Sorting and Endocytosis of Viral Glycoproteins in Transfected Polarized Epithelial Cells

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Abstract. Previous studies (Rindler, M. J., I. E. Ivanov, H. Plesken, and D. D. Sabatini, 1985, *J. Cell Biol.*, 100:136–151; Rindler, M. J., I. E. Ivanov, H. Plesken, E. J. Rodriguez-Boulan, and D. D. Sabatini, 1984, *J. Cell Biol.*, 98:1304–1319) have demonstrated that in polarized Madin-Darby canine kidney cells infected with vesicular stomatitis virus (VSV) or influenza virus the viral envelope glycoproteins G and HA are segregated to the basolateral and apical plasma membrane domains, respectively, where budding of the corresponding viruses takes place. Furthermore, it has been shown that this segregation of the glycoproteins reflects the polarized delivery of the newly synthesized polypeptides to each surface domain. In transfection experiments using eukaryotic expression plasmids that contain cDNAs encoding the viral glycoproteins, it is now shown that even in the absence of other viral components, both proteins are effectively segregated to the appropriate cell surface domain. In transfected cells, the HA glycoprotein was almost exclusively localized in the apical cell surface, whereas the G protein, although preferentially localized in the basolateral domains, was also present in lower amounts, in the apical surfaces of many cells.

Using transfected and infected cells, it was demonstrated that, after reaching the cell surface, the G protein, but not the HA protein, undergoes interiorization by endocytosis. Thus, in the presence of chloroquine, a drug that blocks return of interiorized plasma membrane proteins to the cell surface, the G protein was quantitatively trapped in endosome- or lysosome-like vesicles. The sequestration of G was a rapid process that was completed in many cells by 1–2 h after chloroquine treatment. The fact that in transfected cells the surface content of G protein was not noticeably reduced during a 5-h incubation with cycloheximide, a protein synthesis inhibitor that did not prevent the effect of chloroquine, implies that normally, G protein molecules are not only interiorized but are also recycled to the cell surface.

THE plasma membrane of cells in transporting epithelia is differentiated into distinct apical and basolateral domains that are separated by tight junctions. The functional polarity of these epithelia is largely determined by the presence of different complements of plasma membrane proteins in the two cell surface domains. Such proteins provide specific enzymatic activities, serve as components of transport systems, or function as receptors that mediate the cellular responses to environmental stimuli (c.f. 37). In polarized epithelial cells, the sorting processes that address newly synthesized polypeptides to the different subcellular compartments must also effect the segregation of specific proteins into the two plasma membrane domains. In most cells, including those in polarized epithelia, many plasma membrane proteins undergo extensive recycling through the cytoplasm, which involves their interiorization into endosomes and subsequent return to the cell surface (c.f. 5, 8, 19, 39, 43). Maintenance of the segregation of specific plasma membrane proteins to the different surfaces of epithelial cells must, therefore, either involve the exclusion of certain proteins from the recycling process or the operation of mechanisms that ensure that the interiorized proteins are returned to the surface of origin, possibly by a route that partially overlaps with that taken by the newly synthesized proteins.

The mechanisms involved in the biogenesis and maintenance of distinct plasma membrane domains in epithelial cells can be conveniently studied in a model system that uses cultures of polarized epithelial cells infected with enveloped viruses. These viruses consist of a nucleocapsid core surrounded by a lipoprotein membrane that is derived from the cell plasma membrane during budding. The viral envelope contains virally encoded glycoproteins that are synthesized in the endoplasmic reticulum and are delivered to the cell surface, most likely by the same pathway taken by cellular plasma membrane proteins (c.f. 41). In infected cultures of Madin-Darby canine kidney (MDCK)[1] cells, different types of viruses bud exclusively from one or the other plasma membrane

[1] *Abbreviations used in this paper:* HA, hemagglutinin; MDCK, Madin-Darby canine kidney; MEM, Eagle's minimum essential medium; VSV, vesicular stomatitis virus.

domain. Thus, influenza virions are produced only from the apical surface, whereas vesicular stomatitis virus (VSV) particles assemble exclusively from the basolateral domain (34). The asymmetric assembly of the two types of viral particles is preceeded by an accumulation of the respective envelope glycoproteins at the surface from which budding takes place (33). It seems apparent, therefore, that a study of the sorting of viral envelope glycoproteins in polarized cells should shed light on the biogenetic mechanisms that account for the segregation of cellular proteins in the two different epithelial surfaces.

Recent experiments have demonstrated that the viral glycoproteins G of VSV and hemagglutinin (HA) of influenza are sorted intracellularly and delivered in a polarized fashion to the appropriate domain (21, 23, 28, 29, 32). This intracellular sorting takes place during exit of the glycoproteins from the Golgi apparatus or soon thereafter, since in cells doubly infected with influenza and VSV, glycoproteins of both viruses were found in the same Golgi cisternae (29). It has also been observed that despite its initial polarized delivery to the basolateral surface, during the course of viral infection significant amounts of the VSV G protein also appeared on the apical surface of MDCK cells (28, 29). On the other hand, throughout the infectious cycle, the HA of influenza remained restricted more stringently to the surface to which it was initially delivered. The delayed appearance of G on the apical surface, observed when transport of the glycoprotein to the plasma membrane had been synchronized using a temperature-sensitive mutant (28), suggested that the imperfect segregation of the G protein results from a redistribution of molecules initially delivered to the basolateral domain. This could result from recycling of G through the cytoplasm, or from its lateral diffusion and selective passage through the tight junctions during the course of infection. The capacity of G protein artificially inserted in the apical surface to undergo transcytosis and become incorporated into the basolateral plasma membrane has, in fact, been demonstrated (20, 25).

A study of the sorting of viral envelope glycoproteins in infected cells is complicated by the possible participation in this process of other viral components, such as the matrix protein that appears to mediate the association of the nucleocapsid core with the viral envelope (c.f. 41, 47), as well as by the onset of cytopathic changes that take place during the course of viral infection. We have avoided these complications by investigating the sorting and recycling of the VSV G and influenza HA glycoproteins in cells transfected with expression plasmids that contain the VSV G or HA cDNAs. We observed that in the absence of other viral components, both the HA and G glycoproteins are still segregated to the appropriate cell surface domains of cultured MDCK cells. Other investigators (36) have previously reported that the HA, expressed in primary monkey kidney cells from a gene introduced into an SV40 recombinant virus, accumulates exclusively in the apical domain. We have also found that after arrival to the plasma membrane, the VSV G, but not the influenza HA, recycles through cytoplasmic endocytic vesicles, where it can be quantitatively trapped in the presence of the lysosomotropic drug, chloroquine. A differential recycling of the glycoproteins of the two viruses in infected cells may account for the redistribution of G protein that takes place during the course of infection.

Materials and Methods

Cell Culture and Virus Infection

MDCK cells originally obtained from Dr. J. Leighton, Medical College of Pennsylvania, Philadelphia, were grown in Eagle's minimum essential medium (MEM) (Gibco, Grand Island, NY) with 10% horse serum or fetal bovine serum (Sterile Systems, Logan, UT) and antibiotics, as previously described (6). Cells plated at a density of 10^5 cells/cm^2 on glass coverslips (VWR Scientific Div., San Francisco, CA) placed in 24-well dishes (Falcon Labware, Oxnard, CA) were infected at an moi of 10 pfu/cell 3 d after reaching confluence. Influenza A/WSN virus stocks were prepared in the presence of 2.5 μg/ml trypsin (Gibco) from infected MDCK cells and VSV stocks from infected HeLa cells. For virus infection experiments, cells were incubated with virus for 2 h in 0.2 ml MEM but during infection with VSV, DEAE dextran (100 μg/ml) (Pharmacia Fine Chemicals, Piscataway, NJ) was also present in the medium. The virus inoculum was removed and the infected cultures were incubated with 1 ml MEM that contained serum for an additional 4-h period, in the presence or absence of 25 μM chloroquine (Sigma Chemical Co., St. Louis, MO).

Construction of Expression Plasmids

The expression plasmid pSV2G (35) was provided by Dr. John Rose, Salk Institute. The vector did not yield useful levels of expression in MDCK cells, presumably due to the presence of the small t intron (10, 15). Therefore, the cDNA coding for the G protein was excised and introduced into a related vector that lacks the small t intron (provided by Dr. A. Colman, University of Warwick, UK) (16). To accomplish this (Fig. 1), pSV2G was linearized with BglII, incubated with Klenow polymerase to make the ends blunt, and joined to Hind III linkers. The G cDNA, now containing Hind III sites at both ends, was isolated after cleavage with Hind III and ligated into the Hind III site of the t intronless pSV2 vector.

A cloned cDNA for the HA gene of influenza in pBR322 (48) (strain A/

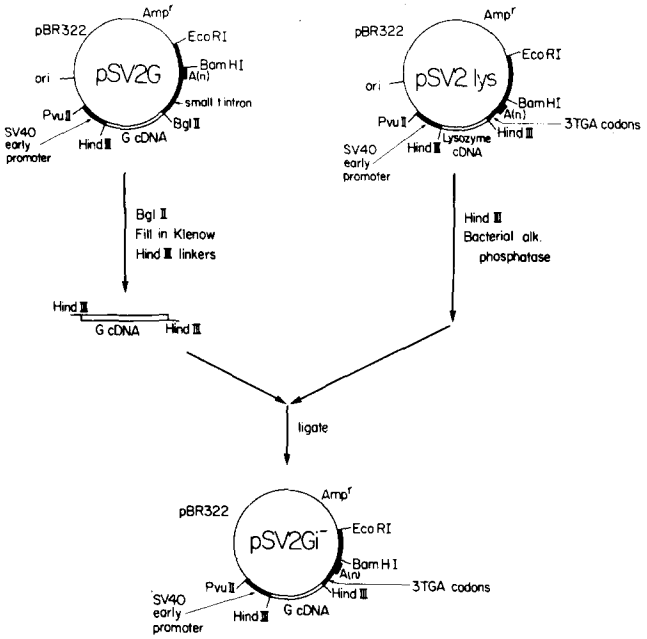

Figure 1. Construction of pSV2Gi$^-$, G protein expression vector lacking the small t intron. The G cDNA insert, removed from the pSV2G vector (see Materials and Methods) and containing Hind III sites at each end, was inserted into the intronless vector pSV21ys, from which the original insert (lysozyme cDNA) had been removed. In the resulting plasmid pSV2Gi$^-$, the G cDNA is placed downstream from the SV40 early promoter that is contained in the Pvu II-Hind III fragment. After the Hind III site at the 3′ end of the G cDNA, a 258-base pair Hind III-Bam HI fragment provides termination codons in all three reading frames as well as the SV40 polyadenylation signal (15). In the diagram for the pSV2Gi$^-$ vector, thin line segments represent sequences derived from the plasmid pBR322, whereas the thicker black lines indicate SV40 DNA. The broad open bar corresponds to the G cDNA.

PR/8/34; HINI), obtained from Dr. Peter Palese (Mt. Sinai Medical Center, New York), was modified as described in Fig. 2, by deletion of the entire 5′ untranslated region and addition of the SV40 polyadenylation signal, before its insertion into a mammalian expression vector. The cDNA (cloned into pUC9HA) was first excised by digestion with Hind III, purified by polyacrylamide gel electrophoresis, and cloned into pUC8PA (30) by blunt end ligation between the Pst I (made flush with Klenow DNA polymerase) and Hinc II sites to obtain the plasmid pUC8PAHA. The pUC8PA plasmid contains a 140-base pair Hinc II-BamHI segment of the SV40 genome, which includes the poly A addition signal for the early transcripts of the SV40 virus. To delete the 5′ untranslated region, pUC8PAHA was linearized with HindIII, digested with exonuclease III, and treated with S1 nuclease. The ends were then made blunt with Klenow polymerase, and HindIII linkers were added. The modified cDNA was removed from pUC8PAHA together with the polyadenylation signal by HindIII-BamHI digestion and ligated to the large Hind III-BamHI fragment of the expression vector pSV2gpt (24), placing it downstream from the SV40 early promoter. The extent of the deletions in each clone was first assessed by restriction mapping using the Pst 1 site downstream from the initiation codon, and then by sequencing using the dideoxy method (38) after subcloning the relevant fragments into M13 vectors (46). A clone, in which the HindIII linker was found to be immediately upstream from the initiation codon of HA, was used for the transfection experiments.

Cell Culture and Transfection

MDCK cells were grown and maintained in T75 flasks with MEM supplemented with 10% horse serum. For transfection experiments, cells in the log phase of growth were lightly trypsinized until the majority of cells were rounded but not yet detached from the flask, as determined by phase microscopy. After at least two washes with medium, cells were incubated in 5 ml of medium at 37°C for 20–30 min. A suspension (0.7 ml) of a calcium phosphate precipitate prepared according to published procedures (40), containing ~75 μg of the appropriate expression vector, was added to the flask of trypsinized cells, which was then incubated for 1 h at 37°C. At that time, 5 ml of supplemental medium was added (40). Cultures used for immunofluorescence or metabolic labeling were then incubated for a total of 8 or 20 h, respectively. After this incubation, the cell layers were treated for 1 min at room temperature with MEM that contained 15% glycerol, and washed twice, once with plain medium and once with a saline solution that contained 1.0 mM EDTA, to dissolve any residual precipitate. The glycerol-shocked cells were incubated overnight (12–16 h) in 20 ml MEM that contained 10% horse serum and 10 mM sodium butyrate (13). The transfected cells were harvested by trypsinization (0.25%) at 37°C until they could be detached from the flasks by tapping the edges and plated on coverslips (1–2 × 10^5 cells/coverslip) for immunofluorescence or in 35- or 60-mm plastic culture dishes (2–3 × 10^6 cells/dish) for metabolic labeling in the presence of 10 mM butyrate. After plating, cultures were incubated for 7–8 h before labeling and for 12–16 h before processing for immunofluorescence. CV-1 cells were transfected in suspension (7) and then plated onto coverslips.

Cell Labeling and Immunoprecipitation

Transfected cells in 60-mm dishes (for G immunoprecipitation) or 35-mm dishes (for HA) were incubated overnight in 1 or 2 ml medium composed of 4 parts methionine-free RPMI (Gibco) and 1 part normal RPMI, supplemented with 10% horse serum and 10 mM sodium butyrate, [^{35}S]methionine (125 μCi/ml; specific activity, 1,000 Ci/mmol) and, when indicated, 25 μM chloroquine. For immunoprecipitation of G, labeled cells in one dish were washed with 1.5 ml of 10 mM Tris-HCl, pH 8.0, and scraped into Eppendorf tubes on ice, where swelling was allowed to continue for 10 min. A lysate was then prepared by sonication and centrifuged (100,000 *g* for 60 min) to obtain a sediment that was resuspended in 1.0 ml of 10 mM Tris-HCl pH 8.0, that contained 1% Triton X-100, 0.4% deoxycholate, and 0.1 mM phenylmethylsulfonyl fluoride. The suspension was kept on ice and vortexed gently over a 20-min period before a second centrifugation was done to remove insoluble material. The supernatant received 5 μl of an anti-G antiserum, was incubated for 2 h at room temperature, and then overnight at 4°C. Protein A-Sepharose (30 μl of pack volume) (Sigma Chemical Co.) was then added and, after a 2-h incubation at room temperature, immunecomplexes were recovered by centrifugation in a microfuge. The pelleted beads were washed at least five times with solution A (2.5% Triton X-100, 50 mM Tris 7.5, 190 mM NaCl, 6 mM EDTA, 10 μl/ml Trasylol) and boiled in sample buffer, and the extracts were analyzed by SDS PAGE (18).

For immunoprecipitation of HA, transfected cells in 35-mm dishes were labeled as described above, but in medium that contained 60 μCi/ml [^{35}S]-methionine. The cells were then washed with saline and solubilized on the plate with 0.25 ml of 20 mM Tris buffer, pH 8.0 that contained 2% SDS. The lysate was scraped into an Eppendorf tube, sonicated, boiled, and diluted 10-fold with solution A. It was then centrifuged in a microfuge to remove insoluble material, and 5 μl of anti-HA antiserum was added to the supernatant. Immunecomplexes were recovered and analyzed as described above.

Immunofluorescence

Cells plated on coverslips were fixed at 23°C for 30 min in 2 or 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) with 0.1 mM $CaCl_2$, washed three times with PBS, and stored at 4°C until immunolabeling. The following primary antibodies were used: a monoclonal to the HA of influenza WSN (1:500 dilution, a gift of Dr. R. G. Webster, St. Jude Children's Hospital, Memphis, TN), a polyclonal rabbit antiserum and a monoclonal to the HA of influenza PR8 (1:100 dilution, gifts of Dr. P. Palese, Mt. Sinai School of Medicine, New York), a rabbit antiserum against the VSV G protein (29), and

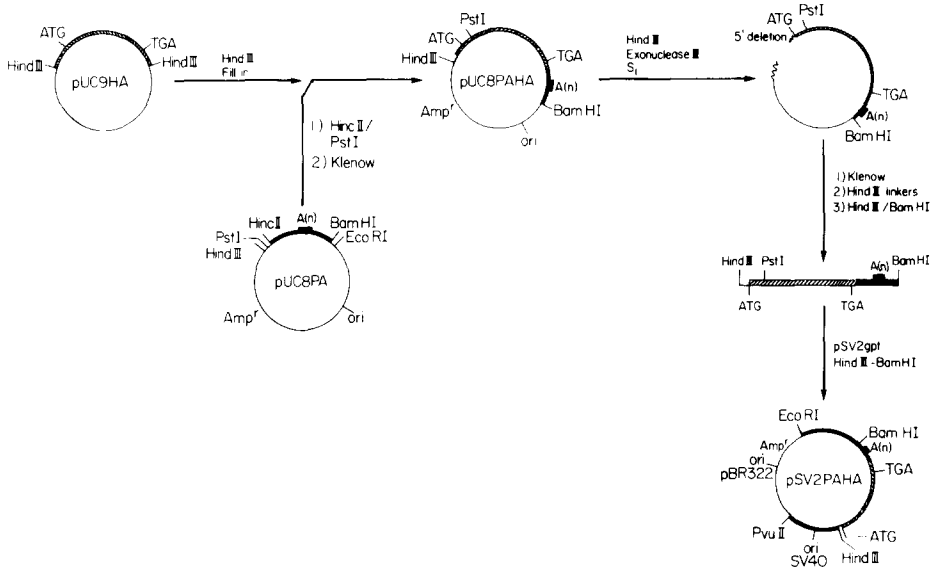

Figure 2. Construction of pSV2-PAHA, an expression vector for the influenza hemagglutinin. The 5′ untranslated region of the HA cDNA was deleted (see Materials and Methods), since its presence has been reported to greatly impair expression of the gene in mammalian cells (10). The HA cDNA, purified from pUC9HA, was blunt-end ligated into pUC8PA to yield pUC8PAHA, in which the SV40 polyadenylate addition signal follows the 3′ terminus of the HA cDNA. The cDNA was then exposed at its 5′ end by Hind III digestion, to remove 5′ untranslated sequences by sequential digestions with exonuclease III and S1 nuclease. The resulting ends were made blunt to allow the addition of Hind III linkers. Digestion with both Hind III and Bam HI yielded a mixture of 5′ deleted HA cDNAs that were fused at their 3′ termini to the SV40 polyadenylation signal. These fragments were ligated to the large Hind III-Bam HI fragment of pSV2gpt (24), a plasmid that provides the SV40 early promoter. Different clones were isolated from bacterial colonies and sequenced. In the one used here, pSV2PAHA, the Hind III linker was immediately adjacent to the initiation codon. In the diagram, DNA segments derived from pBR322 or SV40 are represented by thin and thick black lines, respectively. The hatched bar represents the HA cDNA.

a rabbit IgG raised against the rat kidney Na^+, K^+ ATPase holoenzyme (kindly provided by Prof. Y. Tashiro, Kansai Medical University). The secondary antibodies, rhodamine and fluorescein isothiocyanate-conjugated goat anti-mouse and anti-rabbit IgGs, as well as rhodamine isothiocyanate-conjugated and affinity purified goat anti-rabbit IgG, were purchased from Cooper Biomedical, Inc. (Malvern, PA). The affinity purified second antibodies were used at a dilution of 1:20 and the others at 1:50 to 1:100. Cells were preincubated in PBS that contained 0.2% gelatin (300 Bloom, Sigma Chemical Co.) and, when indicated, 0.1% TX-100 (Kodak) before application of antibody was done as previously described (29). Specimens were observed with a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with epifluorescence and phase optics, and were photographed with a Wild camera (Wild Heerbrugg Instruments Inc., Farmingdale, NY). For each micrograph the plane of focus is indicated in the figure legend.

Results

Synthesis of Viral Envelope Glycoproteins in Transfected MDCK Cells

Synthesis of the HA and G glycoproteins was measured in cells transfected with SV40-based expression vectors in which the early viral promoter directed transient expression of the corresponding cDNAs. Easily detectable levels of expression (Fig. 3, *a* and *a'*) were obtained when the transfected cultures were treated with sodium butyrate, an agent that activates transcription from SV40 early regulatory elements (13). Gel electrophoretic analysis of the ^{35}S-labeled products, recovered by immunoprecipitation with specific antibodies, showed that

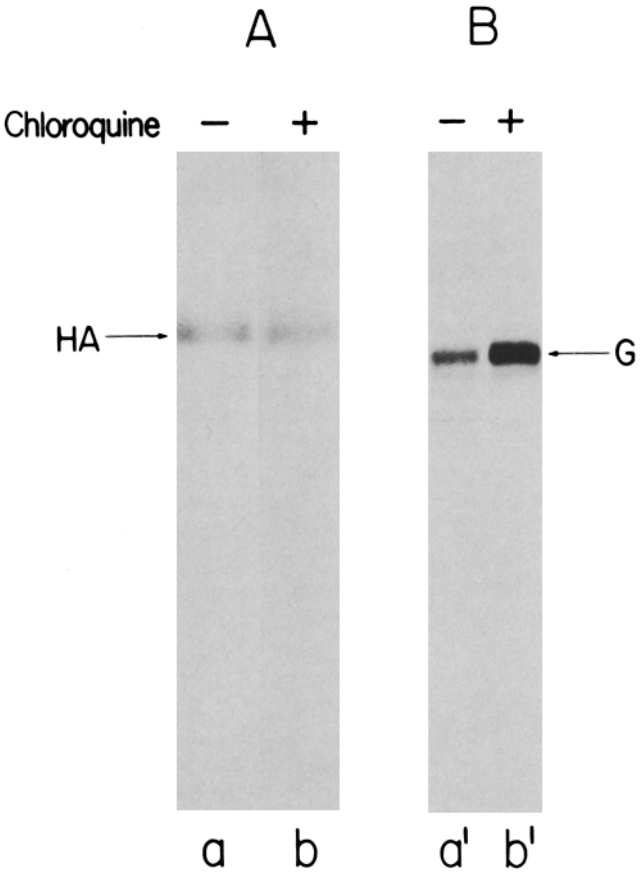

Figure 3. Synthesis of HA and G glycoproteins in transfected cells. MDCK cells were transfected with either pSV2PAHA (*A*) or pSV2Gi$^-$ (*B*), labeled with [^{35}S]methionine, and prepared for immunoprecipitation as detailed in Materials and Methods. Immunoprecipitates obtained from cells labeled in the absence (*a*, *a'*) or presence (*b*, *b'*) of 25 μM chloroquine are shown in adjacent lanes.

the proteins synthesized in transfected cells had the same electrophoretic mobility as those isolated from virally infected cells.

Immunofluorescent Localization of the Envelope Glycoproteins

In confluent monolayers of MDCK cells grown on glass coverslips, a tight junctional network seals the intercellular spaces near the apical surface of the cells. In such monolayers, therefore, only apically located antigens are accessible to antibodies applied without prior permeabilization. The basolateral surfaces of the cells become accessible to antibodies when the fixed monolayers are treated with detergents, a procedure that also allows labeling of intracellular structures. Such detergent treatment does not interfere with the visualization of antigen molecules located in the apical surface. In fluorescent micrographs of permeabilized monolayers, antigens present in the basolateral surface give rise to a polygonal pattern that is generated by the outlines of adjacent cells (9, 21, 23, 25, 28, 29, 32, 33). Using immunoelectron microscopy, previous studies (28, 29) have shown that the appearance of such a contour fluorescence pattern correlates with the arrival of the basolaterally located protein at the cell surface. Optimal visualization of apically and basolaterally located proteins is achieved when the plane of focus is placed at the surface of the monolayer or within the cell, respectively.

Cultures of transfected MDCK cells were plated at high density onto coverslips, and the resulting monolayers were examined 24 h later by indirect immunofluorescence. In cells expressing HA, which represented 2–3% of the total number of cells, surface immunofluorescence was readily apparent without the need of permeabilization with detergent (Fig. 4 *a*). The limits of the individual fluorescent areas corresponded to the borders of the apical surfaces of individual cells visualized by phase microscopy (Fig. 4, *b* and *d*). This indicated the presence of HA in the apical domains. The fluorescence had a punctate distribution that most likely reflected the presence of the glycoprotein in microvilli. After detergent treatment (Fig. 4 *c*), the basolateral domains were not significantly labeled,[2] and the fluorescence of the apical surface did not change substantially.

In cultures transfected with the vector that contained the G cDNA, some cells show weak apical labeling, detectable without detergent treatment (Fig. 5 *a*). However, when such cultures were permeabilized with detergent, the number of labeled cells was substantially higher (~twofold) and labeling of individual cells was more intense. More importantly, it was then apparent that large amounts of the G glycoprotein were concentrated on the lateral and basal surfaces of cells which demonstrated little or no apical labeling (Fig. 5, *b–d*). The coincidence of the contour fluorescent pattern of these cells with the cell periphery, was apparent when the same samples were examined by phase microscopy (Fig. 5, *e* and *f*). In contrast to the case of cells that expressed HA, which were labeled only in the apical surface, all cells that had apical G labeling also contained G protein on the basolateral plasma membranes.

[2] The absence of marked peripheral staining indicates that very little HA protein is located at the basolateral surface. When HA is in fact present on both surfaces, such as after treatment of infected cells with microtubule inhibitors (Rindler, M. J., unpublished results), the labeling of both surfaces is apparent using this immunofluorescence method.

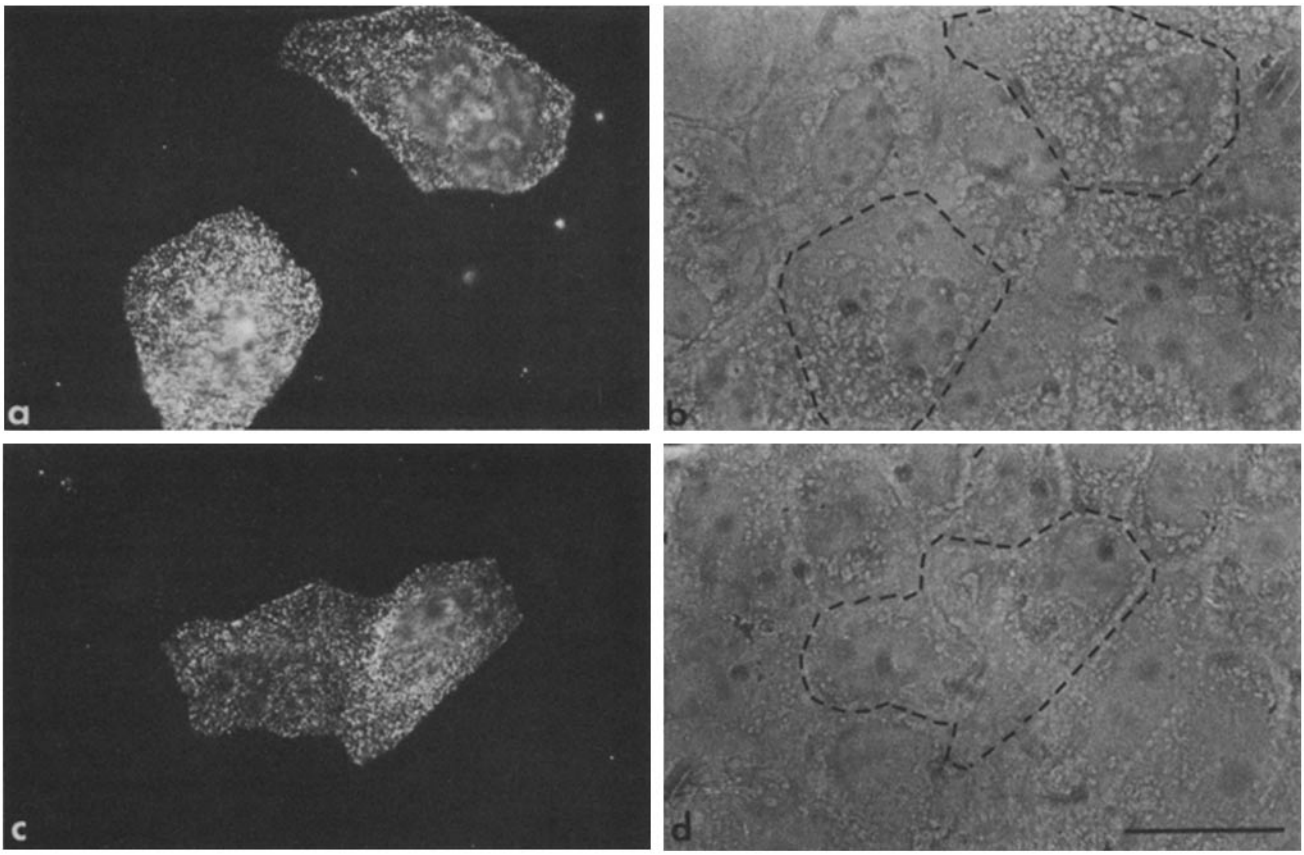

Figure 4. Segregation of HA in the apical surface of transfected MDCK cells. Confluent monolayers of cells transfected with pSV2PAHA were fixed and processed for immunofluorescence, with (*c*, and *d*) or without (*a* and *b*) previous treatment with Triton X-100 and examined with fluorescence (*a* and *c*) or phase optics (*b* and *d*) (*a*) The microscope was focused on portions of the apical surface of the labeled cells. The surface immunofluorescence of these cells, not treated with detergent, indicates the presence of HA in the apical domain. Note the typical punctate appearance that results from labeling of microvilli. (*c*) A group of these cells were labeled after permeabilization of the monolayer with Trition X-100, which allows access of the antibodies to the cell interior and intercellular spaces. The focus of the microscope was placed on the middle of one of the labeled cells. The basolateral surfaces are only very weakly labeled, as indicated by the absence of contour labeling. The fluorescence seen originates from the apical surface, which retains the pattern of HA labeling after the detergent treatment. (*b* and *d*) Phase micrographs demonstrating the completeness of the confluent monolayer in which the borders of the labeled cells in *a* and *c* are outlined. Bar, 10 μm.

A striking demonstration of the independent segregation of both viral envelope glycoproteins was obtained in cells transfected simultaneously with both genes. In this case the simultaneous detection of both proteins was possible using as primary antibodies a mouse monoclonal antibody against HA and a polyclonal rabbit antiserum to the G protein. These were then differentially localized with goat anti-mouse or anti-rabbit antibodies labeled with fluorescein or rhodamine. Many instances were found in which single cells expressed both proteins (Fig. 6). In such cells it was clear that the HA was restricted to the apical surfaces (Fig. 6*a*), which were labeled rather uniformly with the characteristic punctate pattern, whereas the immunofluorescence resulting from the G protein intensely marked the outline of the cells, which correspond to the lateral surfaces (Fig. 6*b*). These observations conclusively showed that the sorting and polarized delivery of the two envelope glycoproteins does not require other viral components.

Selective Endocytosis of G Protein

It is well known (5, 43) that some plasma membrane proteins, but not others, normally undergo a recycling process that involves their interiorization by an endocytic pathway and return to the cell surface. As previously noted, the selective recycling of G protein could account for its delayed appearance in the apical surface of VSV-infected cells and for its imperfect segregation even in transfected cells. The capacity of the two viral glycoproteins to be internalized by endocytosis was examined by immunofluorescence analysis of infected cells treated with chloroquine (Fig. 7). This lysosomotropic drug increases endosomal (22) and lysosomal pH (26), impairs lysosomal function (26), and prevents the return of internalized plasma membrane receptors to the cell surface (11, 12, 29). It was observed that the addition of chloroquine to MDCK cells infected with VSV leads to a marked decrease in the concentration of G molecules at the cell surface and to their concomitant concentration in intensely stained endosome- or lysosome-like cytoplasmic vesicles (c.f. Fig. 7, *a* and *c*). These structures were not visible in nonpermeabilized monolayers (Fig. 7*e*) or in monolayers in which tight junctions were disrupted with EGTA to render the basolateral cell surfaces accessible to the antibody probes (not shown). They were best delineated by focusing in the interior of the cell. On the other hand, chloroquine treatment of influenza-infected

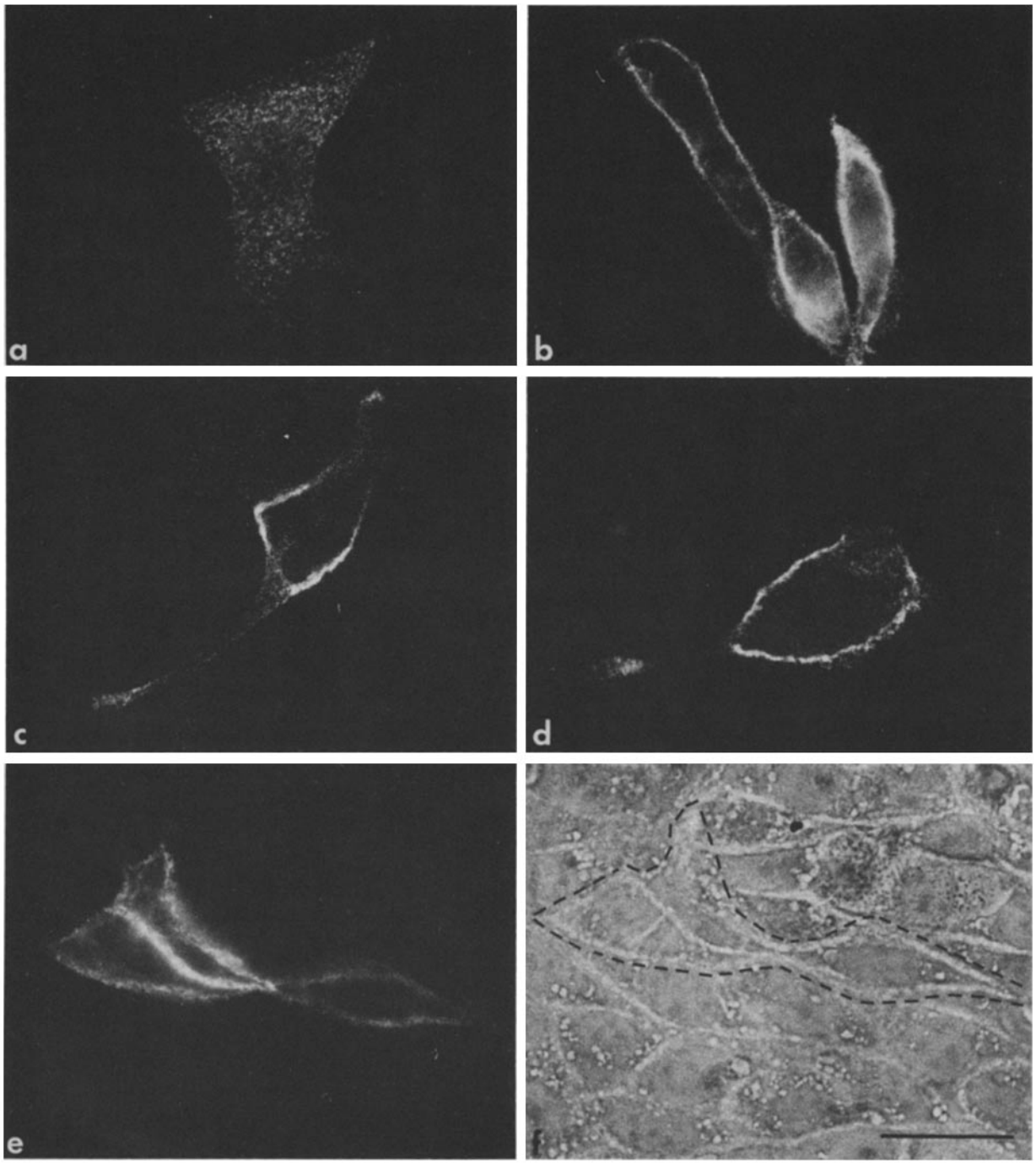

Figure 5. Surface localization of G protein in transfected MDCK cells. Coverslips of confluent monolayers of cells transfected with pSV2Gi$^-$ were fixed and processed for immunofluorescence with (*b–f*) or without (*a*) previous treatment with detergent. (*a*) A cell from a nonpermeabilized monolayer, showing weak labeling of the apical surface, where the plane of focus was placed. Note the typical punctate appearance. (*b–e*) Permeabilized monolayers, examined with the plane of focus in the middle of the cells. A fluorescent pattern of intensely labeled outlines is seen, which indicate a high concentration of G in the basolateral domains. The sodium butyrate treatment, used to enhance expression from the SV40 early promoter, affects the shape of the cells which tend to be elongated or somewhat irregular. (*f*) A phase micrograph demonstrating the completeness of the monolayer examined by fluorescence in *e*. The maintenance of the tight junctional network was verified by electrical resistance measurements. The contours of the fluorescent cells in *e* can be easily traced in this micrograph. Bar, 10 μm.

cells (c.f. Fig. 7, *b* and *d*) caused no accumulation of HA in endosome or lysosome-like vesicles nor did it lead to a substantial reduction in the apical surface immunofluorescence of the cells (Fig. 7*f*). In permeabilized influenza-infected cells treated with chloroquine, however, there was some anti-HA labeling of the cytoplasmic perinuclear region, which may correspond to the Golgi apparatus (Fig. 7*d*), as if chloroquine impaired transfer of the newly synthesized glycoprotein out of this organelle.

These results suggest that the VSV G protein, but not the HA, after its incorporation into the plasma membrane, can be internalized by endocytosis and therefore, in principle, can

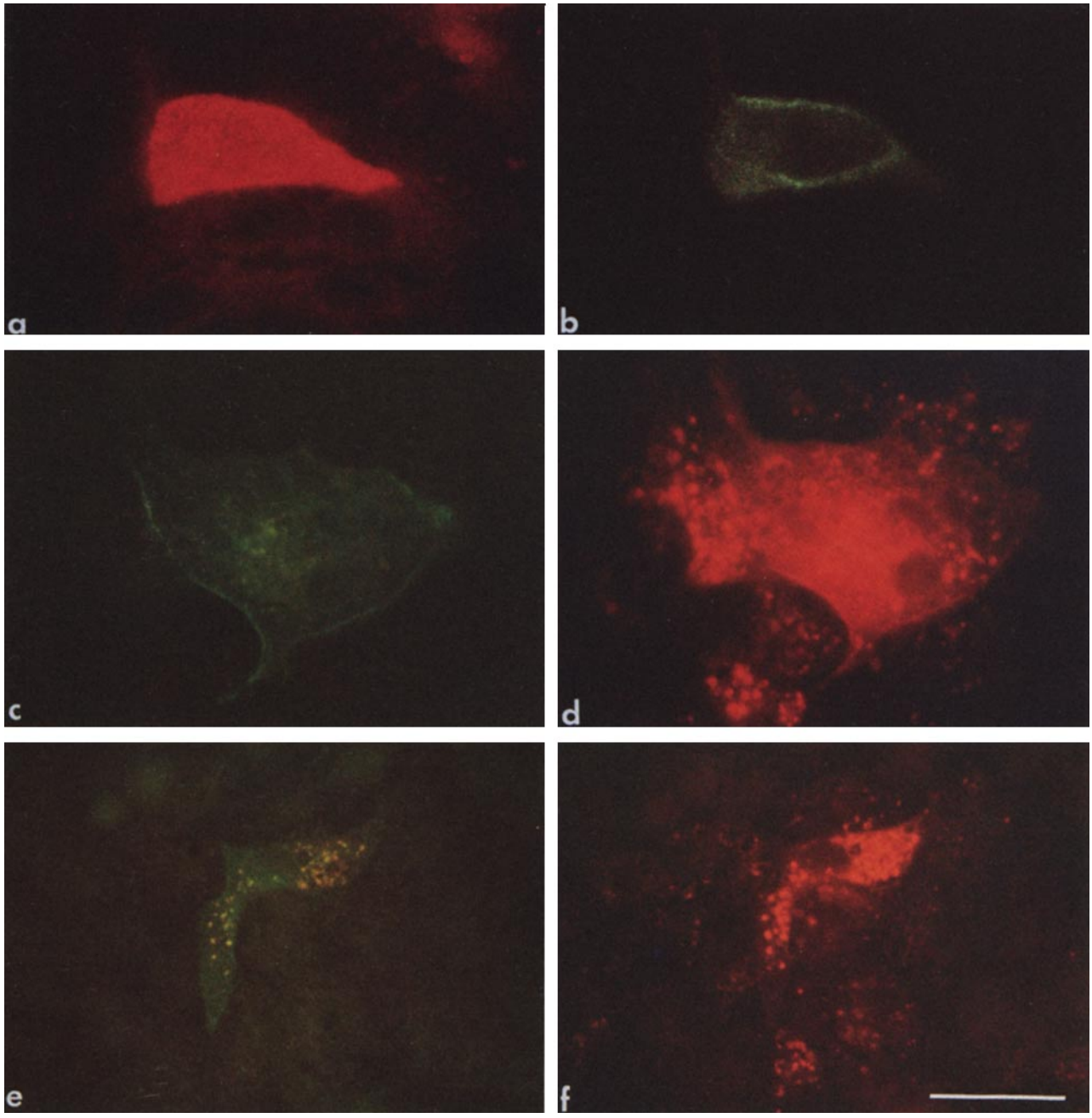

Figure 6. Simultaneous localization of HA and G in MDCK cells co-transfected with the plasmids pSV2PAHA and pSV2Gi⁻. After permeabilization with detergent and labeling with mouse anti-HA and rabbit anti-G antibodies, the distribution of the viral glycoproteins was visualized with secondary goat anti-mouse and anti-rabbit antibodies coupled to rhodamine or fluorescein. (*a* and *b*) In *a*, the plane of focus was placed on the apical surface (*a*) which in this instance has a triangular shape. The intense fluorescence of the HA (labeled with rhodamine) obscures the expected microvillar pattern. (*b*) The same cell was photographed to visualize the fluorescein-labeled G with the plane of focus placed approximately in the cell center. The G fluorescence marks the basolateral outline which does not correspond to the triangular shape of the apical surface seen in *a*. At this level of focus, fluorescence from the right and lower left corners of the triangle delineated in *a* is almost not detectable. (*c–f*) Distribution of HA and G in cells co-transfected with both genes which, 6 h after plating, were incubated overnight with 25 μM chloroquine. The micrographs were taken with the plane of focus placed at the middle of the cell. Whereas HA (labeled with fluorescein) remains on the apical surfaces (*c* and *e*), large amounts of G protein (labeled with rhodamine) are sequestered in intensely fluorescent cytoplasmic vesicles (*d* and *f*). In *e*, the rhodamine fluorescence of some of the vesicles is visible as yellow dots through the fluorescence filter. Some cells in the fields shown only express the G protein. These account for the rhodamine labeled vesicles which lie outside the profile of the double transfected cells in *d* and *f*. Bar, 10 μm.

undergo recycling through the cytoplasm. To determine whether the interiorization of the G protein requires the participation of other viral components, the effect of chloroquine on the distribution of G was examined in cells transfected with the appropriate expression vector. Prolonged chloroquine treatment nearly completely depleted the cell surface of G molecules and led to a striking accumulation of G in cytoplasmic vesicles similar to those found in chloroquine-

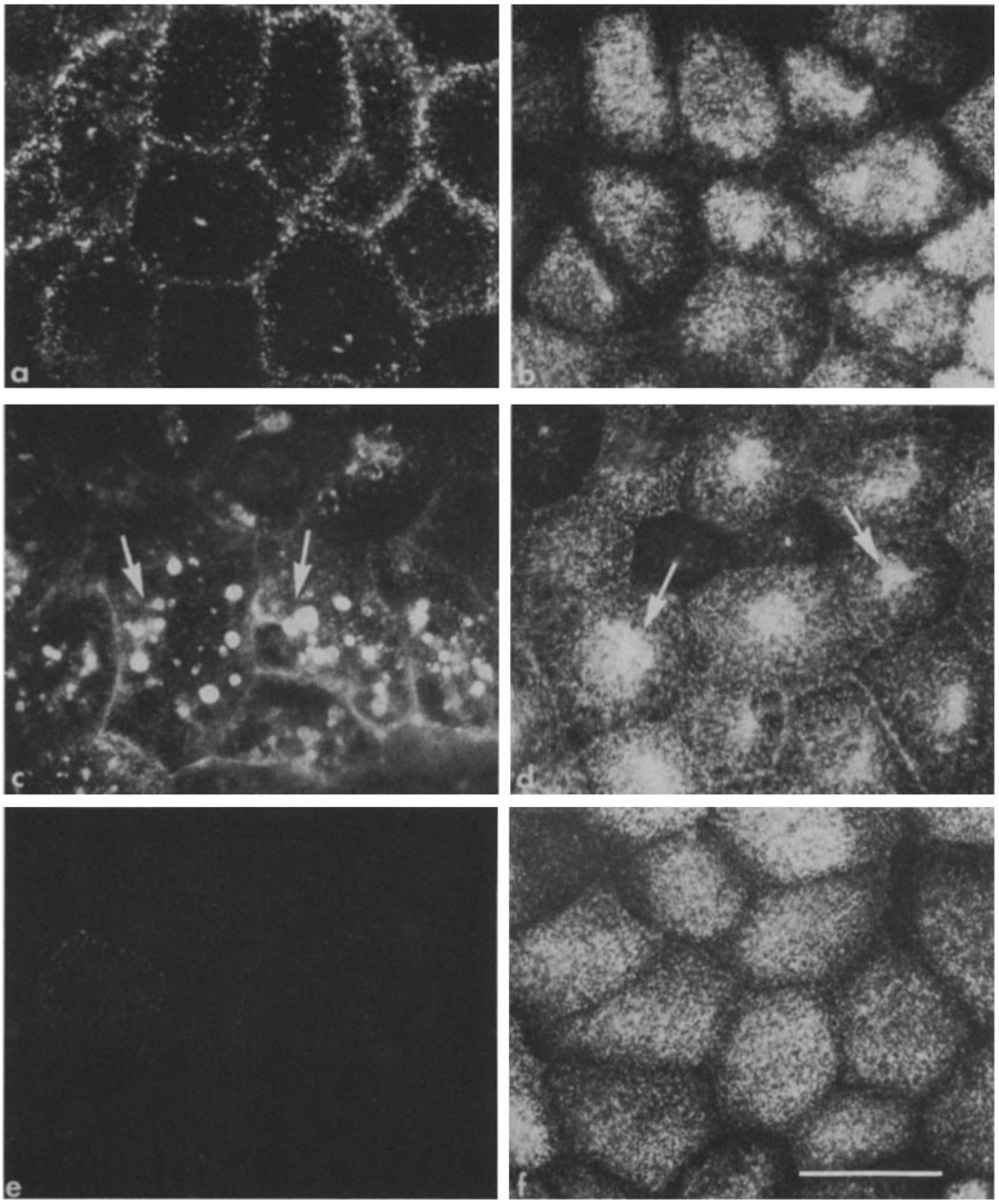

Figure 7. The effect of chloroquine on the distribution of G and HA in virally infected MDCK cells. Confluent monolayers grown on coverslips were incubated (moi = 10) with either VSV (*a*, *c*, and *e*) or influenza A/WSN virus (*b*, *d*, and *f*) for 1.5 h. The inoculum was removed and replaced with medium with (*c* and *d*) or without (*a* and *b*) chloroquine 25 μM. After additional incubation for 4 h (VSV) or 5 h (WSN), monolayers were fixed and examined by immunofluorescence with (*a–d*) or without (*e* and *f*) prior permeabilization with 0.2% Triton X-100. In *a*, *c*, and *d*, the plane of focus was placed in the middle of the cell to visualize the basolateral domain and intracellular structures. In *b*, *e*, and *f* the focal plane was at the apical surface. Typical polygonal (basolateral) and surface punctate (apical) fluorescent patterns of G and HA glycoproteins, are seen respectively in *a* and *b*. (*c* and *d*) After incubation with chloroquine, a decrease in surface fluorescence, accompanied by the appearance of large fluorescent vesicles in the cytoplasm, is observed in VSV-infected (*c*), but not in influenza-infected (*d*) cells. In the influenza-infected cells (*d*), some HA appears to be concentrated intracellularly in a fluorescent juxtanuclear region. (*e*) No labeling is obtained when the anti-G antibodies are applied to a chloroquine-treated monolayer not permeabilized with detergent. (*f*) After treatment with chloroquine the intense apical surface of HA is still observed. Bar, 5 μm.

treated infected cells (Fig. 8, *a–d*). On the other hand, the apical plasma membrane location of the HA of influenza, expressed in transfected cells, was not affected by chloroquine treatment (Fig. 8 *e*), which in this case was done in the presence of cycloheximide to prevent any intracellular accumulation of newly synthesized HA (Fig. 7 *d*). The differential behavior of the two viral glycoproteins was also dramatically demonstrated in doubly transfected cells that expressed both genes and were treated with chloroquine (Fig. 6, *c–f*). Measurements of the levels of the G and HA glycoproteins accumulated in transfected control and chloroquine-treated cells showed that the chloroquine treatment, which inhibits lysosome function, led to a greater than twofold increase in the levels of radioactively labeled G protein (Fig. 3). On the other hand, the amount of labeled HA was not increased. This suggests that normally, after endocytosis, at least a fraction of

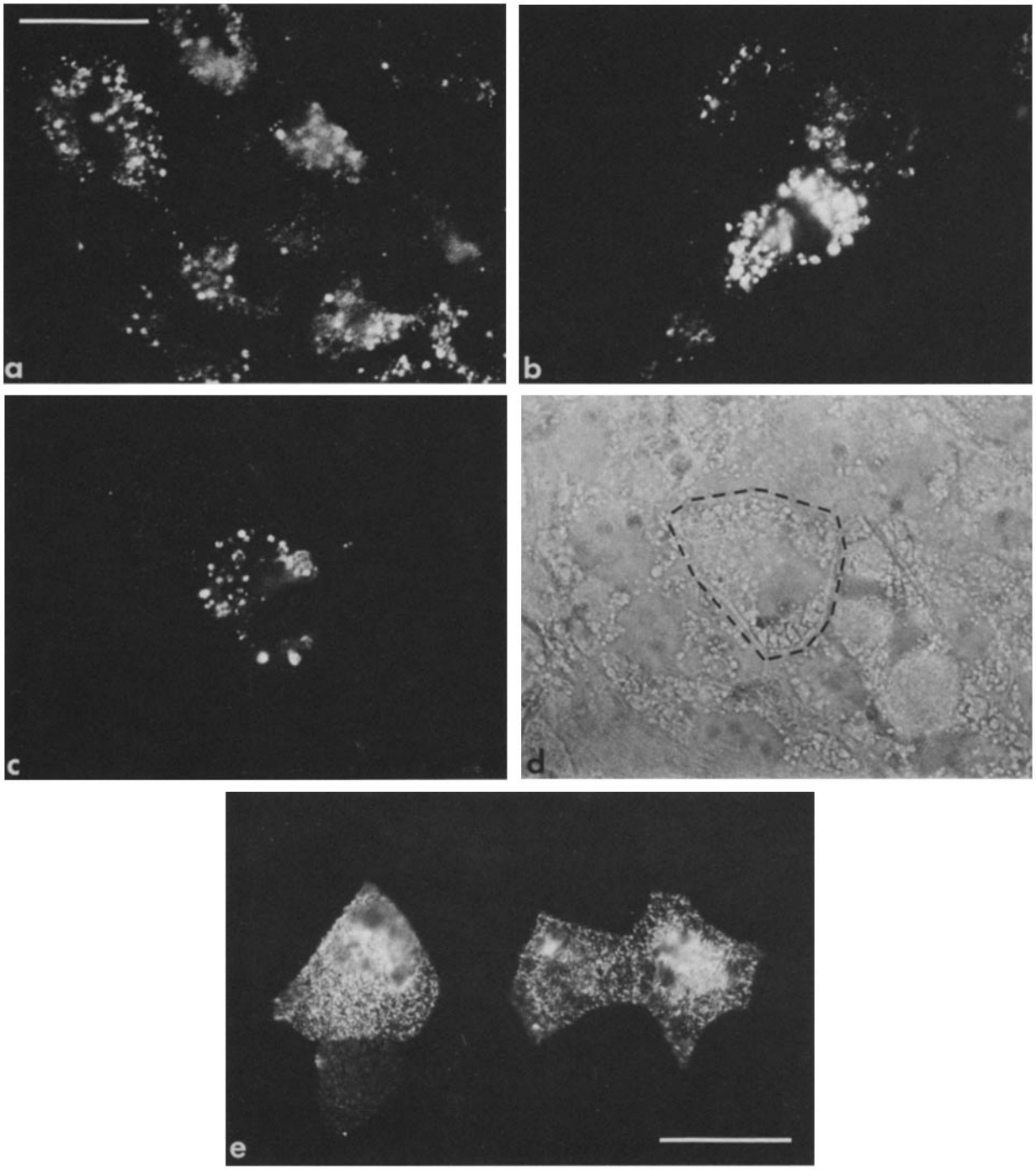

Figure 8. Selective sequestration of the G protein in cytoplasmic vesicles of transfected cells after treatment with chloroquine. 6 h after plating, confluent monolayers of MDCK cells transfected with pSV2Gi$^-$ (*a–d*) were incubated overnight with 25 μM chloroquine. Cells were fixed, permeabilized with detergent, and examined by immunofluorescence (*a*, *b*, or *c*) or phase microscopy (*d*). (*a* and *b*) Groups of transfected cells in which almost all the G protein has been internalized and trapped within cytoplasmic vesicles. (*c* and *d*) A similarly treated monolayer examined by fluorescence (*c*) and phase microscopy (*d*). The nonfluorescent outline of the cell in *c* can be traced in *d*. (*e*) A monolayer of cells transfected with pSV2PAHA was incubated for 6 h with both chloroquine (50 μM) and cycloheximide (5 μg/ml) and processed for immunofluorescence after permeabilization with detergent. The apical fluorescence of HA remains intense and there is no apparent intracellular accumulation of the glycoprotein. In all cases the plane of focus was placed approximately at the middle of the cells. Bar (*a*), 12 μm. Bar in *e* (for *b–e*), 7 μm.

the G protein molecules reaches the lysosomal compartment and undergoes proteolytic degradation. The level of labeling of the proteins was too low to permit a pulse-chase experiment that could have provided definitive evidence that chloroquine inhibits the degradation of the G protein.

In principle, the accumulation of G protein in cytoplasmic vesicles may result from interiorization of surface molecules, or from impaired delivery to the cell surface of G molecules synthesized during the chloroquine treatment (45). These possibilities were examined in transfected cultures that re-

ceived the protein synthesis inhibitor cycloheximide before (45 min) the addition of chloroquine, and were incubated for several hours in the presence of both drugs. We have previously shown (14) that at the concentration used here, cycloheximide reduces protein synthesis in MDCK cells by more than 90% within 3 min of its addition. In these experiments it was clear that cycloheximide had no effect on the chloroquine-induced surface depletion and the concomitant intra-

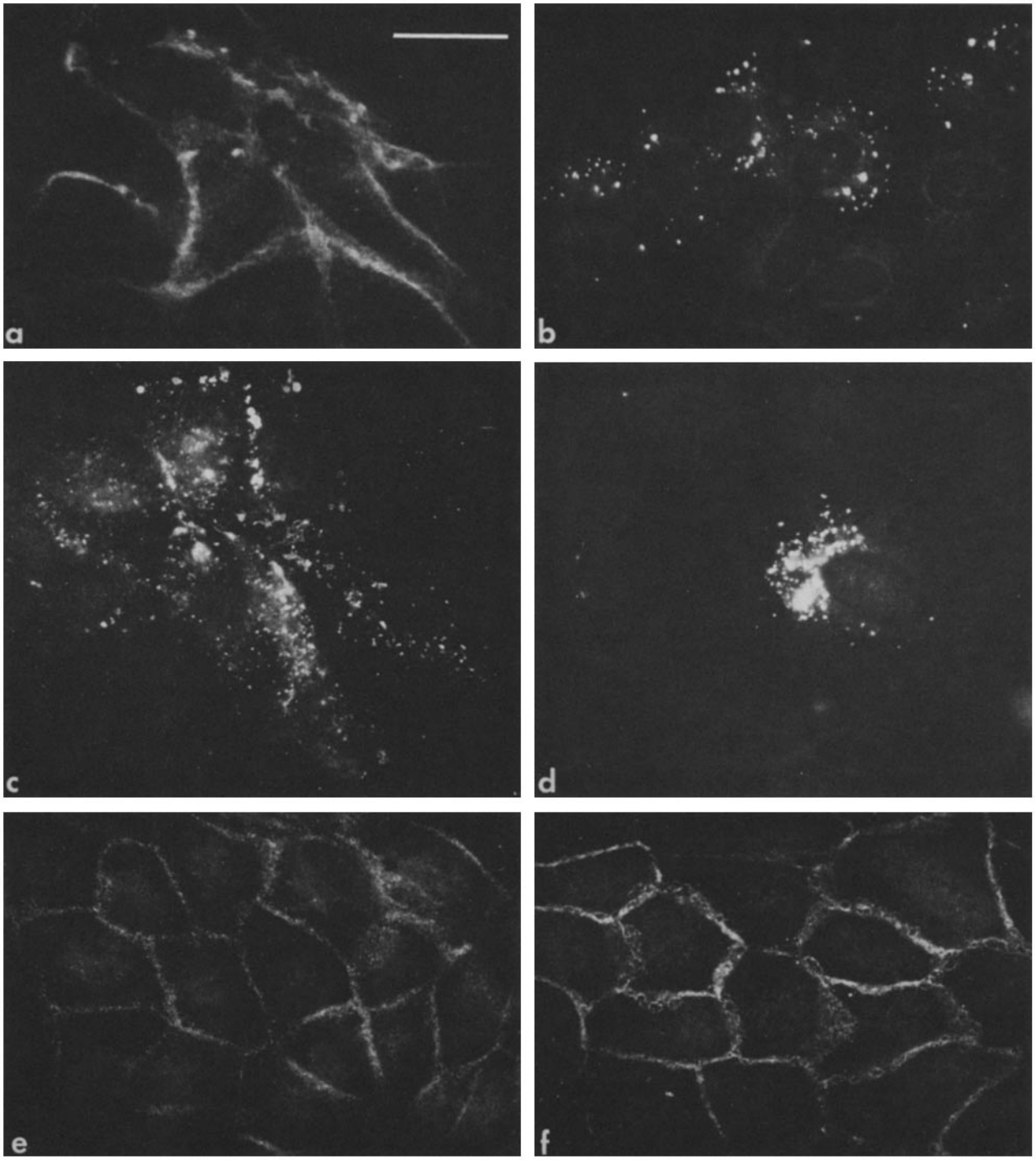

Figure 9. Preexisting surface-associated G molecules, rather than newly synthesized ones, accumulate in cytoplasmic vesicles in the presence of chloroquine. pSV2Gi$^-$ transfected cells, plated on coverslips and incubated overnight, were treated with cycloheximide (5 μg/ml) for 45 min (*a–d*). At this time, chloroquine (50 μM) was added to some of the cultures (*b*, *c*, and *d*), and incubation was continued for 1 h (*b*) or 5 h (*a*, *c*, and *d*). (*a*) In cells incubated with cycloheximide alone for 5 h, G protein remains segregated on the basolateral surfaces. (*b*, *c*, and *d*) After 1 h (*b*) or 5 h (*c* and *d*) in medium containing chloroquine and cycloheximide, most cells have internalized large amounts of their surface G protein into cytoplasmic vesicles. (*d*) In this cell, vesicles containing the interiorized G protein have accumulated in the perinuclear region. (*e* and *f*) Nontransfected confluent monolayers were incubated for 6 h with cycloheximide (5 μg/ml) in the presence (*e*) or absence (*f*) of chloroquine (50 μM) and then examined by immunofluorescence with anti-ATPase antibodies. In all cases cells were permeabilized with detergent, and the plane of focus was placed at the middle of the cell. Bar, 7 μm.

cellular accumulation of G protein (Fig. 9). Thus, complete depletion of the cell surface pool of G and intracellular accumulation of this protein was observed in many cells after 1 h (Fig. 9 *b*), and in almost 70% of transfected cells after 2 h of chloroquine addition. By 5 h (Fig. 9, *c* and *d*) all labeled cells contained G only in intracellular endosome- or lysosome-like vesicles. When cells were treated with cycloheximide alone for several hours, G surface immunofluorescence was perhaps only slightly reduced, but no intracellular accumulation of G was observed (Fig. 9 *a*). This suggests that, normally, the surface G protein does not undergo rapid degradative turnover and that the G protein molecules, which in the presence of chloroquine accumulated in the cytoplasmic vesicles, were those removed by endocytosis from the cell surface. Since cycloheximide did not prevent endocytosis and chloroquine does not increase rates of endocytosis (11), it is possible to conclude that the sustained cell surface levels of G observed in cycloheximide-treated cells reflect a steady state situation in which internalization of the protein is balanced by its efficient return to the cell surface.

To determine if the differential internalization of the two viral envelope proteins was dependent on their segregation to the different cell surfaces, the effect of chloroquine was also examined in nonpolarized CV-1 cells transfected with vectors carrying either the HA or G cDNA. In this case, as expected, both proteins appeared to be homogeneously distributed throughout the entire cell surface (Fig. 10, *a* and *b*). After chloroquine treatment, only the G protein was internalized into the cytoplasmic vesicles (c.f. Fig. 10, *c* and *d*). The structures containing G could not be labeled without previous permeabilization of the cells. Since these cells lack a network of tight junctions and all aspects of the cell surface should be easily accessible to the antibodies even without prior detergent treatment, this finding indicates that the structures that contain G are truly intracellular. The fact that the G protein becomes internalized in both polarized and nonpolarized cells demonstrates that its capacity to be internalized is a property of the molecule itself. The possibility was considered that the capacity to be interiorized is associated with all proteins that in polarized cells become segregated to the basolateral surface. This was shown not to be the case since the basolateral distribution of the Na^+, K^+ ATPase in MDCK cells was not altered by chloroquine treatment (c.f. Fig. 9, *e* and *f*).

Discussion

The experiments presented in this paper examine the capacity of two model plasma membrane proteins of viral origin, synthesized in the absence of other viral components, to be sorted to the different plasma membrane domains of polarized epithelial cells. It was found that both the VSV G and influenza HA glycoproteins produced in confluent monolayers of MDCK cells transfected with the appropriate genes, were segregated into the same plasma membrane domains to which

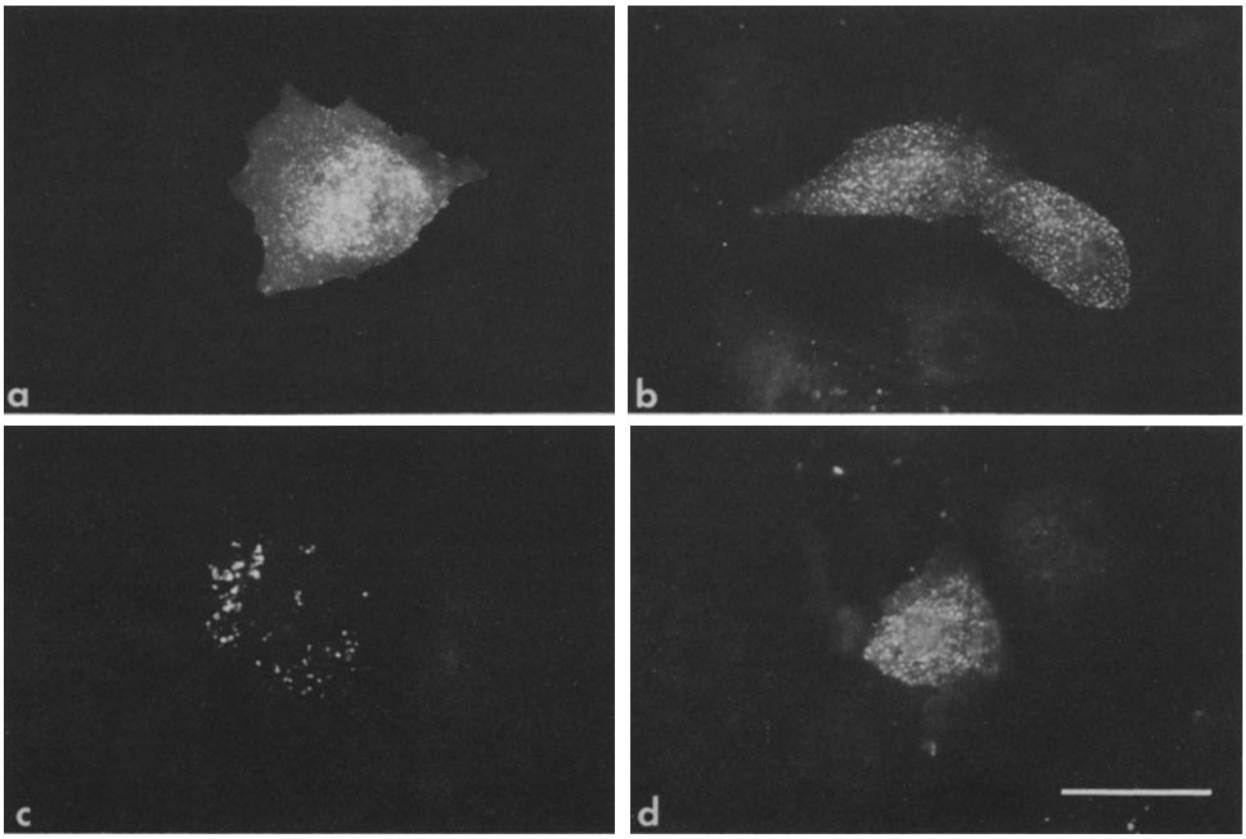

Figure 10. Differential effect of chloroquine on the distribution of HA and G in transfected nonpolarized cells. (*a–d*) CV-1 cells were transfected with the appropriate plasmids, as described in Materials and Methods. 36 h posttransfection, 50 μM chloroquine was added to two samples (*c* and *d*). After 6 h of further incubation, the cells were fixed, permeabilized, and processed for immunofluorescence with anti-G (*a* and *c*) or anti-HA (*b* and *d*) antibodies. In control transfected cells (*a* and *b*), both the G protein (*a*) and HA (*b*) are homogeneously distributed throughout the cell surface. After chloroquine treatment, the G protein was sequestered in cytoplasmic vesicles (*c*), whereas the distribution of HA was unaffected (*d*). Bar, 10 μm.

they are restricted in infected cells, and that this segregation even took place within single cells that expressed both genes. This clearly demonstrates that interaction of each glycoprotein with the matrix protein or the nucleocapsid of the corresponding virus is not required for its segregation, and therefore that the two polypeptides are recognized directly by the cell sorting machinery. For the HA protein, a similar conclusion has been reached (36) from studies with primary African green monkey kidney cell cultures that were infected with SV40 recombinant viruses that contained the HA cDNA.

Using transfected cells, we observed that whereas HA was essentially restricted to the apical surface, the segregation of the G protein was not as strict. Thus, small amounts of G were frequently present in the apical surface, where it acquired a punctate microvilli-associated distribution similar to that of the HA. The imperfect segregation of G in transfected cells is in accordance with our previous finding with infected cells (28, 29), in which it was possible to demonstrate that G first appeared in basolateral surfaces and only considerably later in apical ones, where budding of VSV, however, did not take place. Previous work from this and other laboratories has shown that the localization of the viral envelope glycoproteins in either the apical or basolateral surfaces of infected polarized epithelial cells results from polarized delivery of the newly synthesized polypeptides to each surface domain (20, 23, 28, 29, 32). The appearance of some G protein molecules in the apical surface of transfected cells, in which cytopathic effects of virus infection are eliminated, is therefore likely to reflect a redistribution that takes place after initial incorporation of the polypeptide in the basolateral domain, rather than a breakdown in the process of polarized delivery. This redistribution could, in principle, involve recycling of the viral glycoprotein through the cytoplasm, similar to that which affects many cellular plasma membrane proteins (5, 8, 43) and is required for transcytotic transport in epithelia (17, 31, 44). In fact, Matlin et al. (20) and Pesonen and Simons (25) have shown that when G protein is inserted into the apical domain of MDCK cells by artificial fusion of viral envelopes with the free surfaces of confluent monolayers, a substantial fraction of the molecules undergoes transcytosis through an endosome compartment and is brought to the basolateral surface. Taken together, these observations suggested that the G protein would provide a paradigm for studying the structural features of plasma membrane proteins responsible for their recycling properties.

The capacity of cells to internalize portions of their plasma membrane, and to return specific proteins to the cell surface plays an essential role in many cell physiological processes. The rates of interiorization of individual plasma membrane proteins seem, however, to vary widely. Some proteins, such as the low density lipoprotein receptor, which functions in the uptake of cholesterol for cellular nutrition, appear to recycle continuously through the cytoplasm at high rates, even when not associated with their ligands (2, 5). In contrast to these migrant proteins, other proteins of the plasma membrane, which have been designated resident (5), remain in the cell surface for long periods of time. The rapid interiorization of proteins in the first group appears to require their clustering within coated pits (2), from which resident proteins would be excluded (4). The experiments presented in this paper suggest that the VSV G and HA glycoproteins, respectively, may serve as models for the two classes of plasma membrane proteins, which recycle at high and very low rates. Thus, in both virally infected and transfected cells, chloroquine treatment, which is not known to induce endocytosis (11) but only to block the return to the cell surface of normally interiorized molecules (12), led to a loss of surface G protein molecules that was paralleled by their appearance in large cytoplasmic vesicles. Under the same conditions, and even in the same cells, the amount of HA in the cell surface did not decline significantly and, more importantly, HA did not accumulate in cytoplasmic vesicles. The G protein, which accumulated intracellularly, was clearly derived from the pool of molecules originally contained in the plasma membrane and did not result from a chloroquine-induced arrest of the transport of newly synthesized polypeptides to the cell surface. This can be concluded from the finding that the accumulation of G in cytoplasmic vesicles was not prevented by the presence of cycloheximide during the incubation with chloroquine. The fact that treatment with cycloheximide alone did not lead to depletion of the surface pool of G molecules, whereas simultaneous treatment with both drugs or chloroquine alone did, implies that interiorized G protein molecules normally return to the cell surface.

Chloroquine and other amines are thought to interfere with recycling of plasma membrane proteins by changing the pH of the endosomal compartment (22, 26). A similar effect on the endosomal pH may be exerted by ionophores, such as monensin, which has also been shown to interfere with the transcytotic transport of G protein artifically implanted in the apical surface (25). Indeed, the finding that monensin reduces the level of VSV G that appears on the surface of infected MDCK cells, without a similar effect on the influenza HA (1) may, at least in part, reflect the fact that the ionophore leads to a selective depletion of surface-associated G protein molecules and their entrapment in endocytic vesicles.

The physiological significance of the recycling of G and of the lack of interiorization of HA remains to be elucidated. The selective interiorization of the G protein did not depend on its initial segregation in the basolateral surfaces, since it also occurred in nonpolarized cells, such as CV1, in which both viral glycoproteins were intermingled throughout the cell surface. In addition, the capacity of G to recycle does not represent a common feature of all those proteins which in polarized epithelial cells normally reside in the basolateral surface. Thus, it was also shown that the Na^+, K^+ ATPase, the ion pump responsible for maintaining the intracellular monovalent cation concentration and for driving the ion and water transport activity of epithelia, which is restricted to the basolateral domain, does not accumulate in endocytic vesicles of MDCK cells after treatment with chloroquine.

The previous observation (29) that in infected cells VSV virions did not bud from apical surfaces that contained significant amounts of the G protein, should be considered since such molecules possibly originated from the basolateral domain and reached the apical surface via the recycling pathway. Passage through an acidic endosomal compartment could have caused a conformational change that rendered the G molecules incapable of incorporation into virions. In fact it is known that the G protein can mediate a pH-dependent membrane fusion, which suggests that its structure is altered in an acidic medium (27, 42). In addition, we observed earlier that when in intact infected monolayers, the apical G protein was iodinated in situ by application of lactoperoxidase coupled to

Sepharose beads, most of the labeled G molecules were subsequently degraded, with only a small fraction recovered in virions released into the medium (29).

Although the biological significance, if any, of the appearance of G in the apical surface is not clear, it is possible that it reflects the imperfect polarization in MDCK cells within monolayers that become confluent on impermeable glass surfaces. In this case, in contrast to the situation of cells within natural epithelia that receive nutrients and hormonal signals from the basolateral domain, the cells have access to nutrients only from the apical surface. As previously suggested (9), cell survival under these culture conditions may require the transfer of, at least some, basolateral proteins to the apical surface. This would be the case, for example, for receptor proteins that mediate the uptake of nutrients from the medium and, in carrying out their function, normally recycle through an endosomal compartment. One may therefore speculate that the capacity of the G protein to recycle reflects its evolutionary origin from an ancestral cellular plasma membrane receptor or transport protein. It should also be noted that although under certain conditions recycling of the G protein through the cytoplasm is likely to provide a mechanism for the transfer of this protein to the apical surface, the recycling itself is expected to take place continuously, regardless of the state of polarization of the cell. Thus, in highly polarized cells, recycling should not perturb the surface distribution of the G protein. Indeed, recently, Fuller et al. (9) have studied MDCK cells grown on permeable supports, which therefore have access to nutrients from their basolateral surfaces, and have shown that under these conditions the G protein synthesized during VSV infection is stringently restricted to the basolateral surface domain.

The features of plasma membrane proteins that determine the extent of their interiorization and lead to their intracellular degradation or to their return to the cell surface have not yet been identified. In a recent paper (30), we demonstrate that a chimeric polypeptide that contains the nearly entire growth hormone sequence, linked at its C-terminus to a carboxy terminal 60 amino acid segment of the VSV G, also accumulates in endosome-like vesicles upon treatment of transfected cells with chloroquine. This indicates that information for the interiorization of G is contained in that segment, which includes the transmembrane and cytoplasmic portions of the polypeptide and only 11 amino acids of its luminal domain.

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