

Viral Glycoproteins Destined for Apical or Basolateral Plasma Membrane Domains Traverse the Same Golgi Apparatus during Their Intracellular Transport in Doubly Infected Madin-Darby Canine Kidney Cells

MICHAEL J. RINDLER, IVAN EMANUILOV IVANOV, HEIDE PLESKEN, ENRIQUE RODRIGUEZ-BOULAN,* and DAVID D. SABATINI

*Department of Cell Biology, New York University Medical Center, New York 10016; and *Department of Pathology, Downstate Medical Center, Brooklyn, New York 11203*

ABSTRACT Madin-Darby canine kidney (MDCK) cells can sustain double infection with pairs of viruses of opposite budding polarity (simian virus 5 [SV5] and vesicular stomatitis virus [VSV] or influenza and VSV), and we observed that in such cells the envelope glycoproteins of the two viruses are synthesized simultaneously and assembled into virions at their characteristic sites. Influenza and SV5 budded exclusively from the apical plasma membrane of the cells, while VSV emerged only from the basolateral surfaces. Immunoelectron microscopic examination of doubly infected MDCK cells showed that the influenza hemagglutinin (HA) and the VSV G glycoproteins traverse the same Golgi apparatus and even the same Golgi cisternae. This indicates that the pathways of the two proteins towards the plasma membrane do not diverge before passage through the Golgi apparatus and therefore that critical sorting steps must take place during or after passage of the glycoproteins through this organelle. After its passage through the Golgi, the HA accumulated primarily at the apical membrane, where influenza virion assembly occurred. A small fraction of HA did, however, appear on the lateral surface and was incorporated into the envelope of budding VSV virions. Although predominantly found on the basolateral surface, significant amounts of G protein were observed on the apical plasma membrane well before disruption of the tight junctions was detectable. Nevertheless, assembly of VSV virions was restricted to the basolateral domain and in doubly infected cells the G protein was only infrequently incorporated into the envelope of budding influenza virions. These observations indicate that the site of VSV budding is not determined exclusively by the presence of G polypeptides. Therefore, it is likely that, at least for VSV, other cellular or viral components are responsible for the selection of the appropriate budding domain.

Transporting epithelial cells serve as selective permeability barriers between different physiological compartments. The functional polarization of cells with such epithelia is reflected in the existence of morphologically distinct apical and basolateral plasma membrane domains that contain characteristic enzymatic and transport activities (cf. 20, 25). Little is known about the biogenetic mechanisms that ensure that newly synthesized proteins specific for these domains achieve the specific distribution necessary for the function of polarized

cells. In light of current models for organelle and membrane biogenesis (cf. 44), it is reasonable to propose that these proteins possess addressing signals that determine their intracellular itinerary and ultimate destination. The capacity of plasma membrane proteins of epithelial cells to return to their correct site of function in the course of membrane recycling (26) suggests that such signals are not transient features of the polypeptides but are retained in mature proteins.

Virally infected cultured epithelial cells provide a conven-

ient model system to study sorting-out processes that effect the distribution of plasma membrane proteins to apical or basolateral domains in polarized cells (38). When monolayers of Madin-Darby canine kidney (MDCK)¹ or Madin-Darby bovine kidney (MDBK) cells are infected with enveloped viruses, virions assemble only on specific domains of the plasma membrane. Influenza, sendai, or simian virus 5 (SV5) bud exclusively from the apical surface of the cells, whereas vesicular stomatitis virus (VSV) particles assemble only on basolateral domains. Because polarized budding is preceded by an accumulation of viral envelope proteins in the corresponding plasma membrane domains, it has been suggested (37) that the polarized distribution of envelope proteins is the main determinant of asymmetric budding.

It is widely presumed that viral glycoproteins reach their destination via the same biogenetic pathways used by cellular membrane proteins. These glycoproteins are known to be synthesized on membrane-bound ribosomes and inserted cotranslationally into the endoplasmic reticulum (ER) membranes (12, 18, 19, 22). During their synthesis in the ER and subsequent transfer through the Golgi apparatus to the plasma membrane, the polypeptides undergo several co- and post-translational modifications, such as proteolytic cleavage (47), glycosylation (1, 16, 31, 32), and addition of fatty acids (46), which are also characteristic of cellular proteins.

In this study we compared, in MDCK cells doubly infected with viruses of opposite budding polarity, the intracellular pathways followed by the different viral glycoproteins, from the ER to the apical or basolateral surfaces. Immunocytochemical detection of the viral antigens in ultrathin frozen sections demonstrated that proteins destined to the different plasma membrane domains share a common pathway that includes passage through the same Golgi apparatus. Thus, the necessary sorting steps cannot take place before traversal of this organelle. Preliminary accounts of this work have been presented at meetings (34, 35).

MATERIALS AND METHODS

Cell Culture: Cultures of MDCK (originally obtained from Dr. J. Leighton [Medical College of Pennsylvania]), MDBK (obtained from Drs. R. Krug and P. R. Etkind [both of Memorial Sloan Kettering Cancer Center]) and Vero (ATCC) cells were maintained in plastic tissue culture flasks (Falcon Labware, Oxnard, CA) using minimum essential medium (MEM) supplemented with fetal bovine serum (Gibco Laboratories, Inc., Grand Island, NY), 10% for MDCK and MDBK and 5% for Vero. For experimental work, cells (1.5×10^5) were plated on 35-mm dishes (Falcon Labware). Infection with viruses was always performed at least 3 d past confluence, at which point monolayers were more resistant to the cytopathic effects of viral infection than those which had just reached confluence. For studies involving ultrathin frozen sectioning, MDCK cells (1.5×10^6) were plated in 35-mm dishes containing 25-mm glass coverslips (Fisher Scientific Co., Pittsburgh, PA) coated with rat-tail collagen. For immunofluorescent studies, cells were plated onto 13-mm coverslips (SGA Scientific, Inc., Bloomfield, NJ) in a 24-well dish (4×10^4 cells/well). Electrical resistance measurements were performed on monolayers of cells that were plated on collagen coated nylon disks (5).

Virus Stocks and Infection Protocols: SV5 (originally obtained from Dr. P. Choppin [Rockefeller University]) was plaqued, grown, and titered

on MDBK or MDCK cells, and influenza WSN (provided by Dr. A. Gregoriades [Public Health Research Institute of the City of New York]) on MDCK. VSV (Indiana serotype, from J. Vilcek [New York University Medical Center]) was plaqued and grown on Vero cells and titered on MDCK. The methods used have already been described (38).

Monolayers were infected by incubation with the virus in a small volume of MEM for 1 h at 37°C with frequent shaking. When VSV was used, 75 µg/ml of DEAE dextran (Pharmacia Fine Chemicals, Piscataway, NJ) was also included. After incubation the medium was removed and replaced with an appropriate volume of MEM (containing 2% fetal bovine serum for the SV5 infection).

Successful double infections of MDCK monolayers required adherence to a strict protocol. For SV5 and VSV, it was found that the optimal time for superinfection with VSV was 24 to 42 h after SV5 inoculation. At that time, the medium was removed and a typical infection with VSV was conducted. For influenza and VSV double infections, it was determined that unless influenza was added at least 3 h before VSV little production of influenza proteins could be detected by immunofluorescence or by pulse labeling. If, however, the influenza infection was allowed to proceed for 6 h or more the subsequent infection by VSV was severely curtailed. Optimal results were obtained when VSV infection was carried out 4.5 h after inoculation by influenza. Similar observations were made by Roth and Compans (41) in their studies of phenotypic mixing in doubly infected MDCK.

Antibody Preparations: Rabbit anti-SV5 antiserum was obtained from Microbiological Associates. It was tested by immunofluorescence and shown to react only with the apical membrane of SV5-infected MDCK and not with uninfected cells. An anti-WSN antibody was prepared in rabbits against egg-grown virus and this antibody was specific for influenza infected cells as assayed by immunofluorescence microscopy. Mouse monoclonal antibodies against the hemagglutinin (HA) protein of WSN were a gift of Dr. R. G. Webster, St. Jude Children's Hospital (Memphis, TN). Each of the two different monoclonal antibodies employed reacted strongly with the plasma membrane of influenza infected cells, as determined by immunofluorescence microscopy and ultrathin frozen section immunocytochemistry, but did not react with uninfected cells or VSV-infected cells. Anti-VSV G protein antibody was prepared as described elsewhere (37). This neutralizing antibody specifically immunoprecipitated only the G protein from radiolabeled VSV-infected MDCK cells and showed no reaction with any other viral or cellular proteins. Immunofluorescence and immunocytochemical criteria demonstrated its specificity for VSV-infected cells.

All other antibody preparations were obtained from commercial sources. Rhodamine-conjugated goat anti-rabbit IgG and goat anti-mouse IgG from Cappel Laboratories (Cochranville, PA) and affinity-purified antibodies from Tago Scientific (Burlingame, CA). Goat serum was purchased from Pocono Rabbit Farm and an IgG fraction was prepared by ammonium sulfate fractionation followed by DEAE-cellulose column chromatography.

[³⁵S]Methionine Pulse Labeling: MDCK monolayers infected with the appropriate viruses were transferred to and maintained for 30 min in a 10:1 mixture of methionine-free MEM (made from a Gibco kit) before a 30-min incubation with 0.5 ml of the same medium containing ~25 µCi of [³⁵S]methionine (New England Nuclear, Boston, MA). After labeling cells were removed by scraping, solubilized in detergent (0.5% Nonidet P-40, 0.5% deoxycholate in a hypotonic Tris-HCl buffer) and processed as described by Green et al (11). Samples were analyzed by PAGE on 10% gels followed by fluorography (3) using Kodak XR-5 or Dupont Cronex film.

Immunofluorescence Microscopy: Infected MDCK monolayers on 13-mm coverslips were fixed for 30 min with 4% paraformaldehyde in phosphate-buffered saline (PBS) plus Ca⁺⁺ (0.1 mM), washed and stained for immunofluorescence as described elsewhere (11). Observations were carried out with a Leitz Orthoplan immunofluorescence microscope equipped with a Wild camera.

Preparation of Colloidal Gold: Colloidal gold particles were prepared by the citrate method (18-nm particles) of Horisberger and Rosset (14) and by the white phosphorus technique (5-nm particles) of Frens (8), as outlined Geuze et al. (9) using tetrachloroauric acid (Kodak). Three times the minimal amount of antibody (stored in a borate buffer, pH 8.0) necessary to stabilize a given amount of colloidal gold was diluted in 2 ml of distilled H₂O and the gold solution (first brought to pH 7.8 using K₂CO₃) was added over a period of 1 min. After addition of polyethylene glycol 20,000 (Fisher Scientific Co.) the solutions were centrifuged either at 12,000 g for 60 min to sediment 18 nm or at 50,000 g for 120 min for 5-nm particles. Sediments were resuspended gently in PBS (but at pH 7.8) with 0.02% polyethylene glycol and the procedure was repeated. The material was stored in the same buffer with sodium azide (0.02%) at 4°C. Before use, gold preparations diluted for immunolabeling were centrifuged in an Eppendorf centrifuge for 4 min to remove any large aggregates.

For immunocytochemical studies requiring only a single label, affinity-

¹ Abbreviations used in this paper: ER, endoplasmic reticulum; HA, hemagglutinin; MDBK, Madin-Darby bovine kidney; MDCK, Madin-Darby canine kidney; MEM, minimal essential medium; PBS, phosphate-buffered saline; SV5, simian virus 5; VSV, vesicular stomatitis virus.

purified goat anti-mouse and goat anti-rabbit antibodies coupled to 18-nm gold particles were employed. For double labeling, goat anti-rabbit antibodies coupled to 5-nm gold particles were used, although no significant differences in the amount or distribution of label was observed when the converse combination of antibody-gold complexes was used.

Preparation of Ultrathin Frozen Sections: Infected MDCK cell monolayers on collagen coated coverslips were washed in PBS with Ca⁺⁺, fixed for 30 min at room temperature with 2% glutaraldehyde in the same buffer washed three times in PBS and stored at 4°C until further use. Frozen sections were obtained by a modification of the technique of Tokuyasu and Singer (48, 49). Briefly fragments of the fixed monolayer, infused with 0.6 M sucrose for at least 2 h, were cryosectioned at -80° to 90°C using a MT-2B LKB Sorvall microtome (LKB Instruments, Inc., Gaithersburg, MD) equipped with a freezing attachment (LTC-2). Sections were picked up on carbon-Formvar coated copper grids, washed in PBS containing 10 mM glycine and treated with a 2% gelatin (300 bloom, Sigma Chemical Co., St. Louis, MO) solution in PBS-glycine. Sections were incubated for 30 min on top of 10 µl drops of antibody solutions (a 1:10 dilution of a combination of the two ascites fluids containing monoclonal antibodies to the HA of influenza or a 1 mg/ml solution of rabbit IgG against VSV G protein each containing 2 mg/ml of goat IgG). Grids were then washed with PBS-glycine and incubated for 30 min with the secondary goat IgG antibody complexed to colloidal gold particles, which had been diluted with PBS-glycine until just barely pink. After several washes the grids were fixed in glutaraldehyde and stained for 20 min with 0.3% OsO₄ in cacodylate buffer, washed in cacodylate buffer and H₂O, and placed for 20–30 min over an embedding mixture containing 0.8% methyl cellulose (Fisher Scientific, Inc., 15 centipoises), 0.3% polyethylene glycol 1540 and 0.01% uranyl acetate. Grids were picked up with wire loops and excess embedding mixture was blotted away to leave a film that after drying had a silver-golden interference color. These samples were viewed in a Phillips EM 301 electron microscope operated at 60 kV.

Controls for immunocytochemical experiments (not shown) included uninfected MDCK monolayers treated with antiglycoprotein antibody or infected monolayers incubated with the inappropriate antibody or nonimmune serum. Background labeling in all cases was very low and, in particular, label over the Golgi apparatus was negligible.

Conventional Electron Microscope Studies: Monolayers grown either on 35-mm dishes or on collagen-coated coverslips were fixed in glutaraldehyde for 20 min, scraped, sedimented in an Eppendorf centrifuge, washed in PBS, and processed as described (38).

Iodination Experiments: At various times after infection with VSV, MDCK monolayers grown on 35-mm dishes were washed three times in ice-cold PBS plus Ca⁺⁺, placed on a metal block immersed in ice and iodinated at 4°C as previously described (15, 39). 0.5 ml of iodination mixture containing 1 IU/ml lactoperoxidase-coupled Sepharose beads (P-L Biochemicals, Inc., Milwaukee, WI), 10 mM glucose (Aldrich Chemical Co., Milwaukee, WI), 0.14 mU/ml glucose oxidase (Sigma Chemical Co.), 2.5 nmol/ml NaI, and 0.3 mCi/ml [¹²⁵I]iodine (New England Nuclear) was added to each plate for 30 min. Before use the enzyme-coupled beads were sedimented by centrifugation for 20 s in an Eppendorf centrifuge and washed four times with PBS. After iodination, monolayers were washed three times at 4°C in a 1:1 mixture of PBS with Ca⁺⁺ and 150 mM NaI containing 10⁻⁵ Na₂S₂O₃, and two more times in ice-cold PBS. Samples were then processed as described above for [³⁵S]methionine labeling. Before SDS electrophoresis, samples were precipitated with trichloroacetic acid, ether extracted, and resuspended in gel sample buffer. The incorporation of ¹²⁵I into cell protein was not due to leakage of enzyme from the beads during the incubation period. This was demonstrated by the fact that incubation mixtures that had been previously used for labeling cells but from which the beads had been removed by centrifugation for 20 s in the Eppendorf centrifuge, were no longer capable of labeling untreated monolayers during a second incubation.

Labeled virions released into the medium were recovered by sucrose density gradient centrifugation (11) after adding 60 µg of purified unlabeled virions as a carrier. After sedimentation (100,000 g for 60 min in SW56 Beckman rotor [Beckman Instruments, Inc., Palo Alto, CA]) and resuspension in 10 mM Tris-HCl, pH 7.6, the samples were loaded onto a 7–52% K⁺-tartrate gradient containing the same buffer and centrifuged overnight at 100,000 g. Material in a visible band was recovered by centrifugation, solubilized in gel sample buffer and analyzed by electrophoresis. The data have been corrected for recoveries which typically ranged from 60% to 80%.

Electrical Resistance Measurements: For each time point four disks of cells infected by the standard protocol were used. Measurements were carried out in a modified Ussing chamber as previously described (5). To ascertain that the infection was proceeding normally, we pulse-labeled, 5 h after infection, one infected and one control disk with [³⁵S]methionine for 30 min and analyzed by SDS electrophoresis.

RESULTS

Double Infection of MDCK Monolayers with Viruses of Opposite Polarity

To compare the intracellular pathways that glycoproteins of enveloped viruses with opposite budding polarity follow from their site of synthesis to the eventual site of virion assembly, we developed conditions to allow the double infection of MDCK monolayers (see Materials and Methods).

The simultaneous synthesis of enveloped glycoproteins of both viruses was demonstrated in cultures pulse-labeled with [³⁵S]methionine at various times after VSV addition (Fig. 1). Synthesis of SV5 or influenza proteins was clearly detectable during the first 7.5–8 h following superinfection with VSV, when the rate of synthesis of VSV proteins increased dramatically. In fact, the rate of synthesis of SV5 proteins (Fig. 1*a*), appeared to remain relatively constant during the first 7.5 h of superinfection; on the other hand, the synthesis of WSN proteins reached a peak at 5 h, decreasing somewhat thereafter (Fig. 1*b*).

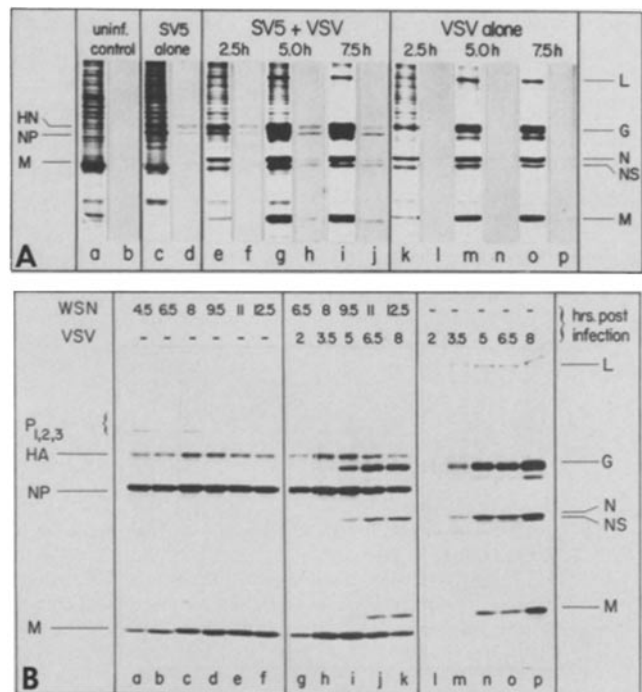


FIGURE 1 Synthesis of viral proteins in doubly infected MDCK cells. Cell monolayers on 35-mm dishes were infected and incubated with [³⁵S]met (50 µCi/ml) for 20 min as described below. The cells were solubilized with detergent (11) and processed for SDS electrophoresis in a 10% polyacrylamide gel followed by fluorography. (A) Cells were infected with SV5 (5 pfu/cell) and superinfected with VSV (10 pfu/cell) 24 h later. Total cell extracts (3-d exposure) were analyzed from cultures which were radiolabeled before each infection (a and c) or at the times indicated after addition of VSV (e, g, i, k, m, and o). The corresponding immunoprecipitates (6-d exposure) obtained with anti-SV5 antiserum from the cell extracts were also analyzed (b, d, f, h, j, l, n, and p). (B) Cells were infected with influenza WSN (10 pfu/cell) and superinfected with VSV (15 pfu/cell) 4.5 h later. Infections were allowed to proceed for the times indicated before radiolabeling. Total cell extracts were analyzed from cultures infected with WSN alone (a–f), with both viruses (g–k) and with VSV alone (l–p).

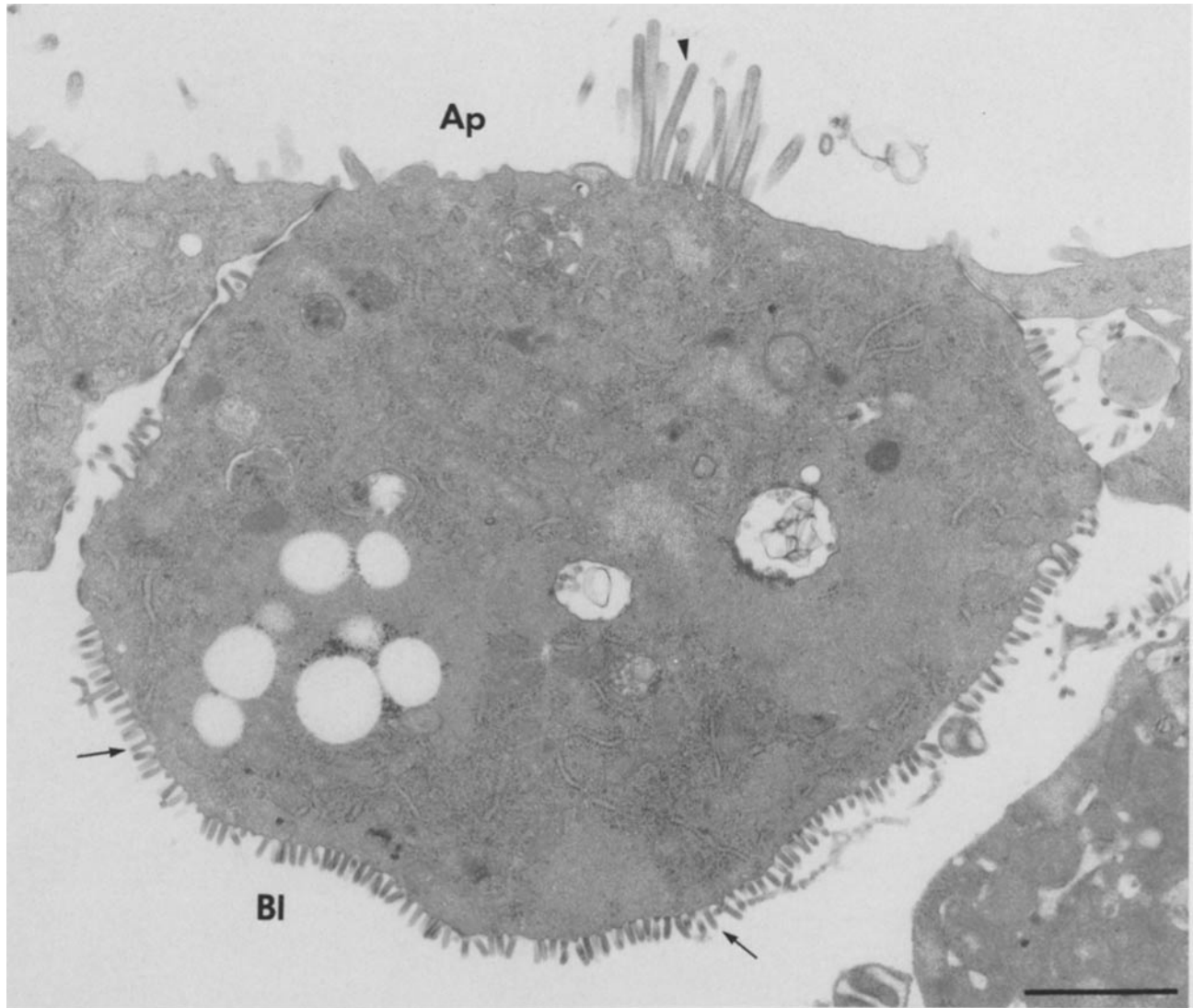


FIGURE 2 Polarized budding of SV5 and VSV virions from different surfaces of the same MDCK cell. Infections were carried out as described for Fig. 1A. Samples taken 11 h after superinfection with VSV were processed for conventional electron microscopy. Filamentous SV5 virions (arrowhead) bud from the apical surface (Ap) while bullet-shaped VSV particles (arrows) assemble on the basolateral domain (Bl) of the cell. Bar, 1 μm . $\times 22,000$.

Electron microscopy revealed that viral assembly proceeded in doubly infected monolayers with the polarity that is characteristic of each virus in singly infected cells. Filamentous SV5 virions, or spherical influenza particles assembled at the apical surfaces of cells that at the same time manifested the bullet-shaped VSV particles budding from lateral and basal domains (Figs. 2 and 3, *a*, *b*, and *c*). With the progression of the double infections budding of VSV predominated and fewer SV5 or influenza virions were produced. After several hours of VSV infection many cells became rounded, tight junctions were disrupted and bullet-shaped VSV virions were seen budding over the entire cell surface. However, even in these cases, when virions from the first infection (SV5 or influenza) were still assembling, this occurred in areas distinct from those producing VSV particles (Fig. 3*d*). In SV5/VSV doubly infected cells, SV5 virions tended to cluster towards the apical pole of the cell, away from areas in the same free surface where patches of VSV budding virions appeared (not shown).

Intracellular Distribution of Viral Glycoproteins in Doubly Infected Cells

The localization of HA of influenza or G of VSV was determined by immunolabeling of ultrathin frozen sections using specific antibodies to either viral glycoprotein and corresponding affinity-purified second antibodies complexed to colloidal gold particles. Intracellularly, the G protein was preferentially localized in the Golgi apparatus (Fig. 4*a*) where it first appeared 3 h after infection, a time when there was practically no labeling on the cell surface. Gold particles were found on the different Golgi cisternae as well as adjacent Golgi vesicles and vacuoles. There seemed to be no preference for the rims of the individual cisternae over the middle regions, and no marked accumulation towards one side or the other side of the Golgi stacks. In contrast to the prominent labeling of the Golgi apparatus, which persisted even when the G protein became more abundant on the plasma membrane, little accumulation of G protein was ever detected over

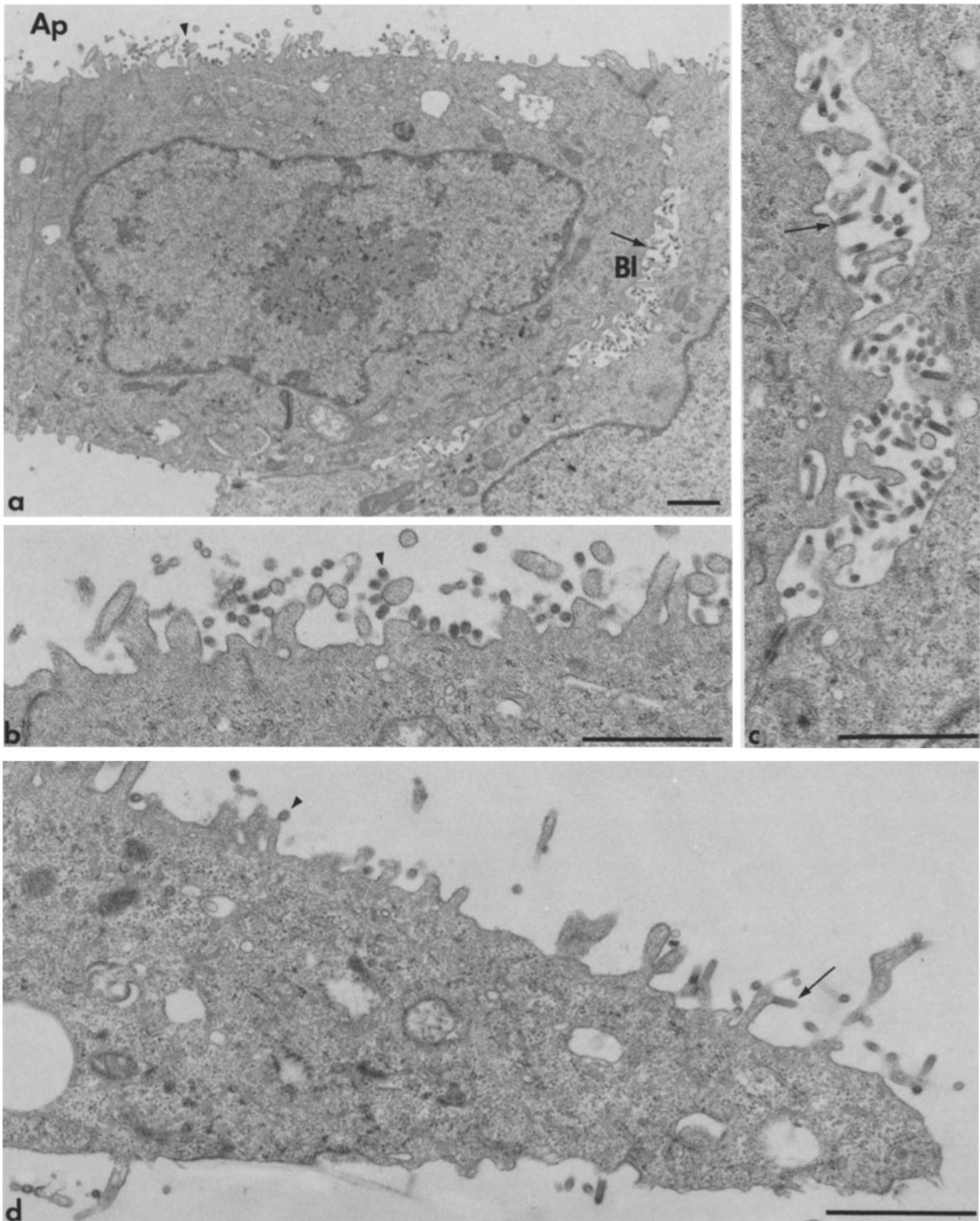


FIGURE 3 Polarized budding of influenza and VSV virions from doubly infected MDCK cells. Monolayers were infected with influenza and 4.5 h later superinfected with VSV and incubated for 5 (a, b, and c) or 9 h (d) before fixation for electron microscopy. (a) Numerous influenza virions (arrowhead) averaging 90 nm in diameter are present on the apical surface (Ap) of the cell, while bullet shaped VSV particles of 65-nm diameter (arrow) budding from the basolateral plasma membrane domains accumulate in the intercellular spaces (Bl). (b and c) Higher magnification views of segments of the apical (b) and lateral (c) surfaces. (d) A cell late in infection, with tight junctions no longer intact. Both types of viruses can be seen budding from nonadherent regions of the plasma membrane. Budding virions of each type, however, do not intermingle completely on the surface. Distinct regions are observed where influenza (arrowhead) or VSV (arrow) particles assemble. Bar, 1 μ m. \times 26,000.

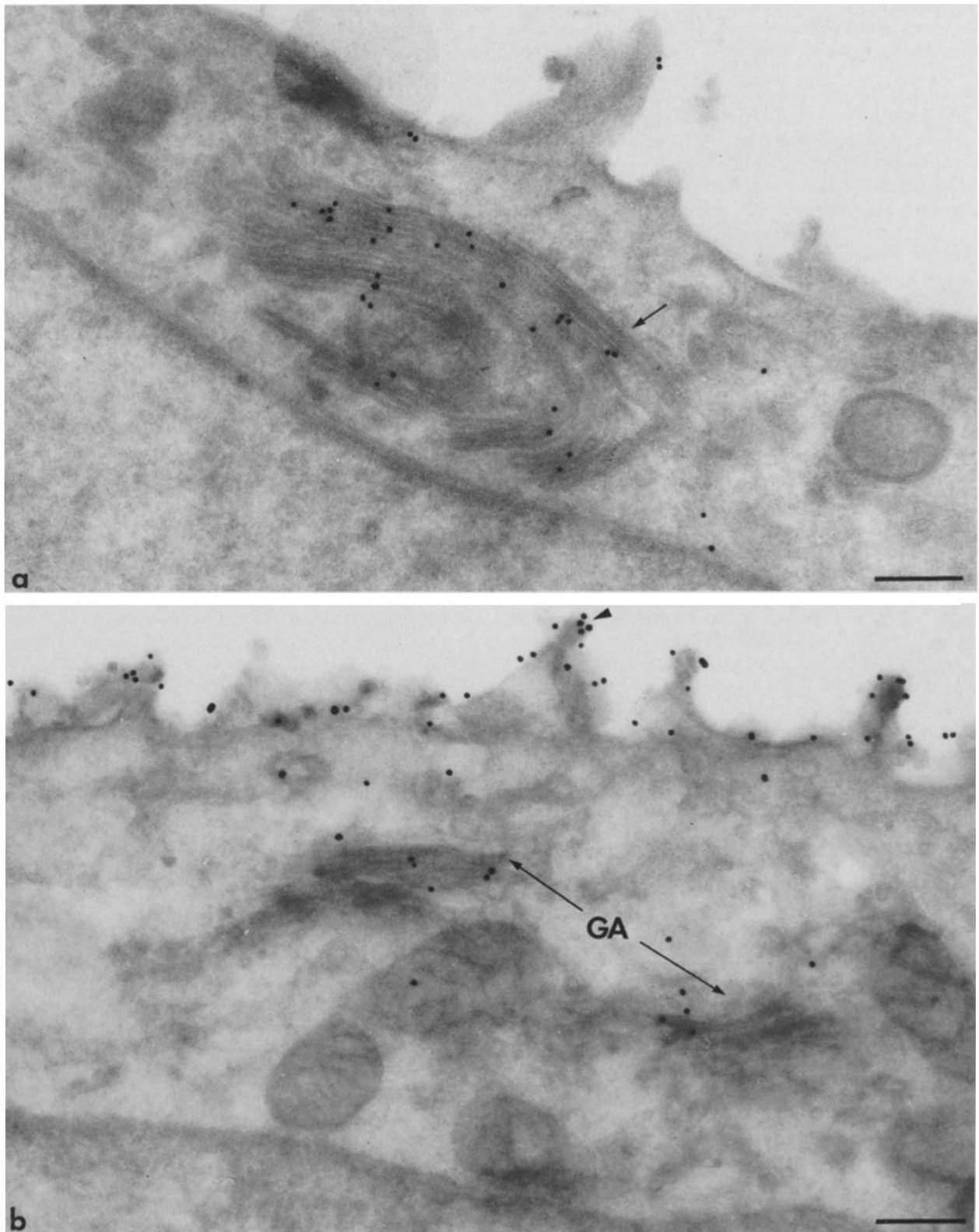


FIGURE 4 Localization of viral glycoproteins in MDCK cells infected with influenza and VSV. (a) Thin frozen sections of doubly infected cells were processed for immunoelectron microscopy using rabbit antibodies to G or mouse monoclonal to HA as described in Materials and Methods. 3 h after superinfection with VSV, before significant amounts of G are detected in the plasma membrane, the G protein is found throughout cisternae of the Golgi apparatus (arrow). (b) 5 h after superinfection with VSV the HA of influenza is found in the Golgi apparatus (GA, arrows) as well as the apical plasma membrane (arrowhead). Bar, 0.25 μm . $\times 60,000$.

the rough endoplasmic reticulum, where the polypeptide is synthesized. Thus, traversal of the Golgi apparatus appears to be a much slower process than exit of the G protein from the ER.

HA was also localized throughout the Golgi apparatus (Fig. 4*b*), although in this case when labeling of the cell surface was intense (5 or more hours after VSV infection), labeling over the Golgi elements decreased. Notably, the remaining few gold particles observed over the organelle appeared to be preferentially located towards the rim of the Golgi cisternae. These immunoelectron microscopic observations are consistent with the changes in the rate of synthesis of the respective viral proteins determined from radioactive pulse-labeling experiments (Fig. 1).

To determine whether the G and HA glycoproteins simultaneously traverse the same Golgi apparatus, we treated thin frozen sections of doubly infected cells with antibodies against both types of viral glycoproteins. In this case, the primary anti-HA mouse monoclonal and anti-G rabbit antibodies were localized by secondary goat anti-mouse or anti-rabbit antibodies coupled to gold particles of different sizes (see Materials and Methods). In cells in which both HA and G were found intracellularly, the Golgi apparatus, regardless of their location within the cell or their proximity to either of the plasma membrane domains, were always labeled with both types of gold particles (Figs. 5–7). Particles of different size classes were often seen singly or in small clusters in proximity within the same cisternae (Fig. 8, *a*, *b*, and *c*). These clusters were either linear or in the form of irregularly shaped clumps of gold particles. The linear clusters clearly indicate the presence of several viral glycoproteins, whereas the irregularly shaped clumps could also be the result of an artifactual aggregation of label around a single antigen. The G protein showed no preferential distribution, but HA was more frequently found over peripheral regions of the cisternae, especially in cases when high concentrations of G were present in the Golgi (Figs. 5 and 8*c*). These observations clearly indicate that envelope glycoproteins of viruses with opposite budding polarity traverse the same organelle.

Anti-viral glycoprotein antibodies were also localized on vesicular elements located in the Golgi area and in the cytoplasm between the Golgi apparatus and the plasma membrane. In most instances these vesicles contained only gold particles of one size (Fig. 6), but occasionally, and in particular in some of the larger vesicles, both types of gold particles were present. It is worth noting that due to the low level of labeling and the small size of most vesicles it cannot be concluded that those vesicles labeled with only one type of antibody did not carry some of the other antigen as well.

Distribution of Viral Glycoproteins in the Cell Surface

As double infection proceeded, both viral glycoproteins were observed in the same Golgi, while HA accumulated predominantly on the apical and G on the basolateral domain of the plasma membrane. However, segregation of the glycoproteins to each domain was not as strict as suggested previously in studies conducted with singly infected cells (37). Even before viral budding began, some labeling of HA was observed in the basolateral domains and this persisted when viral production was intense (4.5–11.5 h after influenza infection, see Fig. 12), but this rarely exceeded 10% of the density of

label found on the apical membrane. The segregation of G was even less strict; the density of anti-G protein label on the apical membrane ranged widely from ~20% of that found on basolateral surfaces to almost 100% in several cells in advanced states of VSV infection. The presence of G protein on the apical surface, which was also observed in singly infected cells (Fig. 9), was only prominent after most of the infected cells had G on the basolateral surface. This observation is in agreement with preliminary results from work in this laboratory (manuscript in preparation) using the VSV temperature-sensitive 045 mutant that permits a better synchronization of the intracellular traffic of G proteins from the Golgi to the plasma membrane. It must be stressed, however, that even in those cases where the density of labeling with anti-G antibodies was equally intense on both surfaces, VSV virions budded exclusively from lateral and basal plasma membrane domains.

The presence of G protein on the apical membrane of singly infected cells was also determined by selectively iodinating the exposed surface of VSV infected monolayers with lactoperoxidase coupled to large (60 μm) Sepharose beads. Only complete opening of tight junctions could allow access of these beads to the basolateral domains. However, as early as 4 h after VSV infection (Fig. 10), soon after the G protein was first detectable on the plasma membrane by immunoelectron microscopy, immunoprecipitable G protein represented one of the predominant species accessible to iodination in the apical membrane. This apical polypeptide was indistinguishable in electrophoretic mobility from the mature G protein detected by Coomassie Blue staining or immunoprecipitation with anti-G antiserum (not shown) in total infected cell extracts and purified virions. When cells were labeled with ^{125}I after 5 h of infection and subsequently incubated at 37°C for 1.5 and 3 h, the amount of labeled G protein recovered in cell extracts diminished to 60% and 40%, respectively. This probably reflects degradation of the apically exposed G protein rather than its incorporation into virions and subsequent release from the monolayer, since virions collected and purified from the medium accounted for only a small fraction (~5%) of the decrease in radioactivity.

The integrity of the junctions at times when G protein was already detected on the apical plasma membrane was monitored by measuring the electrical resistance (5) of infected monolayers (Fig. 11*a*). Despite an active VSV infection (Fig. 11*b*), this began to decrease only 6.5 h after addition of VSV but remained significant even 2 h later. Thus, the appearance of VSV G protein on the apical membrane is unlikely to result from a redistribution of surface protein following disruption of the tight junctions during VSV infection.

Formation of Mixed Virions in Doubly Infected Cells

Cultured cells infected with two different viruses can produce aberrant particles in which the nucleocapsid of one virus is surrounded by an envelope composed partially (phenotypically mixed virions) or totally (pseudotypes) of glycoproteins from the other virus (28). A recent study (41) measured pseudotype and mixed virion formation in polarized MDCK monolayers infected with influenza and VSV by the neutralization of VSV plaque forming units with anti-influenza antibodies. This indicated that the production of aberrant virions containing a VSV nucleocapsid increased dramatically

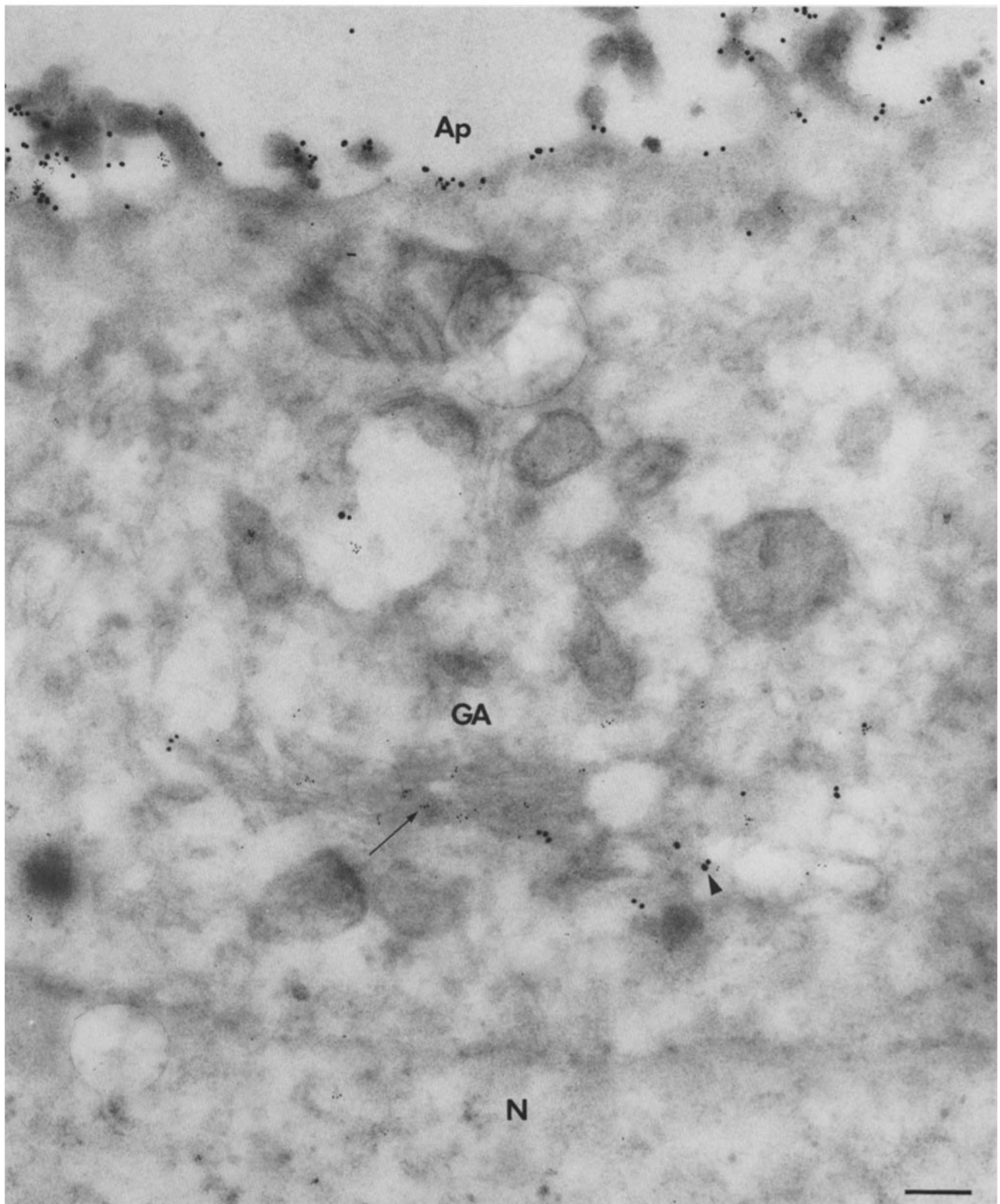


FIGURE 5 Simultaneous localization of VSV and influenza glycoproteins in a doubly infected MDCK cell. Frozen thin sections of doubly infected cells (4 h after superinfection with VSV) were incubated with mouse monoclonal antibodies against the HA of influenza and rabbit anti-VSV G protein antibodies, followed by the appropriate second antibodies complexed to colloidal gold particles of two different sizes. Large gold particles, corresponding to the HA of influenza (arrowhead), and small gold particles localizing the G of VSV (arrow), are found in the same Golgi apparatus (GA). The apical surface (Ap) contains predominantly HA but also some small amount of G. No significant label is observed over the nucleus (N) or the cytoplasm. Bar, 0.2 μm . $\times 57,000$.

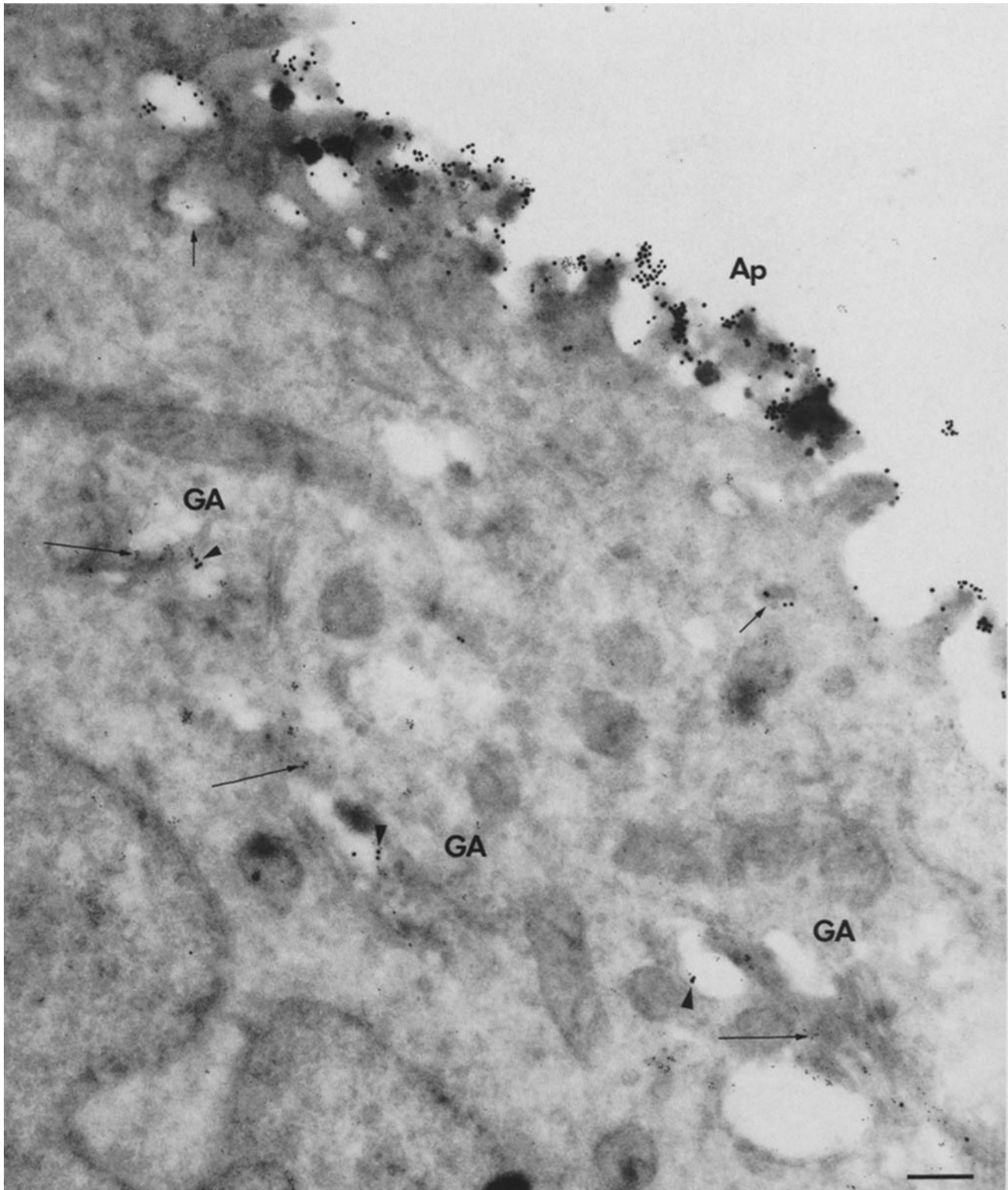


FIGURE 6 Appearance of VSV and influenza glycoproteins in multiple Golgi stacks of the same doubly infected cells. Frozen thin sections were prepared and labeled as described in Fig. 5. Large gold particles (arrowheads) representing the HA of influenza, are distributed in each Golgi apparatus (GA) depicted, as well as on the apical surface (Ap). Small gold particles (long arrows) are found within the same Golgi stacks, showing that the VSV G protein and the HA of influenza are sharing the same organelle. Most cytoplasmic vesicles (short arrows) are labeled with only one type of antibody. Bar, 0.25 μm . $\times 45,000$.

with the course of infection and with the onset of cytopathic effects, suggesting that opening of the tight junctions promoted the intermingling of viral glycoproteins in the cell membrane.

The formation of mixed virions was demonstrated by immunolabeling in frozen sections of doubly infected MDCK cells (Figs. 12 and 13). Bullet-shaped virions (i.e., with VSV nucleocapsids) containing varying amounts of label corre-

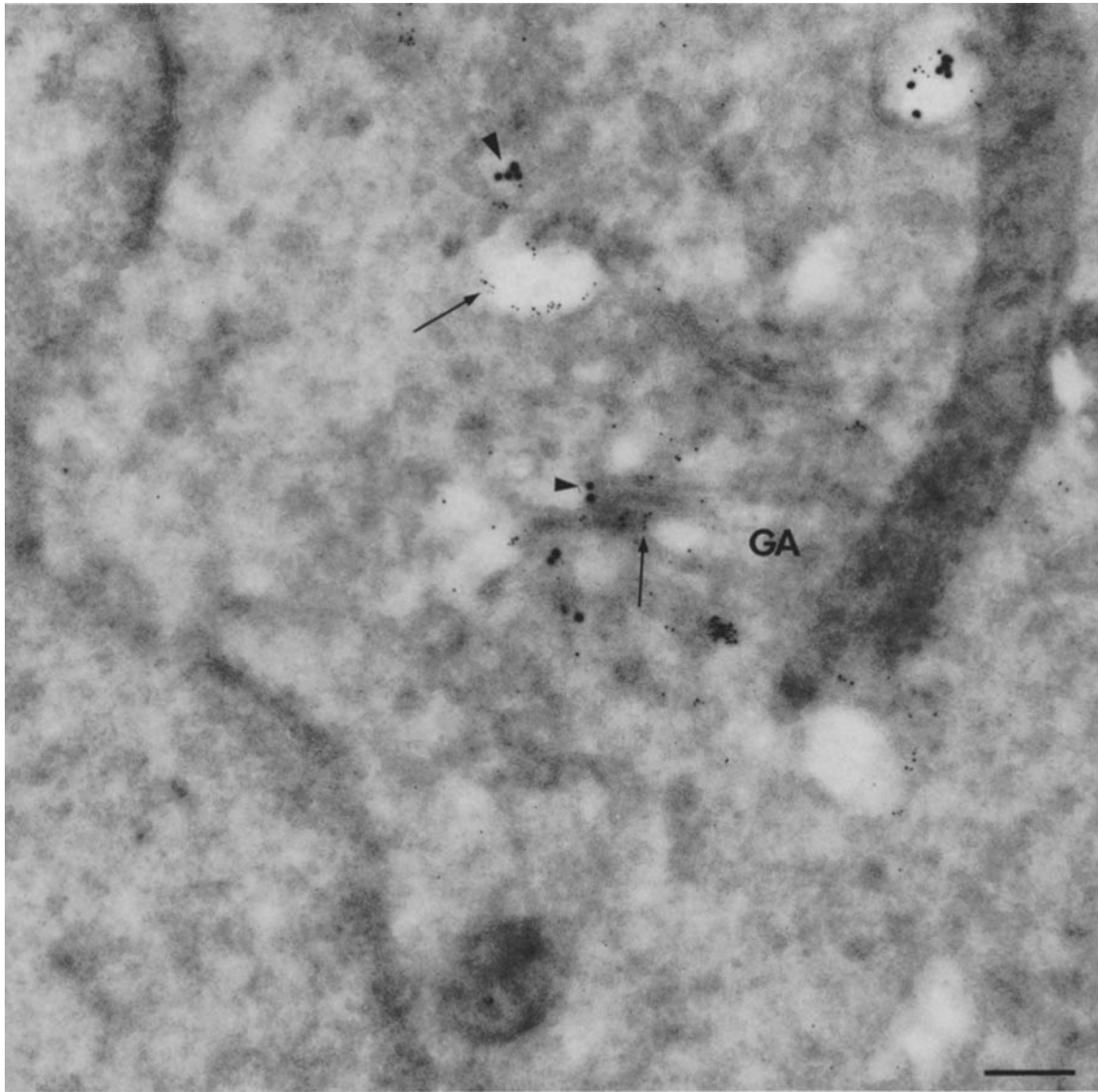


FIGURE 7 Distribution of HA and G within the same Golgi apparatus of a doubly infected MDCK cell. Infected monolayers were prepared and processed as described in Fig. 5. Each Golgi apparatus (GA) contains both large gold particles (arrowheads) representing HA of influenza and small ones (arrows) corresponding to the G of VSV. The two types of glycoproteins are found in the same cisternae and in associated vesicular structures, some of which are dilated. Bar, 0.2 μm . $\times 63,000$.

sponding to the HA glycoprotein were commonly seen both free in the intercellular spaces and budding from the lateral membrane, well before there was any evidence for the loss of junctional integrity (Figs. 12 and 13*a*). Mixed-type virions with a rounded influenza nucleocapsid but labeled with anti G antibodies were rarely observed (Fig. 13*b*), despite the relative abundance of G protein on the apical membrane, where influenza virions assembled.

DISCUSSION

The realization that many plasma membrane proteins are glycoproteins bearing complex oligosaccharides (cf. 45) known to be processed by enzymes located in Golgi membranes (4, 13) suggested that these proteins must traverse the Golgi apparatus on their way to the cell surface. This has only recently been demonstrated directly by immunoelectron microscopy for the viral envelope glycoproteins of VSV (2) and SFV (10), and as a result of the work presented here, for the

HA of influenza. We observed that in cells labeled during the productive stages of infection, HA of influenza and G protein of VSV could be detected throughout all cisternae within a Golgi stack. This most likely represents the distribution of molecules in passage through the organelle from the cis to the trans face, since it is now clear that the Golgi apparatus is a polarized structure in which different enzymatic activities are confined to specialized regions (cf. 7, 40).

Even though the viral glycoproteins are synthesized in the ER, we detected little labeling of this organelle. This may reflect the low concentration of newly synthesized glycoprotein in ER membranes, resulting from their rapid transit to the Golgi apparatus. Somewhat higher levels of ER labeling for the glycoproteins of SFV (10) and VSV (2) have been observed by other investigators in nonpolarized cells.

In many secretory cell types the Golgi apparatus has a characteristic disposition and orientation, which appears well suited for the directional transfer of its contents to the cell

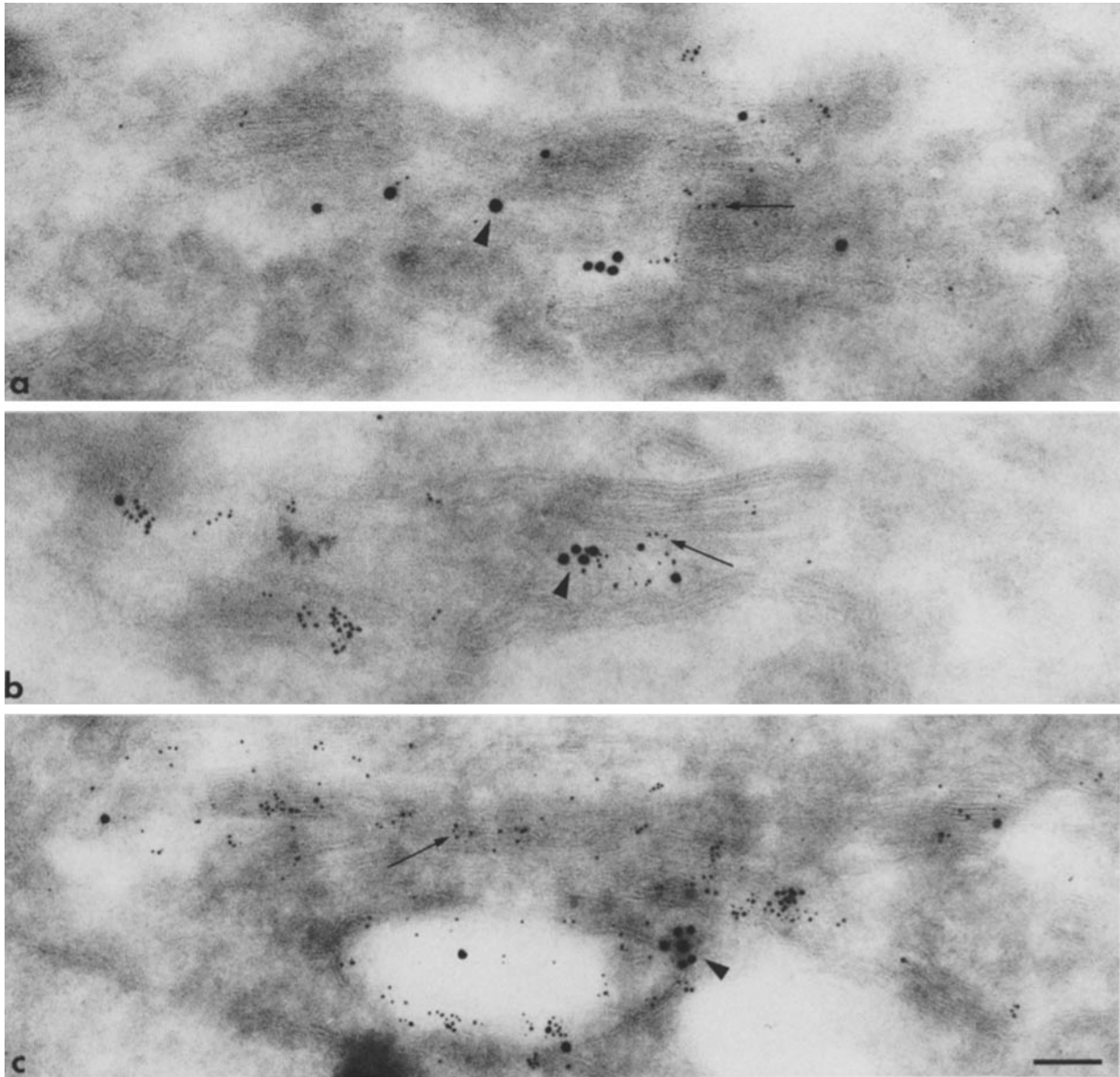


FIGURE 8 Localization of viral glycoproteins in Golgi apparatus of doubly infected MDCK cells. After 4 h of infection with VSV, samples were sectioned and labeled as outlined in Fig. 5. Three Golgi apparatus (a-c) taken from different doubly infected cells are depicted at higher magnification than in Figs. 5, 6, and 7. Often glycoprotein molecules of the same type are concentrated in specific regions within Golgi stacks and in separate vesicles. Groups of HA molecules (large gold particles, arrowheads) are seen in areas distinct from those containing G (small gold particles, arrows). Bar, 0.1 μ m. \times 100,000.

surface domain where secretory proteins are ultimately discharged (17). We considered the possibility that, in polarized cells, separate Golgi apparatus, or portions of a single complex, function in the transfer of proteins exclusively to either the apical or basolateral plasma membrane domains. This would imply that sorting of proteins destined to the two domains takes place in the ER or when proteins exit from this organelle. This notion can now be discounted because of our findings that in doubly infected MDCK cells the HA and G glycoproteins traverse the same Golgi apparatus, although they ultimately accumulate predominantly in different plasma membrane domains. Both types of viral antigens were even found in proximity within the same cisternae where, however, they rarely intermingled but rather appeared to concentrate in areas containing either one type of glycoprotein or the other.

Vesicles that were found in the Golgi area containing viral glycoprotein molecules may be involved in several stages of the intracellular transport. Some may represent coated vesicles that transfer the newly synthesized polypeptides from the ER to Golgi cisternae (42, 43), although the presence of a clathrin coat could not be established under the conditions employed for immunolabeling. Other vesicles distributed in the cytoplasm between the Golgi apparatus and the plasma membrane may be presumed to be in transit between these two organelles. Although it appears that only one or the other type of glycoprotein was carried by most of these vesicles, the relatively low level of immunolabeling and the small size (\sim 100 nm) of most vesicles does not exclude the possibility that in many instances the glycoprotein of the other type was also present. Thus, even though the formation of clusters of each protein within Golgi cisternae suggests that sorting of glyco-

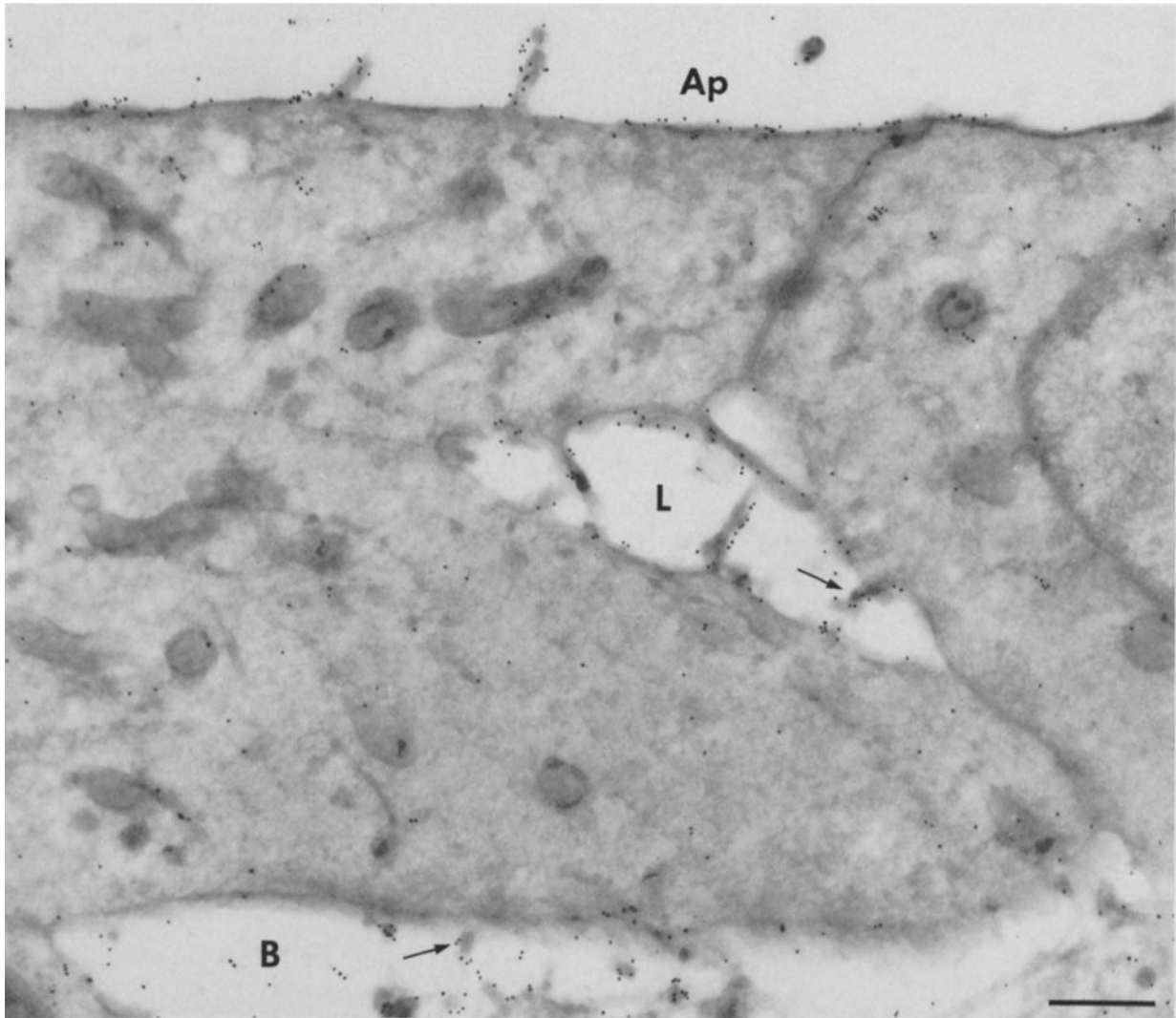


FIGURE 9 Presence of VSV G protein on the apical plasma membrane of MDCK cells. Frozen thin sections from monolayers which had been infected only with VSV (15 pfu/cell) for 5 h were labeled with anti-G protein antibodies. In this cell significant labeling is observed on the apical surface (*Ap*), comparable to that on the basal (*B*) and lateral (*L*) plasma membrane domains. However budding virions (arrows) are only observed on the lateral and basal surfaces. Bar, 0.2 μm . $\times 57,000$.

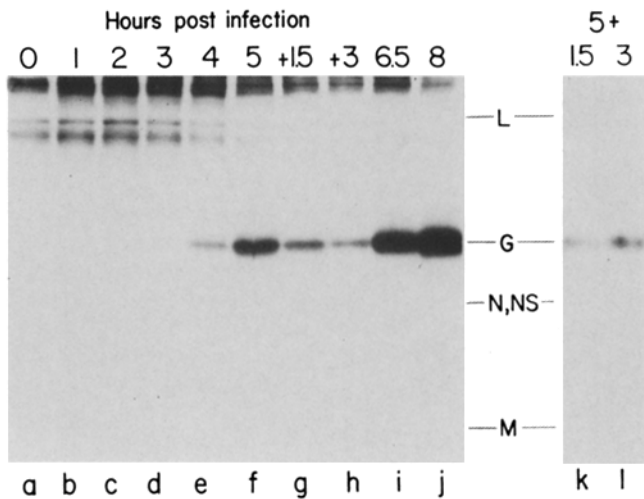


FIGURE 10 Lactoperoxidase iodination of VSV G protein exposed on the apical surface. MDCK monolayers on 35-mm dishes were infected with VSV for the times indicated and incubated for 30 min at 4°C with lactoperoxidase coupled to Sepharose beads and ^{125}I , as described in Materials and Methods. Cell extracts were analyzed in 10% polyacrylamide gels which after staining with Coomassie Blue to determine the positions of the viral proteins were processed for fluorography (1-d exposure). Labeling of the G protein is evident 4 h after infection (track e) and is very intense by 8 h (track j). In tracks g and h, cells labeled 5 h after infection were further incubated for the times indicated before cell extracts were prepared. Quantitative analysis of the gels showed that labeling in the G protein band decreased by 40% at 1.5 h and 60% at 3 h. Virions recovered from the respective media of these two samples (tracks k and l, 7-d exposure) contained labeled G protein, but this only represented <5% of the decrease in radioactivity in the cellular G protein that occurred during the postlabeling incubation.

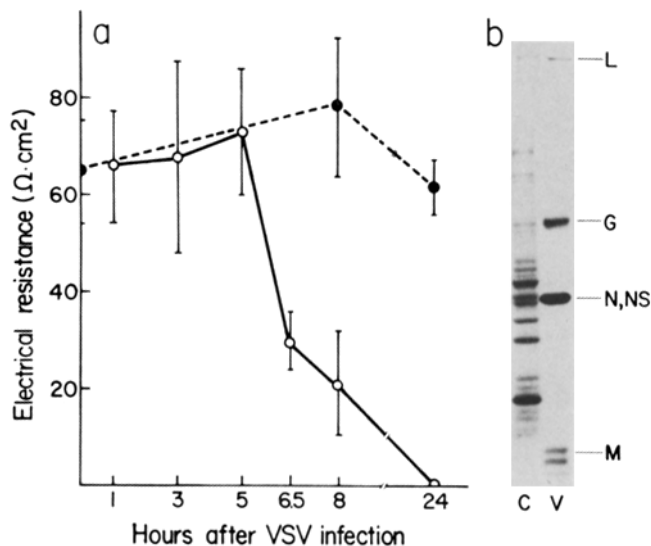


FIGURE 11 Changes in the transepithelial electrical resistance of VSV-infected MDCK monolayers during the course of infection. Monolayers plated on collagen coated nylon disks were infected with VSV (15 pfu/cell). Measurements of the electrical resistance were made at various times after infection using four disks for each time point (open circles). The electrical resistance of parallel uninfected control cultures is also shown (closed circles).

proteins begins during traversal of the Golgi, the segregation into separate vesicles may not always be complete. Indeed, the incomplete segregation of the two viral glycoproteins that we observed in the apical and basolateral domains may reflect the direct transfer of some glycoproteins from a population of mixed vesicles to the cell surface. In addition, the contribution that recycling of those glycoprotein molecules already incorporated into the cell surface may make to the pool of intracellular vesicles cannot easily be assessed. Recycling may play an important role in refining an initially imperfect sorting. Recently, work from Simons' laboratory (27, 33) has shown that a significant fraction of G protein, artificially incorporated into the apical surface of MDCK cells through the fusion of viruses induced by acid pH, is subsequently transferred by an intracellular route to the basolateral surface.

Immunolabeling of frozen sections of virally infected cells allowed us to assess the distribution of envelope glycoproteins in the cell surface, without complications arising from the inaccessibility of antibody probes to basolateral domains in intact monolayers. As previously reported for singly infected cells (37), each glycoprotein accumulated in the plasma membrane domain from which the corresponding virus assembled. However, in the current studies the G protein appeared on the apical surface of cells at earlier times than it was previously observed (37). This was probably due to the fact that the much higher viral multiplicities required for successful double infections accelerated the overall process that led to the mis-addressing of G. It should be noted that this did not correlate with a detectable disruption of the tight junctions or with a loss of polarity of viral budding, which was only affected much later.

When the G protein located in the apical surface was labeled by lactoperoxidase with ^{125}I , only a very small fraction of the labeled protein was found in viruses released into the medium. Instead, the apical G protein appeared to be degraded within fairly short times (with a half life of ~2 h). Whether or not some of the iodinated G protein found in the apical plasma membrane was eventually transferred by recycling to the basolateral surface and into virions that formed at this site was not determined, and it is possible that changes introduced by iodination rendered the glycoprotein incompetent in envelope assembly and subsequent budding.

A consequence of incomplete sorting of viral envelope glycoproteins was the formation of mixed virions in which the envelope of each virus contain some glycoprotein molecules encoded by the genome of the other virus. Mixed virions were previously detected in doubly infected MDCK cells using antibody neutralization of viral infectivity as an assay (6, 41), and such studies suggested that opening of the junctions played an important role and allowing the mixing of glycoproteins. Our immunocytochemical studies indicate that bullet-shaped VSV containing HA molecules were found (but only at the basolateral surface) even at the onset of viral budding, well before disruption of the junctions. They also confirmed previous reports that the nucleocapsid of VSV is far less discriminating with respect to the protein composition of its envelope than that of influenza (51) but indicate that this is not due to exclusion of the VSV G protein from the preferred domain of influenza virion assembly.

The discrepancy between the strict polarity with which viral budding takes place and the less stringent distribution of viral glycoproteins in the cell surface raises the question of whether

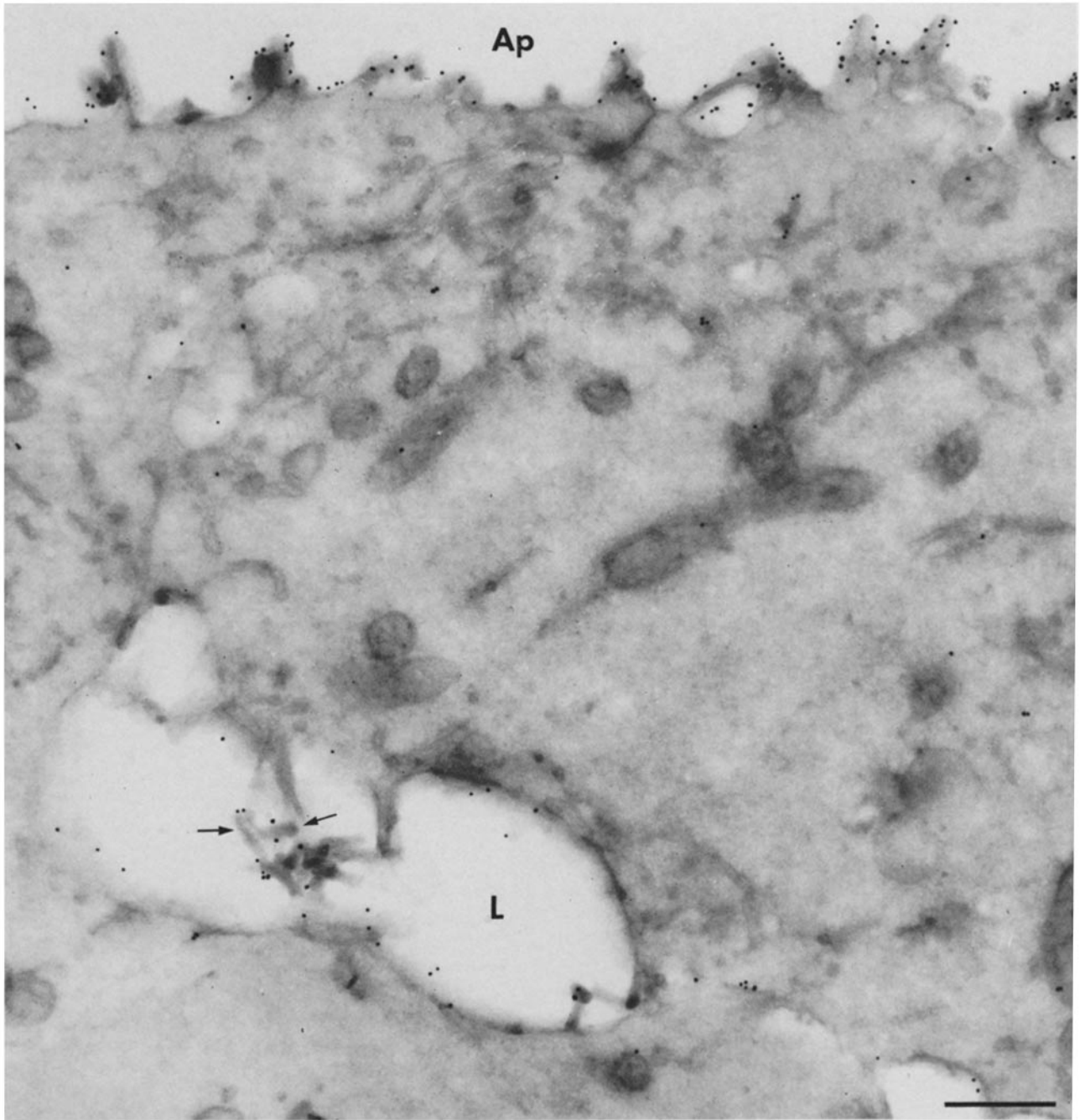


FIGURE 12 Incorporation of the HA of influenza into phenotypically mixed bullet-shaped (VSV) virions in doubly infected MDCK cells. Frozen thin sections of monolayers infected with influenza and superinfected with VSV fixed 5 h after superinfection were labeled with anti-HA antibodies. While the label is localized predominantly on the apical plasma membrane (Ap), some HA is found on the lateral surface (L), where it is often incorporated in the envelope of VSV virions (arrows). Bar, 0.5 μm . $\times 35,000$.

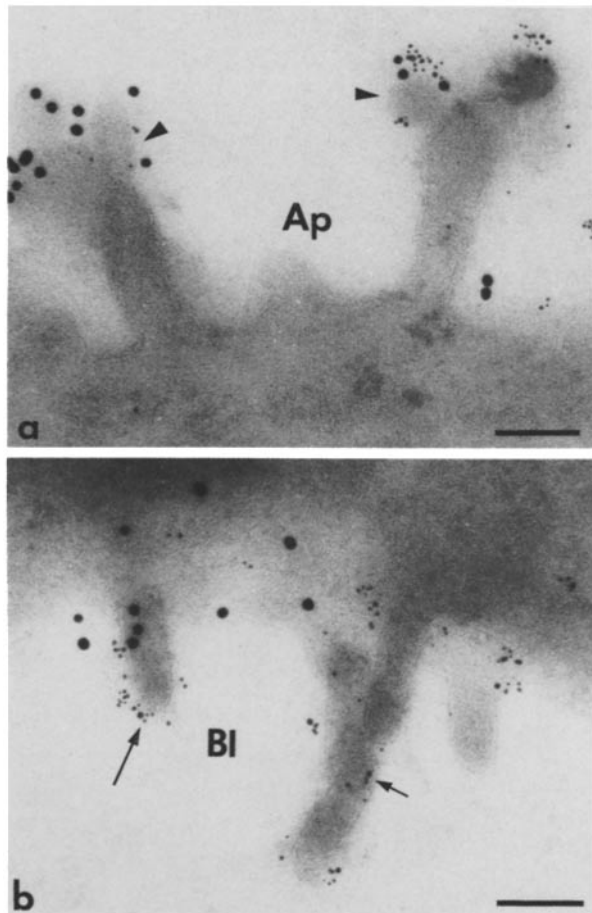


FIGURE 13 Double labeling of phenotypically mixed virions budding from the surfaces of doubly infected MDCK cells. Frozen thin sections of cells fixed 4 h after superinfection with VSV were doubly labeled with antibodies complexed to colloidal gold particles of two different sizes, as indicated in Fig. 5. (a) Influenza virions (arrowheads) with a mixed envelope containing the VSV G protein (small particles) are occasionally seen budding from the apical plasma membrane (Ap). (b) VSV virion (arrow) containing HA molecules (large gold particles) is observed assembling on the basolateral surface (Bl). Other virions are labeled only with anti-G protein antibodies (short arrow). Bar, 0.1 μm . $\times 110,000$.

cellular membrane proteins or other viral components such as the matrix protein (M), play a role in determining the site of budding. The M protein, which is a peripheral membrane protein synthesized on free polysomes (21, 30), mediates the interaction of the nucleocapsid with the viral envelope glycoproteins and therefore may be important in this regard. The incorporation of host membrane proteins into the viral envelope has also been reported (23, 24) and while it is not clear whether these proteins play any role at all in virion assembly, the polarized distribution of a required host assembly protein in epithelial cells remains an intriguing possibility. In addition, other factors, such as differences in the membrane composition (50) or in the cytoskeleton underlying the plasma membrane domains (29) may be significant, since we observed that the regional nature of viral budding is maintained even after junction disruption (Fig. 3b) and, under appropriate conditions, in sparsely plated cells (36).

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