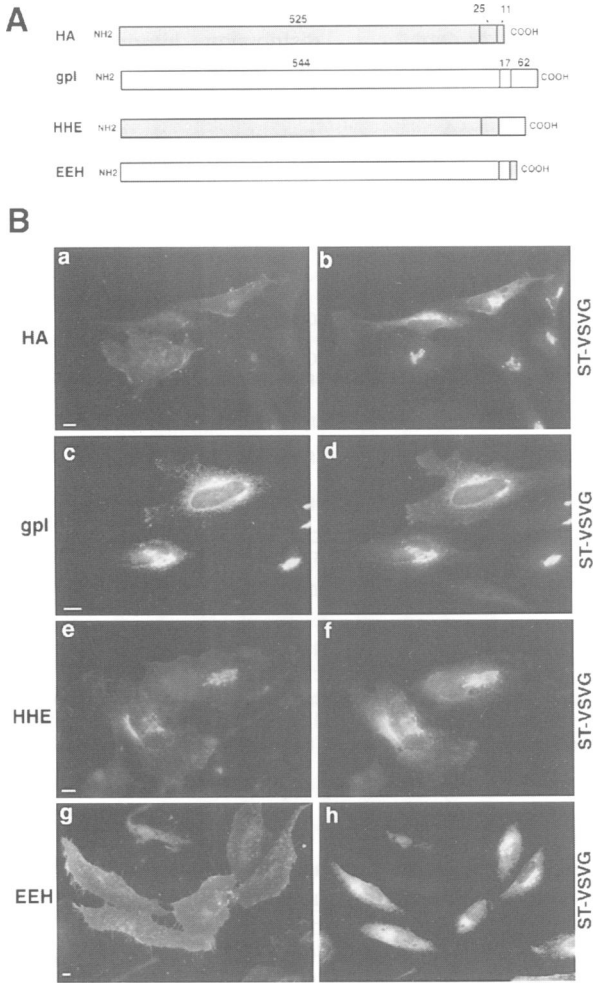

Fig. 7. Intracellular distribution of HA–gpI chimeric molecules. (**A**) Schematic representation of the chimeric molecules used in this study drawn to scale: HHE, luminal and transmembrane domains of HA fused to the cytoplasmic domain of gpI; and EEH, luminal and transmembrane domains of gpI fused to the cytoplasmic tail of HA. The length of the different topological domains is indicated above the sequences. (**B**) HeLa cells were double transfected with VSV-G epitope-tagged sialyltransferase and the wild-type HA (a and b), or the wild-type gpI (c and d), or the HHE (e and f) and EEH (g and h) chimeras. After fixation and permeabilization, the cells were labelled with a mixture of a polyclonal antiserum against the VSV-G epitope and the HC269 that recognizes an epitope in the luminal domain of HA, to detect wild-type HA and the HHE chimera, or anti-VSV-G and mAb SG1 to detect wild-type gpI and the EEH chimera. a, c, e and g show the distribution of wild-type HA, wild-type gpI and the HHE and EEH chimeras respectively, whereas the localization of STVSV-G in the same cells is shown in b, d, f and h. Bars: 20 μm.

Steady-state localization and intracellular traffic of gpI

Conventional and confocal light microscopy clearly show that gpI is present at steady-state in the TGN, since it co-localizes with three different TGN markers, STase (Roth *et al.*, 1985; Munro, 1991), furin (Bosshart *et al.*, 1994; Molloy *et al.*, 1994; Schäfer *et al.*, 1995) and TGN38 (Luzio *et al.*, 1990; Humphrey *et al.*, 1993). A similar conclusion has been reached previously by Zhu *et al.*, based on the co-localization of gpI with the CI-MPR and NBD-ceramide (Zhu *et al.*, 1995). Occasionally, gpI could also be detected at the cell surface. Similar observations have been made with TGN38 (Humphrey *et al.*, 1993; Wong and Hong, 1993; Reaves and Banting, 1994) and furin (Takahashi *et al.*, 1995), a phenomenon most likely associated with a massive overexpression of the proteins, an inherent problem of transient transfection. In any case, this mislocalization suggests that the mechanisms involved in TGN localization of type-I transmembrane proteins can become saturated. Although gpI and the CI-MPR show a certain degree of co-localization, their steady-state distributions are different, as seen in cells treated with drugs affecting the integrity of membrane-bounded compartments. Our results, although restricted at the light microscope resolution level, indicate that, in HeLa cells, a large fraction of the CI-MPR localizes to a structure closely located to but different from the TGN, presumably the late endosomes, as shown at the electron microscope level for different cell types (Griffiths *et al.*, 1990; Klumperman *et al.*, 1993). It has been suggested that gpI localizes to the same compartment as the MPRs, both in VZV-infected cells (Gabel *et al.*, 1989) and in gpI-transfected cells (Zhu *et al.*, 1995). Despite the variations found in the MPR distribution among different cell types, the co-localization between MPRs and gpI observed by others in gpI-transfected cells probably reflects either interspecific cell differences or the inability to discern at the optical microscopy level between late endosomes, where the MPRs mostly resides, and TGN where gpI is found at steady-state.

We provide evidence that gpI cycles between the TGN and the cell surface, a property shared with furin (Molloy *et al.*, 1994; Jones *et al.*, 1995; Takahashi *et al.*, 1995; Voorhoes *et al.*, 1995) and TGN38 (Bos *et al.*, 1993; Chapman and Munro, 1994), but not with STase. As for TGN38 (Humphrey *et al.*, 1993; Chapman and Munro, 1994; Ponnambalam *et al.*, 1994; Reaves and Banting, 1994) and furin (Voorhoes *et al.*, 1995), the endocytosed gpI appears in endosomes before it can be detected in the TGN. An important feature in the intracellular traffic of gpI that our study also illustrates is its ability to promote AP-1 recruitment onto TGN membranes. The simplest interpretation of this finding is that this interaction, as it occurs for the MPRs, represents the initial step leading to the incorporation of gpI into CCVs for subsequent transport to the endosomes. So far, the pathways that TGN-resident, type-I transmembrane proteins follow on their way to the cell surface remain uncharacterized. It is possible that the traffic of furin and TGN38 also involves their transit through endosomes via clathrin-coated vesicular carriers. Indeed, morphological analyses have revealed the presence of furin in clathrin-coated buds and CCVs (Schäfer *et al.*, 1995). Furthermore, the cytosolic tail of TGN38 has been shown to interact genetically in the yeast two-hybrid system with the 47 kDa subunit of the AP-1 complex (Ohno *et al.*, 1995), and overexpression of TGN38 leads to some redistribution of γ-adaptin (Reaves and Banting, 1994). Alternatively, TGN-resident, type-I transmembrane proteins could recruit AP-1 transiently without producing an efficient sorting event through the formation of a vesicle. In this way, AP-1 could have a more structural role and contribute in the maintenance of the boundaries of this compartment. In this respect, it is interesting to note that BFA, which prevents the insertion of ARF in membranes (Donaldson *et al.*, 1992; Helms and Rothman, 1992) and the subsequent binding of AP-1 (Robinson and

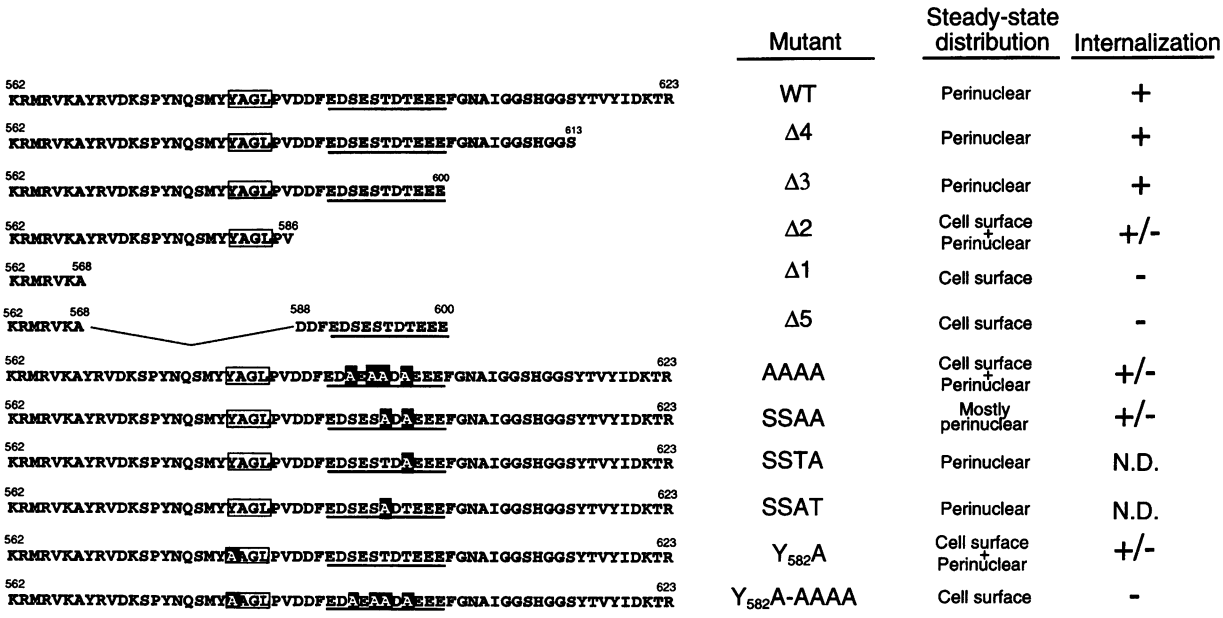

Fig. 8. Representation of the cytoplasmic tail of wild-type gpI and of the different mutants used in this study. The amino acids are shown in one-character code. Numbering above the sequences denotes the amino acid position in the gpI precursor. The acidic stretch containing the four hydroxylated residues and the two casein kinase II phosphorylation sites is underlined, and the potential tyrosine-based motif is boxed. Positions where point mutations were introduced are shown by white characters inside black boxes. The intracellular localization and internalization ability of the different mutants, as judged by immunofluorescence analysis and antibody uptake experiments, is shown on the right. Key for internalization: +, mostly perinuclear; +/–, cell surface and endosomal; –, cell surface only; N.D., not determined.

Kreis, 1992; Stammes and Rothman, 1993), leads to the mixing of TGN and endosomal markers (Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991).

A tyrosine-based motif and two casein kinase II phosphorylation sites determine the TGN localization of gpI

The TGN localization of gpI relies exclusively on determinants of its cytoplasmic domain, as also shown by Zhu *et al.* (1995) using chimeric molecules between gpI and the interleukin-2 receptor (Tac). Thus, gpI behaves like the convertase furin (Bosshart *et al.*, 1994; Schäfer *et al.*, 1995; Takahashi *et al.*, 1995). However, TGN38 appears to contain some additional signals in its transmembrane domain (Ponnambalam *et al.*, 1994), and STase is retained in the TGN by means of signals exclusively present within or in the proximity of its transmembrane domain (Munro, 1991). Interestingly, several yeast proteins are apparently retained in a late Golgi compartment by means of their cytosolic tails (Cooper and Bussey, 1992; Wilcox *et al.*, 1992).

The first important determinant in the gpI tail is the 581YAGL584 peptide, a consensus tyrosine-based motif of the kind YXXZ (Y is tyrosine, X is any amino acid and Z is a hydrophobic residue). The single point mutation of this tyrosine leads to a partial mislocalization of the protein to the cell surface. This type of signal, first identified in several plasma membrane molecules internalized via CCVs (Trowbridge *et al.*, 1993), has also been found to determine the TGN localization of furin (Jones *et al.*, 1995; Schäfer *et al.*, 1995; Takahashi *et al.*, 1995; Voorhoes *et al.*, 1995) and TGN38 (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993). The role that tyrosine-based motifs play in the intracellular traffic of TGN-resident molecules is not completely clear. Whereas the mutation of the tyrosine or of the whole signal in the furin tail has no effect on the steady-state distribution of this molecule (Schäfer *et al.*, 1995; Takahashi *et al.*, 1995), similar mutations in the TGN38 tail induce the accumulation of the protein at the cell surface (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993). One has to consider that the sequences of the tyrosine motifs found in the different TGN proteins are not totally identical and, therefore, their relative contributions to the overall localization of the protein could vary. For example, the YKGL sequence in the furin tail, in the absence of other signals, can only partially retain the protein in the TGN, while its replacement by the YQRL sequence of TGN38 confers complete TGN retention (Schäfer *et al.*, 1995). Although the sequence found in the tail of gpI (YAGL) lacks both the bulky amino acid in position 2 and the positively charged residue in position 3, it is able to confer a fair TGN localization as shown by the localization of the gpI mutant that contained only this signal (Δ2 mutant).

The second important determinant regulating gpI trafficking is the highly acidic stretch of amino acids (587-DDFEDSESTDTEEE602) that contains four hydroxylated residues (Ser593, Ser595, Thr596, Thr598), two of which (Thr596 and 598) fall into consensus sites for casein kinase II phosphorylation. Previous work showed that threonines 596 and 598 can be phosphorylated *in vivo* and *in vitro* by purified casein kinase II (Yao *et al.*, 1993b). Accordingly, the removal of two threonines leads to a considerable redistribution of gpI to the cell surface, whereas the single mutation of any one of the two threonines does not affect the steady-state distribution of the protein. Furthermore, the two adjacent serines are also important since the simultaneous mutation of the four hydroxylated amino acids leads to a more drastic mislocal-

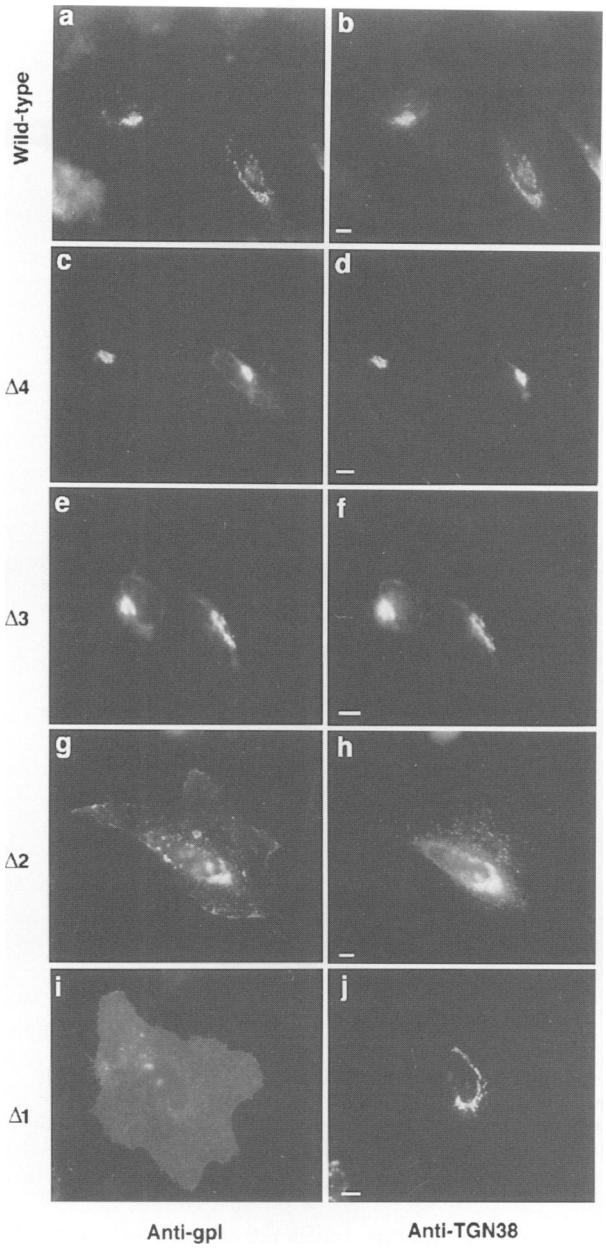

Fig. 9. Subcellular localization of gpI truncation mutants. HeLa cells were transfected with plasmids encoding TGN38 and either the wild-type gpI (**a** and **b**), or one of the four truncation mutants: Δ4 (**c** and **d**), Δ3 (**e** and **f**), Δ2 (**g** and **h**) and Δ1 (**i** and **j**). After fixation and permeabilization, cells were double labelled with the mAb SG1 anti-gpI and anti-TGN38 antibodies, followed by fluorescein- and rhodamine-conjugated secondary antibodies. Wild-type gpI and mutants are shown in (a), (c), (e), (g) and (i) (fluorescein channel) and the corresponding TGN38 labelling is shown in (b), (d), (f), (h) and (j) (rhodamine channel). Bars: 20 μm.

ization of gpI. Thus, this suggests that the intracellular traffic of gpI could be regulated by a phosphorylation–dephosphorylation process. Furin, but not TGN38, contains in its cytosolic tail a similar acidic peptide containing serine residues which can be both phosphorylated *in vitro* by casein kinase II (Jones *et al.*, 1995; Takahashi *et al.*, 1995) and *in vivo* (Jones *et al.*, 1995), and which is important for the steady-state distribution of the protein (Jones *et al.*, 1995; Takahashi *et al.*, 1995). Whereas the furin acidic peptide has been shown to be sufficient for conferring TGN localization to a reporter molecule

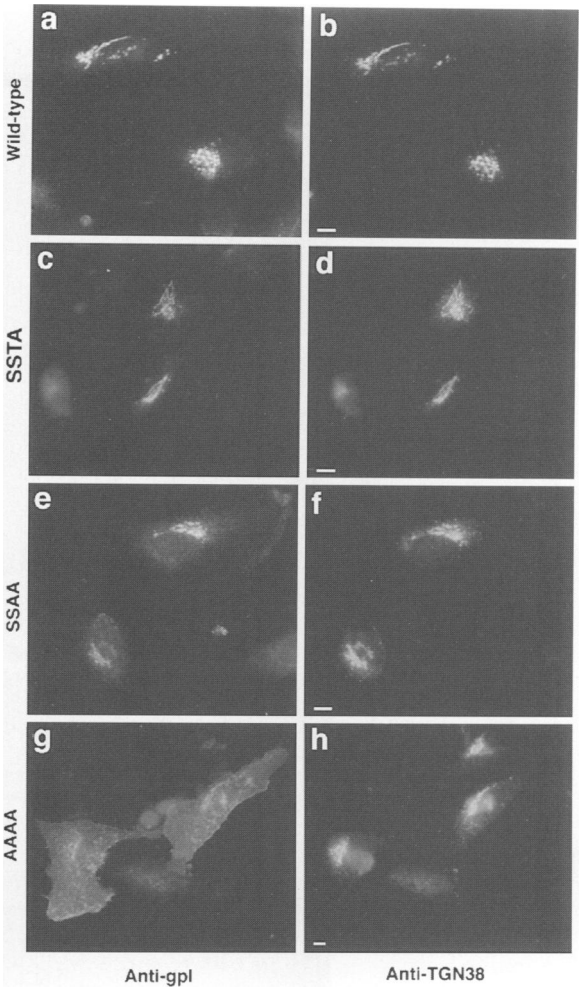

Fig. 10. Changes in the intracellular distribution of gpI upon mutation of the hydroxylated residues within the acidic stretch. HeLa cells transiently expressing TGN38 and either wild-type gpI (**a** and **b**) or the phosphorylation site mutants SSTA (**c** and **d**), SSAA (**e** and **f**) and AAAA (**g** and **h**) were processed for immunofluorescence as indicated in Figure 9. The same cells are shown in the fluorescein channel, for detection of wild-type gpI or mutants (a, c, e and g) and in the rhodamine channel for TGN38 labelling (b, d, f and h). Bars: 20 μm.

(Schäfer *et al.*, 1995), our experiments, in which the acidic peptide from gpI was placed directly after the first seven residues of the tail, failed to reveal its direct role in TGN localization (result not shown).

According to the results obtained in our antibody uptake experiments, gpI appears to recycle between the TGN and the cell surface and, therefore, its proper TGN localization must depend on its ability to be sorted from, and retrieved back to this compartment. Our antibody uptake experiments as well as the steady-state distribution of gpI mutants show that both the tyrosine-based motif and the casein kinase II phosphorylation site are required for efficient endocytosis of gpI and its recycling back to the TGN. Mutation of these two determinants completely impairs the endocytosis of gpI which then accumulates at the cell surface. However, the mutation of the tyrosine-based motif or the casein kinase II phosphorylation sites are not sufficient to prevent internalization of gpI from the cell surface, suggesting that they can mediate endocytosis of gpI independently. Similar results have been reported for furin, whose efficient internalization also

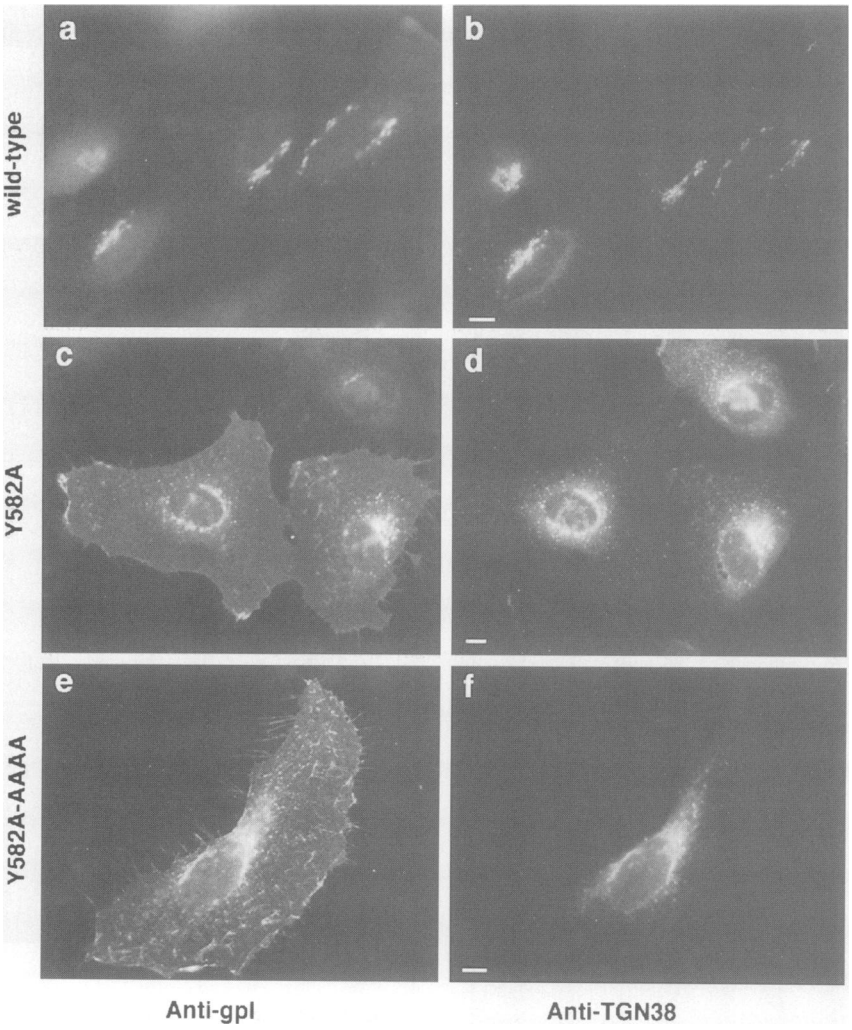

Fig. 11. Effects of mutations in the tyrosine-based motif in the cytosolic tail of gpI on the intracellular distribution of this molecule. HeLa cells transfected with TGN38 and either the wild-type gpI (**a** and **b**), the Y582A mutant (**c** and **d**) or the Y582A-AAAA (**e** and **f**) were prepared for immunofluorescence microscopy as described in Figure 9. The localization of wild-type gpI and mutants is shown in (a), (c) and (e) (fluorescein channel), and the distribution of TGN38 in the same cells appears in (b), (d) and (f) (rhodamine channel). Bars: 20 μm.

relies on the presence of both a tyrosine-based motif and a casein kinase II phosphorylation site present in its cytoplasmic domain (Voorhoes *et al.*, 1995). In addition, it has been proposed that this latter determinant could control the transport of furin from endosomes to the TGN (Jones *et al.*, 1995; Takahashi *et al.*, 1995). Thus, the recycling of both proteins from the plasma membrane to the TGN is controlled by similar protein determinants. However, the recycling of TGN38 from the plasma membrane back to the TGN only requires the presence of a tyrosine-based motif (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993).

Although gpI and the MPRs reside in different intracellular compartments, these two molecules share the ability to trigger AP-1 recruitment. The re-expression of cation-dependent MPR (CD-MPR) mutants in MPR-negative cells has shown that the casein kinase II phosphorylation site is a key determinant for high affinity binding of AP-1 to membranes (Mauxion *et al.*, 1996). However, this latter study could not detect any role for the other known trafficking signals in the CD-MPR tail, particularly the tyrosine-based and dileucine-based motifs which could function independently for efficient AP-1 binding. We show here that the overexpression of a gpI mutant (Δ2 mutant) containing the tyrosine-based motif but devoid of the casein kinase II site can still trigger AP-1 recruitment on membranes. Since the method employed does not allow us to quantitate the kinetic parameters of the interaction, we cannot exclude the possibility that the casein kinase II phosphorylation site also contributes directly or indirectly to AP-1 binding, as seen for the CD-MPR. Although this aspect requires further investigation, our findings are consistent with the notion that tyrosine-based motifs can contribute to AP-1 recruitment, as proposed in other studies (Ohno *et al.*, 1995; Heilker *et al.*, 1996).

During the course of this study, Zhu *et al.* (1996) have shown that gpI cycles between the TGN and the plasma membrane and have characterized the signals in the gpI tail responsible for its TGN localization. These authors agree with the importance of the acidic patch for TGN retention, but they have identified an additional sorting element within the sequence: the peptide 568AYRV571. This additional signal could account for the increased

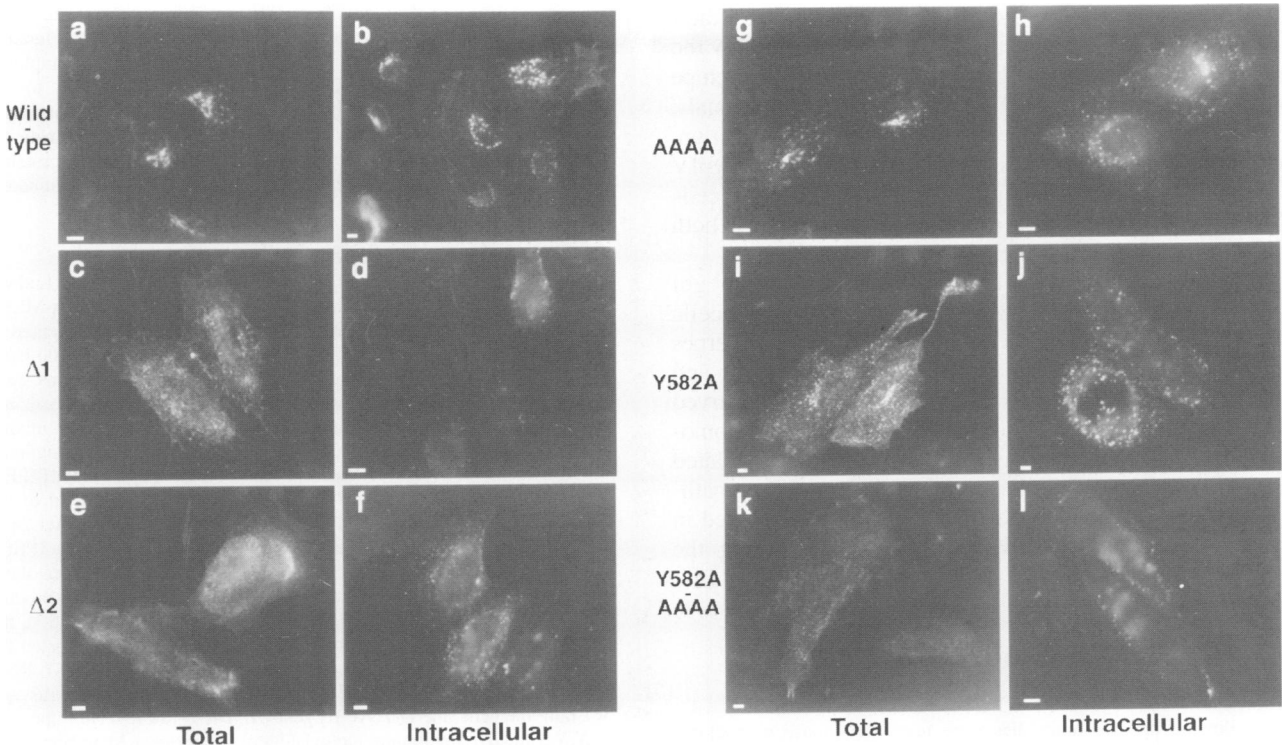

Fig. 12. Effects of mutations in the gpI tail on the return of the protein to the TGN. HeLa cells were transfected in duplicate with either wild-type gpI (**a** and **b**) or the following mutants: Δ1 (**c** and **d**), Δ2 (**e** and **f**), AAAA (**g** and **h**), Y582A (**i** and **j**) and Y582A-AAAA (**k** and **l**). At 48 h after transfection, cells were incubated for 1 h at 0°C with a human anti-gpI antibody, extensively rinsed with PBS and returned to a 37°C chamber for an additional hour. Cells were either directly fixed (a, c, e, g, i and k) or subjected to acid wash and then fixed as described in Materials and methods (b, d, f, h, j and l) to reveal intracellular labelling. Fixed cells were decorated with a fluorescein-conjugated anti-human IgG antibody. Bars: 20 μm.

TGN localization that we have observed in the mutant Y582A-AAAA in comparison with the Δ1 mutant. It seems likely that both the AYRV and the YAGL signals contribute to maintain gpI in an intracellular location.

Implications for the biogenesis of varicella-zoster and other herpes viruses

Earlier studies have illustrated that the envelope glycoproteins of VZV as well as the gD glycoprotein of the herpes simplex virus can acquire mannose 6-phosphate residues on their *N*-linked oligosaccharides, like the classical soluble lysosomal enzymes (Gabel *et al.*, 1989; Brunetti *et al.*, 1994). These findings, together with substantial morphological evidence (Gershon *et al.*, 1994), led to the proposal that these viruses could form by budding of the nucleocapsids in the TGN (Gershon *et al.*, 1994). In this compartment, the mature viruses would then bind to the MPRs and be transported to endosomes, where mature viruses can be detected. More recent studies have demonstrated, however, that the biogenesis of the herpes simplex virus is not impaired in cells either lacking the MPRs or unable to synthesize the mannose 6-phosphate signal, suggesting that additional mechanisms must exist for virus biogenesis (Brunetti *et al.*, 1995). An alternative and non-exclusive model for VZV biogenesis is that the viral glycoproteins are transported to endosomes where virus budding could also take place. This would imply that the viral glycoproteins must reach this location, probably using the same sorting mechanisms as the MPRs. Our study, showing that gpI has the potential to be transported within the same intracellular compartments as the MPRs, could provide some support for the latter model. However, our findings and those of Zhu and co-workers (Zhu *et al.*, 1996), showing that gpI mostly localizes to the TGN when expressed in HeLa cells, are more consistent with a model in which the viruses bud into the TGN, provided that this localization faithfully reflects that of gpI in virus-infected cells, a point which remains to be firmly established. Indeed, overproduction of gpI in virus-infected cells could result in the saturation of sorting machineries leading to a different distribution of this protein. It is interesting to note, however, that the hydroxylated amino acids contained in the acid cluster of the gpI tail regulate the intracellular trafficking of gpI. Interestingly, the genome of the VZV encodes two Ser/Thr protein kinases (Davidson and Scott, 1986), that could potentially phosphorylate those sites. Therefore, these kinases could promote a more efficient sorting of gpI to endosomes and control the steady-state distribution of this protein in VZV-infected cells. Similar differences in the regulation of cycling of type-I transmembrane proteins could also explain the different steady-state distribution of gpI and MPRs. Experiments are currently in progress to address these points.

The genome of VZV encodes several other integral membrane glycoproteins that are apparently devoid of sorting signals (Grose, 1990). If VZV budding were to occur in endosomes, these envelope glycoproteins should also be delivered to the same cellular compartment by so far uncharacterized means. Although the mechanisms that could ensure co-segregation of the different envelope glycoproteins remain unknown, one could envisage that

the different glycoproteins could interact through their luminal domains, and be transported collectively to the same destination. Since gpI is the most abundant envelope glycoprotein and contains the appropriate sorting signals, it could represent a good candidate to fulfil this function. In this regard, it has been reported that gpI and gpIV can be isolated as a complex from VZV-infected cells (Montalvo *et al.*, 1985), or upon co-transfection of both molecules (Yao *et al.*, 1993a).

Sequence comparison of the cytoplasmic tails of gpI homologues from related herpes viruses (simian varicella virus, pseudorabies virus, equine herpes virus and herpes simplex virus) revealed that similar acidic stretches and tyrosine-containing motifs are by far the most conserved elements within all the sequences considered. This homology most probably reflects the fact that gpI and related glycoproteins follow similar intracellular trafficking pathways, and that related molecular mechanisms involved in the viral assembly are likely to be shared among the different herpes viruses.

Materials and methods

Materials

The following primary antibodies were used: the mouse monoclonal antibodies SG1 (Viro Res. Inc., Rockford, USA) and VL8 (generous gift of Dr B.Rentier, University of Liege, Belgium) against VZV gpI; a human convalescent zoster serum (a generous gift of Dr A.Arvin, Standford University, Standford, USA) and a rabbit polyclonal against the recombinant gpI produced in *Escherichia coli*; rabbit polyclonal antibodies against the full-length TGN38 (kindly provided by Dr G.Banting University of Bristol, Bristol, UK) and against the cytoplasmic tail of furin (provided by Dr W.Garten, University of Marburg, Marburg, Germany); a mouse monoclonal P5D4 against the VSV-G protein epitope; mouse monoclonal 100.3 against the human and bovine γ-adaptin (Sigma, Diesenhofen, Germany); an affinity-purified rabbit polyclonal serum raised against the hinge region of the mouse γ-adaptin (kindly provided by Dr M.S.Robinson, University of Cambridge, UK); a monoclonal antibody against the human transferrin receptor (Boehringer Mannheim, Mannheim, Germany); the mouse monoclonal HC269 against the influenza HA (Japan strain) (kindly provided by Dr J.Skehel, NIMR, London, UK); a rabbit polyclonal against the VSV-G protein epitope and a rabbit polyclonal against the luminal domain of the CI-MPR. All the secondary antibodies against the Fc of mouse, rabbit, human and bovine IgGs coupled to FITC or rhodamine were purchased from Dianova (Hamburg, Germany).

Constructs for eukaryotic expression of TGN markers were provided by Drs P.Luzio (pCMV-TGN38, for expression of the rat TGN38), W.Garten (pSG5-furin, for expression of the bovine furin) and T.Nilsson (pSRα-STVSVG, for expression of a VSV-G epitope-tagged version of the rat STase).

Recombinant DNA methods

VZV gpI was cloned by PCR from a lysate of VZV (Dumas strain)-infected cells, using the Expand high-fidelity system (Boehringer Mannheim, Mannheim, Germany) employing the following primers: forward primer, 5′-GCTCTAGAATGGGGACAGTTAATAAACCTG-TGG-3′; reverse primer, 5′-GCAAGCTTTCACCGGGTCTTATCTAT-ATACACCG-3′.

Introduction into the primers of *Xba*I and *Hin*dIII sites (in the forward and reverse PCR primers, respectively) allowed the cloning of the PCR product on the same sites into the pSFFV6 (Chen *et al.*, 1993) and pGEM1 vectors. For generation of the different truncation mutants (Δ1, Δ2, Δ3 and Δ4), primers were designed that contained the coding sequence of the last 6–8 amino acids to be included followed by a stop codon and a *Hin*dIII site. These primers were used together with the forward primer to amplify the corresponding fragments from the infected cell lysate and these were cloned in the different expression vectors.

The different single or multiple point mutations were introduced by a PCR-based approach using the native gpI as a template and synthetic oligonucleotides that contained the desired mutations within their sequence. Construction of the influenza HA–gpI chimeric molecules was also performed using conventional PCR techniques. Briefly, the luminal and cytosolic domains of gpI and HA (Japan strain) were amplified using synthetic oligonucleotides that contained, in addition to the complementarity region, the whole coding sequence of the transmembrane domain of the other molecule. The PCR fragments were denatured, annealed and subjected to a second PCR, yielding the chimeric proteins. All the mutants and chimeric molecules were verified by dideoxy sequencing.

Cell culture and transfections

HeLa cells or immortalized MPR-deficient mouse fibroblasts (Ludwig *et al.*, 1994) were maintained in α-minimal essential medium (α-MEM) or Dulbecco's modified Eagle's medium, respectively, supplemented in each case with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For transient transfections, cells were split and plated onto coverslips the day before. For calcium phosphate-mediated transfection, a precipitate was prepared by mixing with constant agitation a 250 mM $CaCl_2$ solution containing 0.018 μg/μl of DNA with an equal volume of 2× HBS (50 mM HEPES, 280 mM NaCl, 1.43 mM NaH_2PO_4, pH 7.07). One hundred μl of the precipitate were added per well containing 1 ml of medium. After 24 h, the cells were washed extensively with phosphate-buffered saline (PBS), and incubated for another 16 h with fresh medium. Under these conditions, the mature form of gpI (mol. wt 100 kDa) was the only product detected by Western blotting. For vaccinia T7-DOTAP transfection, the cells were washed with medium devoid of FCS and then incubated for 1 h with a recombinant vaccinia virus that expresses the T7 RNA polymerase gene (Fuerst *et al.*, 1986). After removing the virus and washing the cells, the DOTAP reagent (*N*-[1-(2,3- dioleoxyloxy)propyl-*N*,*N*,*N*-trimethyl ammonium methylsulfate) (Boehringer Mannheim) was used to transfect the cells with the different gpI constructs cloned in the pGEM1 vector, following the manufacturer's instructions. The cells were washed and allowed to express for 2–3 h in the presence of 5 mM hydroxyurea.

For generation of stable cell lines, a modified version of the calcium phosphate procedure was used (Mauxion *et al.*, 1996). A calcium phosphate precipitate was made containing 20 μg of the desired construct in pSFFV/pSFFV6 (previously linearized with *Fsp*I) and 1 μg of pBHyg, a plasmid containing the hygromycin resistance gene. After the cells were incubated with the precipitate for 24 h, they were washed, split and plated in the presence of 350 μg/ml of hygromycin B (Boehringer Mannheim). After 12–14 days of selection, colonies were picked, expanded and tested for expression of the gpI, either by immunofluorescence or by Western blot.

Indirect immunofluorescence and image processing

Cells were fixed with 3% paraformaldehyde for 15 min at room temperature and the excess of this reagent was neutralized by a 10 min incubation in 50 mM ammonium chloride. The cells were permeabilized with 0.1% Triton X-100, incubated for 30 min in 10% normal goat serum and then for 30 min with each of the antibody mixtures, previously diluted to the working concentration with 10% goat serum. When indicated, the cells were treated before fixation with either 10 μg/ml BFA or 10 μM nocodazole for the indicated times. Stock solutions of 5 mg/ml BFA or 10 mM nocodazole were diluted to the working concentration in α-MEM and pre-warmed at 37°C before being added to the cells. Treatment was stopped by removing the drug-containing medium and adding the paraformaldehyde fixing solution. Samples were visualized either in an Axiophot or in the EMBL compact confocal laser microscope. Each immunofluorescence image shows a representative pattern observed in at least 90% of the transfected cells. Unless otherwise indicated, the figures were obtained with the mAb SG1. The steady-state distribution of wild-type gpI was tested with the four different anti-gpI antibodies and was found to be identical in each case.

For the quantitation of the fluorescent signal, selected fields were captured using a cooled charge-coupled device CH250 from Photometrics (Tucson, AZ) having a Kodak KAF 1400 CCD chip (Grade 2) for 12-bit image collection (4096 intensity levels) and that was controlled by the KHOROS software package running in a SUN 10/41 workstation. In each field, regions containing the gpI-labelled TGN area were selected, and the fluorescence in the rhodamine channel was calculated using the AIM program developed by J.C.Olivo from the EMBL microcomputing and data acquisition group. On the same coverslips, areas of similar size in non-transfected cells were also selected and processed in an analogous manner. After estimating the fluorescence intensity from 20–50 transfected and non-transfected cells, the mean and standard error of the

mean were calculated. The confidence limits of the results obtained were assessed by a Student's t test and shown in each case to be >99% ($P<0.01$) .

Internalization assays

Cells grown on coverslips and transiently transfected 2 days in advance were washed with PBS containing 0.05% bovine serum albumin (BSA) and incubated with a 1:100 dilution of the human anti-VZV serum for 1 h at 0°C. The cells were washed again and returned to the 37°C incubator for the desired amount of time, after which they were fixed directly and processed for immunofluorescence following the above described procedures. The acid wash to remove surface-bound antibody was performed essentially as described in Corvera *et al.* (1994), by incubating the cells for two sequential 2 min periods in a solution containing 100 mM glycine, 20 mM magnesium acetate and 50 mM potassium chloride (pH 2.2). The cells were washed with PBS–0.05% BSA and processed for immunofluorescence.

Acknowledgements

We would like to thank Drs A.Arvin, G.Banting, W.Garten, T.Nilsson, B.Rentier, M.S.Robinson, J.Skehel and E.Ungewickel for providing us with antibodies and cDNAs, and R.Saffrich and J.-C.Olivo for assisting us with image collection and processing. We are indebted to Drs T.Nilsson, G.Griffiths, R.Le Borgne and M.Zerial for critical reading of the manuscript and to members of the Hoflack's lab for helpful discussions. A.A. was supported by a post-doctoral fellowship from the Spanish Ministerio de Educación y Ciencia. Work in the authors' laboratory is supported by grants from 'Vaincre les Maladies Lysosomales' and the European Community (Grant B102-CT93-0205).

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Received on July 11, 1996; revised on August 13, 1996