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A sorting signal for the basolateral delivery of the vesicular stomatitis virus (VSV) G protein lies in its luminal domain: Analysis of the targeting of VSV G–influenza hemagglutinin chimeras

(membrane protein targeting/viral glycoprotein hybrids/epithelial cell polarity)

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ABSTRACT **When synthesized in polarized epithelial cells, the envelope glycoproteins hemagglutinin of influenza and G of vesicular stomatitis virus are targeted to the apical and basolateral plasma membranes, respectively. To determine which portions of these transmembrane proteins contain information necessary for their sorting, the behavior of two different G–hemagglutinin chimeric polypeptides, consisting of all or nearly all the luminal portion of the vesicular stomatitis virus G protein linked to C-terminal segments of influenza hemagglutinin that included its transmembrane and cytoplasmic domains, was studied in MDCK cells transformed with the corresponding cDNAs. Both chimeras were transported from the endoplasmic reticulum to the Golgi apparatus and from there to the cell surface with the same rapid kinetics as the intact G protein. By using a cell surface immunoprecipitation assay with monolayers cultured on permeable filters that allows the recovery of labeled protein molecules present in each cell surface domain, it was found that both chimeric proteins as well as the intact G protein were delivered almost exclusively to the basolateral surface. This polarized distribution of the polypeptides did not change during a subsequent 90-min chase period, although during this time a large fraction of the glycoprotein molecules underwent degradation. In addition, a small fraction of the cell surface-associated glycoprotein molecules shed their ectoplasmic segments into the basolateral compartment, apparently as a result of a proteolytic cleavage. Immunofluorescence on transverse frozen sections and immunoelectron microscopy revealed a prominent accumulation of the chimeric polypeptides in the lateral cell membranes, with lesser amounts on the basal and apical surfaces. These results indicate that information specifying the basolateral transport of the G glycoprotein is located within the first 426 N-terminal amino acids of its ectoplasmic portion.**

During the process of plasma membrane biogenesis in polarized epithelial cells, a specialized sorting apparatus must operate to ensure that the sets of proteins characteristic of the two surface domains of these cells are correctly targeted to their ultimate destinations. When such cells are infected with enveloped viruses, the viral glycoproteins are segregated to the specific plasma membrane domains where virion assembly takes place (1). Thus, hemagglutinin (HA) molecules accumulate in the apical surface of influenza-infected cultured Madin–Darby canine kidney (MDCK) cells, whereas, in vesicular stomatitis virus (VSV)-infected cells, G molecules are segregated to the basolateral plasma membrane (2–4). The viral glycoproteins are appropriately sorted, even when synthesized in the absence of other viral components, in cells transfected with recombinant plasmids containing the viral envelope genes (5–7). This implies that the signals mediating the sorting process lie in the polypeptides themselves.

The viral glycoproteins influenza HA and VSV G are oriented similarly with respect to the phospholipid bilayer in the membrane, with large N-terminal domains (509 and 446 amino acids, respectively) residing on the luminal or ectoplasmic side of the membrane. These are followed by short transmembrane segments and by cytoplasmic domains consisting only of 11 (HA) or 29 (G) amino acids. Putative sorting signals in these proteins may be found in any of their three domains. Evidence for the presence of sorting information in the luminal domain of HA has been obtained (8, 9) from the effective sorting to the apical surface of hybrid proteins containing the ectocytoplasmic domain of HA and the membrane and cytoplasmic portions of the VSV G protein. Additional support for a role of the luminal domain of HA in sorting is provided by the finding (8) that an anchorless version of this protein is secreted from the apical surface of monkey kidney cells infected with a recombinant simian virus 40 containing the HA cDNA. On the other hand, a similar anchorless HA was found to be released indiscriminately from both surfaces when expressed in permanently transformed MDCK cells (10).

Anchorless versions of two basolateral proteins, the VSV G and the murine leukemia virus glycoprotein gp70, when expressed in MDCK cells, were also indiscriminately secreted from both surfaces (10, 11). While these results would be consistent with the notion that sorting information for basolateral proteins lies in their cytoplasmic or membrane-spanning segments, the nonpolarized secretion may only indicate that, because they are removed from the membrane, the truncated proteins do not easily attain the conformation required for their recognition by the sorting receptor.

Conflicting results have been obtained with respect to the sorting of hybrid molecules comprising the luminal domain of the VSV G protein and the cytoplasmic domain of the HA. In one case (12), a chimeric protein containing the luminal and transmembrane domains of the VSV G but the cytoplasmic domain of the HA was found to accumulate in both the basolateral and apical surfaces of permanent MDCK transformants expressing the chimeric gene. In the other (13), a chimeric protein containing the luminal domain of VSV G and both the transmembrane and cytoplasmic domains of HA, when expressed in cells infected with recombinant vaccinia viruses, accumulated exclusively on the basolateral surface of MDCK cells. In the work presented in this paper, we examined, in permanently transformed MDCK cells, the

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Abbreviations: HA, hemagglutinin; VSV, vesicular stomatitis virus; endo H, endoglycosidase H.

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targeting and distribution of chimeric proteins that comprise the ectoplasmic domain of the VSV G protein combined with the membrane-spanning and cytoplasmic domains of the HA. This work has previously appeared in abstract form (14).

MATERIALS AND METHODS

Construction of G–HA Hybrid Genes. To generate precise fusions of VSV G and influenza HA sequences, the G protein cDNA and a 3′ portion of the HA cDNA were cloned in tandem into the vector M13mp19, and intervening sequences were deleted by oligonucleotide-mediated *in vitro* mutagenesis. Briefly, pSV2G (a gift of J. Rose) (6) was digested with *Bgl* II, made blunt-ended with the Klenow fragment of DNA polymerase, and then digested with *Hin*dIII to excise the G cDNA. pSV2HA (6) was cleaved at a unique *Eco*RI site within the cDNA, made blunt-ended, and digested with *Bam*HI to excise a 3′ 600-base-pair fragment of HA. The isolated cDNA fragments were ligated together into pUC9 that had been digested with *Hin*dIII and *Bam*HI. For site-specific mutagenesis, the linked cDNAs were excised with *Kpn* I and *Bam*HI, and the antisense strand was subcloned into M13mp19. Mutagenesis was performed essentially as described by Zoller and Smith (15) by using specific mutagenic primers, as well as the M13 universal sequencing primer. G–HA1 was generated with a 48-mer oligonucleotide complementary to the last 24 bases of the G cDNA segment encoding the luminal domain of G and 24 bases of the HA cDNA segment encoding the 3 juxtamembrane luminal amino acids and the first 5 residues of the transmembrane domain of HA (see Fig. 1). G–HA2 was generated by using a 40-mer oligonucleotide complementary to the coding bases 1337–1356 of the G cDNA and bases 1563–1582 of the HA cDNA. The encoded protein lacks the last 20 amino acids of the ectoplasmic portion of the G but contains instead 19 luminal amino acids of HA followed by the transmembrane and cytoplasmic domains of the protein. The sequence at the junctions of each of the hybrids was determined by dideoxynucleotide sequencing (16). The chimeric cDNAs were excised from the replicative form of the M13 phage vector with *Kpn* I and *Bam*HI and ligated into a pSV2-based plasmid containing the bacterial neomycin-resistance gene under the control of the herpes simplex virus thymidine kinase promoter. Permanent transformants expressing the chimeric proteins were obtained after G418 selection and were screened by immunofluorescence, as described (6, 10, 17).

Cell Surface Immunoprecipitation. MDCK cells expressing G, G–HA1, or G–HA2 were plated at 8×10^5 cells per 30-mm permeable disk (Transwell, 0.4-μm size; Costar). To induce expression of the transgene, 10 mM sodium butyrate was added to the medium 50–55 hr after plating. Prior to metabolic labeling (72 hr after plating), the cells were incubated in methionine-free minimal essential medium (MEM) for 30 min. For pulse labeling, the filter devices were inverted and placed in a wet chamber, and 200 μl of methionine-free MEM containing [^{35}S]methionine (625 μCi/ml; 1 Ci = 37 GBq; specific activity of 1000 Ci/mmol) was added to the bottom of the filter. At the end of the pulse period (generally 15 min), filters were repositioned, washed, and incubated with chase medium (MEM containing 20 mM methionine). Immunoprecipitation of molecules exposed at the cell surface was carried out by a modification of a published procedure (7). The chambers were placed on ice, washed three times with ice-cold Dulbecco's phosphate-buffered saline (PBS) containing 0.1 mM Ca^{2+}, and incubated for 1 hr with 200 μl of anti-VSV rabbit antiserum (diluted 1:10 in PBS) applied to either the apical or basolateral surface. After three washes with PBS, the cells were gently scraped into 1 ml of ice-cold lysis buffer (150 mM NaCl/50 mM Tris·HCl/20 mM EDTA/1% Triton X-100/1% deoxycholate/0.1% SDS, pH 7.5) containing unlabeled cell extract to prevent postextraction interactions with unreacted antibody-binding sites. The lysate was transferred, together with the excised filter, to a 1.5-ml Eppendorf tube and sonicated briefly. Cell and filter debris were removed by centrifugation, and the supernatant was incubated with protein A-Sepharose beads for 1 hr at 4°C. The beads were recovered, washed four times with lysis buffer, resuspended in sample buffer, and boiled. The eluates were analyzed by SDS/PAGE on 10% acrylamide gels, followed by fluorography using EN^{3}HANCE (New England Nuclear). Control samples were treated with lysis buffer before addition of antibody.

Immunofluorescence and Immunoelectron Microscopy. For immunofluorescence, transverse semithin sections (0.5–1 μm) of monolayers fixed in 4% (vol/vol) paraformaldehyde, together with their underlying filters, were prepared by the method of Tokuyasu (18). For immunoelectron microscopy, thin frozen sections were prepared from pellets of monolayers fixed in 0.5% glutaraldehyde and scraped from the filters. Immunolabeling with anti-VSV or anti-VSV G protein antiserum using rhodamine-conjugated secondary antibodies or protein A coupled to 10-nm gold was conducted as described (3, 4).

RESULTS

Polarized Delivery of G–HA1 and G–HA2 to the Basolateral Surface of MDCK Cells. Two different G–HA hybrids (Fig. 1) were studied. The first (G–HA1) contains the entire luminal portion of the VSV G protein (446 amino acids after cleavage of the signal sequence) and a C-terminal segment of HA containing the transmembrane and cytoplasmic domains. The second (G–HA2) lacks not only the membrane-spanning and cytoplasmic domains of the G but also the 20 luminal amino acids adjacent to the membrane-spanning segment. These have been replaced by 19 amino acids from the corresponding region of HA.

The G–HA1 and G–HA2 hybrids synthesized in permanent transformants of MDCK cells were transported efficiently from the endoplasmic reticulum to the Golgi apparatus, as

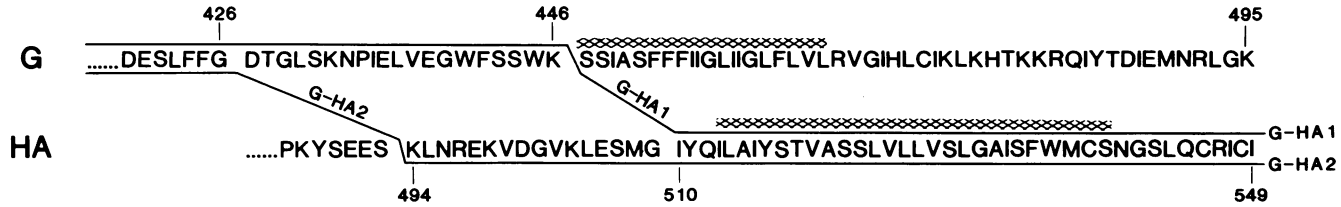

FIG. 1. Structures of the G–HA1 and G–HA2 hybrid molecules. The sequences of the C-terminal portions of the G and HA proteins are shown; the transmembrane domain of each protein is marked by the crosshatched area. The two lines that connect the G and HA sequences indicate the portions included in the fusion proteins G–HA1 and G–HA2, as marked. Note that in G–HA1 the entire luminal domain of G is linked to a C-terminal segment of HA encompassing the 3 juxtamembrane luminal residues and the entire transmembrane and cytoplasmic domains of HA. In G–HA2, the portion of the chimera derived from HA includes an additional segment of 16 amino acids of the luminal domain, which replaces the 20 juxtamembrane residues of the G protein.

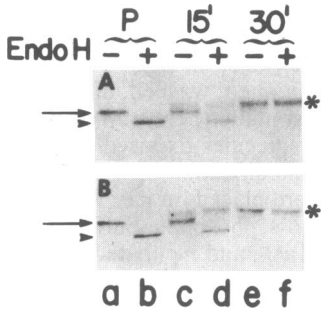

FIG. 2. Kinetics of acquisition of endo H resistance by the G-HA1 and G–HA2 chimeric polypeptides. Permanent transformants of MDCK cells expressing the G–HA1 or G–HA2 polypeptides were pulse-labeled for 15 min with [^{35}S]methionine and then incubated for 15 or 30 min in chase medium. After the pulse (P) and chase periods, cell lysates were prepared, and the chimeric proteins were immunoprecipitated with anti-VSV G antibodies. Immunoprecipitates incubated with (+) or without (−) endo H were analyzed by SDS/polyacrylamide gel electrophoresis and fluorography. (*A*) G–HA1. (*B*) G–HA2. Endo H digestion (lanes b, d, and f) converts the newly synthesized proteins (arrows in lanes a and c) to more rapidly migrating species (arrowheads in lanes b and d) but has no effect on the mature proteins (lanes e and f). The arrows indicate the position of the high mannose-containing forms present in the endoplasmic reticulum, and the asterisks indicate the positions of the mature glycoproteins modified in the Golgi apparatus.

assessed in pulse–chase experiments by the kinetics of acquisition of complex oligosaccharides resistant to removal by endoglycosidase H (endo H) (Fig. 2). Transfer to the Golgi apparatus was completed by 30 min of chase, as was previously observed for the G protein itself (19). This can be contrasted with the slow acquisition of endo H resistance observed for a G–HA chimera that contained only the cytoplasmic domain of HA (20) and was not sorted effectively in MDCK cells (12).

A surface immunoprecipitation procedure was used to examine the delivery and accumulation of the intact G and hybrid proteins on the apical and basolateral surfaces of the permanent transformants. The cells were grown as monolayers on permeable filters, and expression of the proteins was induced by addition of sodium butyrate to the medium. After pulse labeling with [^{35}S]methionine and various times of chase, the chambers were cooled to 4°C and incubated with antibody to VSV G added to either the apical or the basolateral bathing medium (see *Materials and Methods*). As shown in Fig. 3, all three proteins were delivered in a polarized fashion to the basolateral surface, where after 30 min of chase they could be captured by antibodies. Very low amounts of the labeled proteins, 10–20% as assessed by densitometric scanning, were detected at the apical surface.

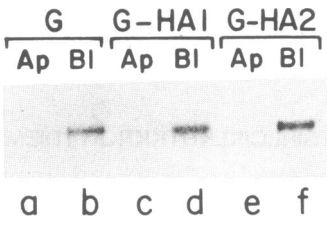

FIG. 3. Preferential delivery of newly synthesized G, G–HA1, and G–HA2 polypeptides to the basolateral surface. Monolayers of MDCK transformants were grown on permeable filters and labeled by a 15-min pulse with [^{35}S]methionine, followed by a 30-min chase. Anti-VSV antibody was added to the apical (Ap; lanes a, c, and e) or basolateral (Bl; lanes b, d, and f) chambers of individual filters, and immunoprecipitation of labeled molecules present at the cell surface was carried out as described. Immunoprecipitates were analyzed by SDS/polyacrylamide gel electrophoresis and fluorography.

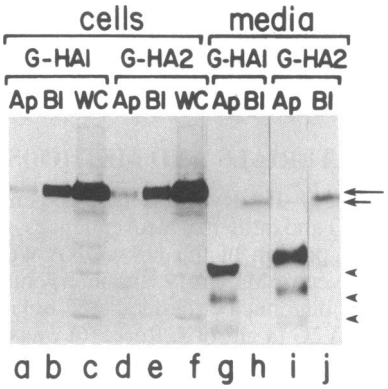

FIG. 4. Accumulation of the G–HA chimeric proteins on the basolateral surface and shedding of ectoplasmic domains into the basolateral medium. Permanent transformants of MDCK cells grown on filter chambers were pulse-labeled for 15 min with [^{35}S]methionine and chased for 2 hr. Surface molecules (long arrow) were immunoprecipitated from the apical (Ap; lanes a and d) or basolateral (Bl; lanes b and e) sides, or intact monolayers were solubilized with lysis buffer prior to immunoprecipitation (WC; lanes c and f) to recover the chimeric protein from the whole cell. Medium from each compartment collected after the chase period was also analyzed by immunoprecipitation (lanes g–j). The three bands marked by arrowheads, detectable only in the apical compartment (lanes g and i), represent the subunits of the endogenous secretory product of MDCK cells (17), which are found in large amounts in this compartment and are recovered as a contaminant in the immunoprecipitates. The band (short arrow) present in the basolateral medium (lanes h and j) represents the shed ectodomain of the G–HA proteins.

This polarized distribution was maintained after a 2-hr chase period (Fig. 4), even though a substantial portion of the cell-associated proteins appeared to turn over during this time with a half-life on the order of 1 hr (Fig. 5). Part of the apparent turnover, however, was due to shedding of the extracellular portions of the transmembrane proteins into the basolateral medium where they could be detected by immunoprecipitation (Fig. 4, lanes h and j). The fragments detected in the extracellular medium were not present intracellularly and were clearly derived from the plasma membrane-associated molecules. They were, therefore, different from the secretory truncated form of the G protein detected intracellularly in VSV-infected baby hamster kidney cells, which is thought to represent a primary translation product (21).

Fig. 4 shows that even after a 2-hr chase, when nearly all the molecules synthesized during the pulse should have reached the cell surface, only ≈70% of the cell-associated labeled molecules could be recovered by the surface immunoprecipitation procedure. This low recovery may in part be due to interiorization of surface glycoproteins, which we have shown takes place for the G protein (6), as well as for

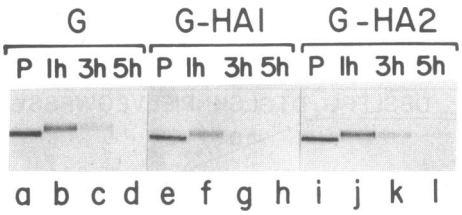

FIG. 5. G and the G–HA hybrids turn over rapidly in permanently transformed MDCK cells. Cultures of permanent transformants of MDCK cells were labeled for 15 min (lanes a, e, and i) with [^{35}S]methionine and chased in MEM medium for 1 (lanes b, f, and j), 3 (lanes c, g, and k), or 5 (lanes d, h, and l) hr, as indicated. At the end of the pulse (P) and chase periods, cell lysates were prepared, and the viral glycoproteins were immunoprecipitated and analyzed by gel electrophoresis and fluorography.

the chimeric molecules (T.G., unpublished observations). Moreover, the presence of the filter may hinder access of the antibodies to the adherent basolateral plasma membrane.

Surface Localization of the G–HA Chimeric Proteins. The polarity of the steady-state distribution of the chimeric proteins in the transformed cells was examined by immunofluorescence and immunoelectron microscopy in transverse frozen sections of monolayers grown on filters. As can be seen in Fig. 6, both G–HA1 and G–HA2 were heavily concentrated on the lateral surfaces. It should be noted, however, that some labeling of the apical surfaces of the monolayers could always be detected. This distribution is similar to that previously reported for the VSV G protein (3, 6). Immunoelectron microscopy confirmed the basic findings made by immunofluorescence. G–HA1 (Fig. 7) and G–HA2 (data not shown) were predominantly localized to lateral plasma membranes, with less striking labeling of basal surfaces. A variable, and in some cells significant, amount of the hybrid proteins was also detected on the apical surfaces.

DISCUSSION

The results presented in this paper, with two chimeric constructs, lead to the conclusion that the 426 luminal amino acids of the VSV G protein contain information sufficient to ensure the polarized delivery of the polypeptide to the basolateral surface of MDCK cells and its accumulation in that domain. Using a surface immunoprecipitation technique to quantify the amounts of newly synthesized glycoprotein molecules on the apical or basolateral domains, we found that the vast majority of the pulse-labeled molecules that reached the cell surface after a 30-min chase were confined to the basolateral aspect of the plasma membrane. This distribution did not change significantly after a further 90 min of chase and must be very close to the steady-state distribution of the viral glycoproteins on the surface of the cell, since the labeled products were shown to turn over rather rapidly (with a half-life on the order of 1 hr).

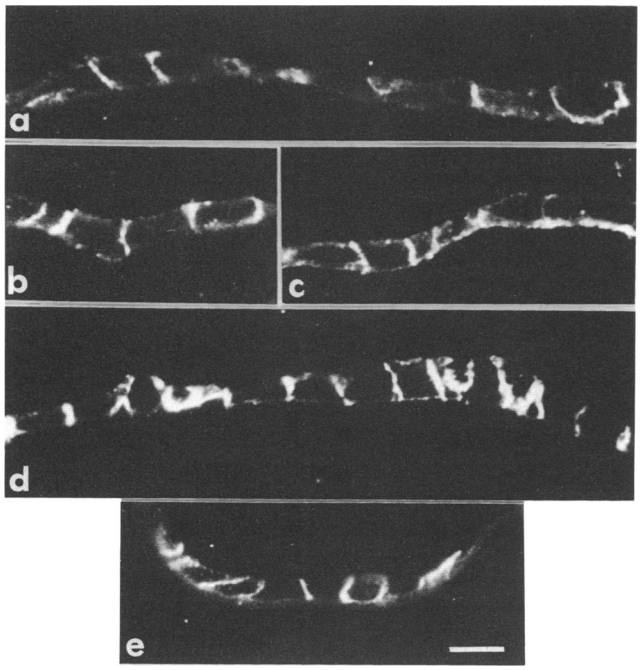

FIG. 6. Immunofluorescence localization of G–HA1 and G–HA2 in MDCK cells. Semithin sections of filter-grown, formaldehyde-fixed, MDCK cell transformants expressing G–HA1 (*a–c*) or G–HA2 (*d* and *e*) were processed for immunofluorescence with anti-VSV primary antibodies and rhodamine-labeled secondary antibodies. In both cases the hybrid proteins are seen to be concentrated on the lateral membranes. (Bar = 10 μm.)

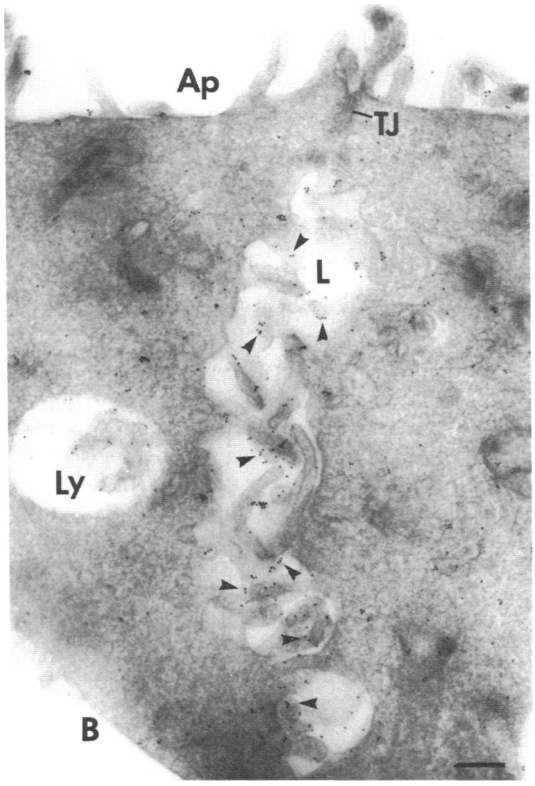

FIG. 7. Immunoelectron microscopic localization of G–HA1 in MDCK cells. Filter-grown transformants expressing G–HA1 were fixed in glutaraldehyde, and the cells were scraped from filters and sedimented. Thin frozen sections were prepared and immunolabeled with anti-VSV G antiserum and subsequently with protein A-gold (10 nm) conjugates. Gold particles (arrowheads) are concentrated primarily on the lateral plasma membrane (L) as well as in lysosomes (Ly), whereas little label is bound to the apical (Ap) and basal (B) surfaces. Similar results were obtained with G–HA2 transformants (data not shown). (Bar = 0.2 μm.) TJ, tight junction.

The striking basolateral segregation of the chimeric glycoproteins was also apparent by immunofluorescence and immunoelectron microscopy. Our findings with permanently transformed cells, are, therefore, in accord with those of McQueen *et al.* (13) who, by using recombinant vaccinia viruses, examined the distribution of a chimeric protein similar to G–HA1 but containing an HA segment from a different influenza virus strain. In that work, however, a direct demonstration of polarized delivery was not carried out, and the steady-state distribution of the chimeric protein was assessed using "en face" immunofluorescence and a surface radiolabeling technique.

On the other hand, a G–HA chimera that incorporated only the cytoplasmic domain of HA, when expressed in permanently transformed MDCK cells (i.e., the same system used for our studies), was found to accumulate in both the apical and basolateral surfaces (12). In contrast to those chimeras that included both the cytoplasmic and transmembrane domains of HA studied by McQueen *et al.* (13) and us, this chimeric glycoprotein, when expressed in Chinese hamster ovary (20) or COS cells (22), was transported very slowly from the endoplasmic reticulum to the Golgi apparatus, judging from the rate of its acquisition of endo H resistance. Although it has been shown that the G–HA protein that contains only the cytoplasmic domain of HA undergoes proper trimerization, a process that takes place in the endoplasmic reticulum and appears to be essential for transport out of the organelle (23–25), the slow interorganellar trans-

port of this chimera suggests that it had an abnormal conformation that could also have been responsible for its failure to undergo proper sorting as it exited from the Golgi apparatus.

The sorting of another basolaterally directed membrane protein, the polymeric immunoglobulin receptor, has also been examined in MDCK cells (26–28). It was reported that an anchorless form of this protein and a form lacking the cytoplasmic segment were directly transferred to the apical surface, where the native protein normally appears only as a result of transcellular transfer. This led to the suggestion that the cytoplasmic segment of the polymeric immunoglobulin receptor contains a positive signal that directs the molecule to the basolateral surface (27). Indeed, it is possible that, whereas some proteins, such as the polymeric immunoglobulin receptor, are capable of sorting by themselves by the direct interaction of their cytoplasmic domains with cellular transporting elements, other proteins, such as the viral glycoproteins, are sorted in a "piggyback" fashion through interaction of their luminal domains with sorting receptors that, like the polymeric immunoglobulin receptor, can sort by themselves.

We have also made the observation that a fraction of the intact G protein molecules, as well as the G–HA chimeras synthesized in MDCK cells, undergo a proteolytic cleavage that leads to the release of the luminal portions of these proteins exclusively into the basolateral medium. It is not yet clear if the cleavage takes place intracellularly, following endocytosis, or if it is effected by a cell surface protease. Nevertheless, the exclusive release of the fragment from the basolateral surface supports the conclusion that the chimeras are sorted as efficiently as the intact G protein.

Another striking observation is that both the intact G protein and the chimeras undergo rapid turnover in the permanent transformants. After a 5-hr chase period, the pulse–labeled proteins had almost completely disappeared from the cells. Since only a small percentage of the membrane-associated molecules was shed into the medium, the rapid turnover implies that the chimeras are internalized by endocytosis, which is a prerequisite for the degradation, as efficiently as the intact G protein. This is in striking contrast to the behavior of the intact HA molecule, which is the source of the cytoplasmic and transmembrane domains of the chimeras and is rather stable in transfected cells (T.G., unpublished results).

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