Polarized Delivery of Viral Glycoproteins to the Apical and Basolateral Plasma Membranes of Madin-Darby Canine Kidney Cells Infected with Temperature-sensitive Viruses

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ABSTRACT   The intracellular route followed by viral envelope glycoproteins in polarized Madin-Darby canine kidney cells was studied by using temperature-sensitive mutants of vesicular stomatitis virus (VSV) and influenza, in which, at the nonpermissive temperature (39.5°C), the newly synthesized glycoproteins (G proteins) and hemagglutinin (HA), respectively, are not transported out of the endoplasmic reticulum.

After infection with VSV and incubation at 39.5°C for 4–5 h, synchronous transfer of G protein to the plasma membrane was initiated by shifting to the permissive temperature (32.5°C). Immunoelectron microscopy showed that under these conditions the protein moved to the Golgi apparatus and from there directly to a region of the lateral plasma membrane near this organelle. G protein then seemed to diffuse progressively to basal regions of the cell surface and, only after it had accumulated in the basolateral domain, it began to appear on the apical surface near the intercellular junctions. The results of these experiments indicate that the VSV G protein must be sorted before its arrival at the cell surface, and suggest that passage to the apical domain occurs only late in infection when tight junctions are no longer an effective barrier.

In complementary experiments, using the temperature-sensitive mutant of influenza, cultures were first shifted from the nonpermissive temperature (39.5°C) to 18.5°C, to allow entrance of the glycoprotein into the Golgi apparatus (see Matlin, K. S., and K. Simons, 1983, Cell, 34:233–243). Under these conditions HA accumulated in Golgi stacks and vesicles but did not reach the plasma membrane. When the temperature was subsequently shifted to 32.5°C, HA rapidly appeared in discrete regions of the apical surface near, and often directly above, the Golgi elements, and later diffused throughout this surface. To ensure that the anti-HA antibodies had access to lateral domains, monolayers were treated with a hypertonic medium to dilate the intercellular spaces. Some labeling was then observed in the lateral plasma membranes soon after the shift, but this never increased beyond 1.0 gold particle/μm, whereas characteristic densities of labeling in apical surfaces soon became much higher ($\sim$10 particles/μm). Our results suggest that the bulk of HA follows a direct pathway leading from the Golgi to regions of the apical surface close to trans-Golgi cisternae.

Confluent monolayers of cultured Madin-Darby canine kidney (MDCK)[1] cells are functionally and morphologically po-

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; G protein, glycoprotein; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; MEM, minimal essential medium; VSV, vesicular stomatitis virus.

larized (7, 17, 24) and provide a useful model to study the biogenesis of epithelial cell plasma membranes. After infection with enveloped viruses, the polarized nature of the cells is manifested in the assembly of specific virions on either one or the other plasma membrane domain (33). Influenza, simian virus 5, or Sendai virions assemble exclusively on the

apical surface, whereas vesicular stomatitis virus (VSV) and certain RNA tumor virus particles bud only from the basolateral regions of the plasma membrane (1, 28, 31, 32, 35, 37).

The asymmetric budding of viruses from polarized epithelial cells is thought to be determined by a preferential accumulation of the envelope glycoproteins in the corresponding membrane domain, which precedes viral assembly (32). It appears that segregation of the glycoprotein does not require other viral components, in that it has been reported that accumulation of hemagglutinin (HA) in the apical surface takes place in cells infected with recombinant SV40 viruses carrying the HA gene, but not encoding the other influenza polypeptides (36). Thus, most likely, the mechanisms that ensure the distribution of viral envelope glycoproteins in the cell surface are the ones utilized for the segregation of cellular proteins to specific plasma membrane domains.

Theoretically, plasma membrane proteins could be sorted intracellularly by a mechanism that directs them to one or the other plasma membrane domain, or sorting would take place after arrival at the cell surfaces. The delivery of protein molecules to the plasma membrane need not be restricted to the domain where the proteins eventually accumulate, because a redistribution could be effected either through recycling mechanisms or by controlled lateral diffusion.

Using MDCK cells doubly infected with VSV and influenza we have recently demonstrated (28) that glycoproteins of both viruses can be found within the same Golgi apparatus. We, therefore, concluded that their sorting cannot take place before arrival to this organelle. To determine whether the glycoproteins are sorted intracellularly or only after they have reached the cell surface, we have now employed temperature-sensitive mutants of VSV and influenza, in which, at the nonpermissive temperature, the envelope glycoproteins accumulate in the endoplasmic reticulum (ER). Their transfer to the cell surface was synchronized in one of two ways, by either directly shifting the temperature to the permissive one or by introducing an intermediate cold temperature block, which leads to intracellular accumulation of the glycoprotein after its delivery to the Golgi apparatus (22). Our results suggest that sorting takes place intracellularly, because the bulk of the accumulated protein appeared to be transferred directly to the domain from which viral budding takes place. A recent study with cells infected with the same temperature-sensitive mutant of influenza, but using a different immunocytochemical procedure, provided a similar conclusion for the distribution of HA (31).

MATERIALS AND METHODS

Cell Cultures and Viruses: The MDCK and Vero cell lines were cultured by standard procedures as previously described (7, 28). The ts045 mutant (16) of VSV (a gift of Dr. J. Lenard, Rutgers University, New Brunswick, NJ) and the ts61s mutant (42) of influenza (a gift of Dr. P. Palese, Mount Sinai School of Medicine, New York) were plaqued at 32.5°C on Vero and MDCK cells, respectively. For both mutants, viruses from different plaques were grown on Vero cells at 32.5°C and used to determine in microtiter plates the viral cytopathic effects on 50% (CPE_{50}) of Vero and MDCK at the nonpermissive (39.5°C) and permissive (32.5°C) temperatures. Only plaqued viruses that showed at least a 10^4 differential CPE_{50} were utilized.

MDCK cells were infected with ts045 (15–20 plaque-forming units/cell) or ts61s (5–20 plaque-forming units/cell) in a small volume of minimal essential medium (MEM), which in the case of ts045 contained 75 μg/ml DEAE Dextran (Pharmacia Fine Chemicals, Piscataway, NJ). Cultures were kept for 1–1.5 h at 32.5°C in a 5% CO_2 incubator. The medium was then replaced with prewarmed MEM and incubation continued at 39.5°C for 4–5 h. To block exit from the Golgi apparatus by incubation at 18.5°C (22), the medium was replaced with MEM minus HCO_3^- (from a Gibco Laboratories [Grand Island, NY] kit), but containing 20 mM HEPES. Cultures were then transferred to an 18.5°C incubator without CO_2 for 80–90 min. When cultures were shifted to the permissive temperature, the medium was replaced with prewarmed new medium at 32.5°C and incubation continued at this temperature for the indicated time intervals. For immunofluorescence studies, infection was carried out on cells that were grown on 13-mm coverslips placed in 24-well dishes (Costar, Cambridge, MA). For preparation of frozen thin sections, cells that had been plated on collagen-coated 25-mm glass coverslips (~3 × 10^5 cells/cm²) were washed and then fixed with 2% glutaraldehyde (Polysciences Inc., Warrington, PA) in Dulbecco's phosphate-buffered saline (PBS) with $CaCl_2$ (0.1 mM) for 60 min at 4°C. Coverslips were washed in PBS and stored at 4°C until use.

Immunofluorescence Microscopy: Specimens fixed in 4% formaldehyde in PBS for 30 min were treated with 0.2% Triton X-100 and incubated with an IgG fraction (100 μg/ml) of rabbit anti-VSV glycoprotein (G protein) antibody, or a mixture of two mouse monoclonal anti-HA antibodies (a gift of Dr. R. G. Webster, St. Jude Children's Hospital) applied at a 1:300 dilution. Two preparations of rabbit anti-G protein antibody were used (one of which was the gift of Dr. J. Lenard, Rutgers University). Both were reactive with only the VSV G protein, as demonstrated by immunoprecipitation and Western immunoblots, and gave very low background labeling on uninfected cells. In a second step, samples were labeled with tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit or anti-mouse IgG (Cappel Laboratories, Cochranville, PA) as previously described (28). Preparations were examined in a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with a Wild camera (Wild Heerbrugg Instruments, Inc., Farmingdale,NY).

Immunoelectron Microscopy: Monolayers were scraped, infused with 0.6 M sucrose, and cryosectioned at −90°C using a Sorvall MT-2 ultramicrotome (DuPont Instruments-Sorvall Biomedical Div., Newtown, CT) with a DuPont freezing attachment (FTS). Sections 60–180 nm thick (estimated from the interference color after embedding and drying) were prepared essentially by the procedure of Tokuyasu and Singer (40, 41) with modifications described previously (15, 28). Frozen sections attached to grids were incubated in a first step with either 1 mg/ml of the rabbit anti-G protein IgG fractions or a 1:15 dilution of mouse monoclonal ascites fluid. In a second step, affinity-purified goat anti-rabbit or sheep anti-mouse IgG (Cappel Laboratories) conjugated to 18-nm colloidal gold particles was applied. The conjugation procedures used were similar to those described by Geuze et al. (11) with modifications detailed elsewhere (15, 28). Sections were examined in a Philips 301 microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) operated at 60 kV.

[³⁵S]Methionine Labeling: Infected cells on 35-mm dishes were preincubated at 39.5°C for 30 min with methionine-free MEM (from a Gibco Laboratories kit). Labeling was carried out in 0.5 ml of fresh medium containing [³⁵S]methionine (50 μCi/ml). After 30 min, cells were washed three times in MEM and incubated for an additional 30 min at 39.5, 32.5, or 18.5°C. Samples were solubilized with 0.5% NP-40, 0.5% deoxycholate in a 10 mM Tris-HCl buffer at pH 7.6. After removing nuclei by sedimentation, samples were analyzed by electrophoresis in a 10% polyacrylamide gel. Fluorography (5) was performed using Enhance (New England Nuclear, Boston,MA) and autoradiography with Kodak X-Omat film (Eastman Kodak Co., Rochester, NY).

RESULTS

Localization of the G protein in MDCK Cells Infected with the ts045 Mutant of VSV

IMMUNOFLUORESCENT LABELING: The pattern of immunofluorescence suggested that when MDCK cells infected with the ts045 mutant were maintained at 39.5°C, the newly synthesized G protein was retained in the ER. The cytoplasm of permeabilized cells was diffusely labeled, and the periphery of the nucleus was well marked, but neither the Golgi apparatus nor the plasma membrane were discernible (Fig. 1 a). Electrophoretic analysis of cultures incubated with [³⁵S]methionine (not shown) showed that the G protein synthesized at 39.5°C was of higher mobility than that found in infected MDCK cells incubated at the permissive temperature, and corresponded in apparent molecular weight to the core glycosylated form of G protein (18).

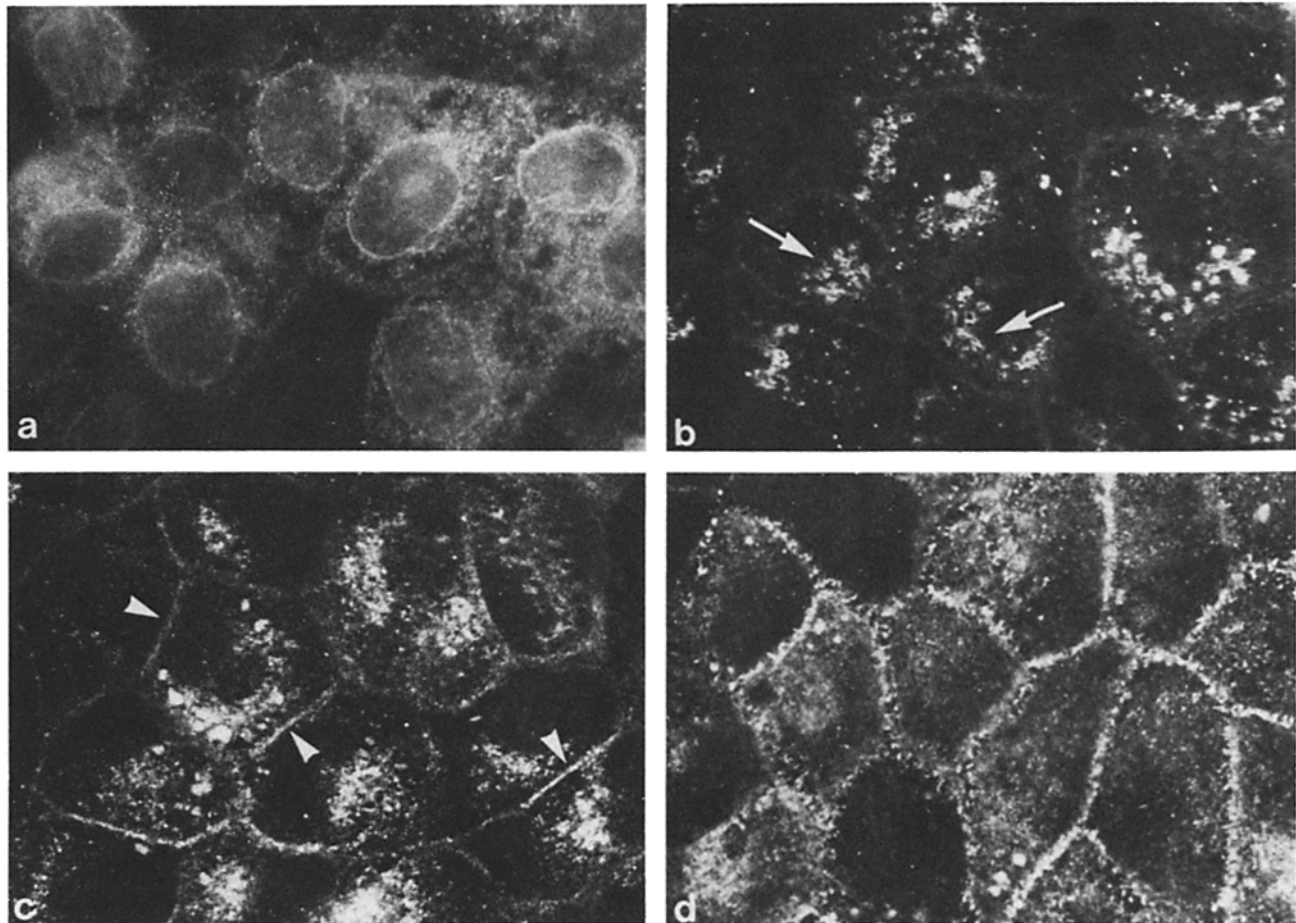As soon as 10 min after cells incubated at the nonpermissive

FIGURE 1   Immunofluorescent localization of G protein in cells infected with the ts045 mutant of VSV. MDCK cells were infected by incubation with the virus for 1 h at 32.5°C, maintained for 1 h at this temperature, and then incubated at 39.5°C for 4.5 h before a temperature shift to 32.5°C (the permissive temperature). At different times, monolayers were fixed in formaldehyde, permeabilized with 0.2% TX-100, and treated with rabbit anti-G protein antibody followed by rhodamine-conjugated goat anti-rabbit IgG. (a) In cells maintained at 39.5°C, fluorescence is observed around the nucleus and in a lacework of threadlike cytoplasmic elements, as expected from an accumulation of G protein in perinuclear and cytoplasmic ER cisternae. (b) After 15 min at 32.5°C, the G protein is concentrated in the crescent shaped Golgi region surrounding the nucleus (arrows). (c) At 30 min, significant label is also apparent in the lateral domain of the plasma membrane (arrowheads). (d) By 120 min, lateral domains are predominantly labeled. Photo exposure times were (a) 1 min, (b and c) 30 s, and (d) 20 s. × 2,900.

temperature were transferred to 32.5°C, a crescent-shaped juxtanuclear region of the cytoplasm became intensely fluorescent, as expected from the transfer to and concentration of the G protein in the Golgi apparatus. The intensity of labeling in the juxtanuclear region continued to increase for the next 5–10 min (Fig. 1 b), when fluorescence in the lateral plasma membrane began to be detected. By 30 min after the temperature shift, the accumulation of G protein in lateral regions of the plasma membrane was very apparent (Fig. 1 c), and intense labeling of the lateral plasma membranes was the most notable feature of cells that had been incubated for 2 h at 32.5°C before fixation (Fig. 1 d).

IMMUNOELECTRON MICROSCOPIC OBSERVATIONS: Examination of ultrathin frozen sections immunolabeled with the gold-particle technique showed that in infected cells maintained at 39.5°C the G protein was present in tubulovesicular and cisternal elements, some of which could be identified as belonging to the rough ER, as well as in the nuclear envelope and in small round dense bodies resembling lysosomes (Fig. 2a). Very few gold particles were found on Golgi cisternae, although they were frequently seen in vesicular elements that

surrounded the Golgi area. Labeling of the plasma membrane was minimal and when this occurred it took place preferentially on lateral membranes.

The distribution of the G protein changed soon after shifting to the permissive temperature: by 15 min, gold particles labeled the Golgi stacks (Fig. 2b). As was previously shown in cells infected with the wild-type virus (28), the G protein seemed to be randomly distributed over the Golgi cisternae. Beginning at 15 min and becoming more prominent at later times, labeled small vesicular structures (60–120 nm) were seen near the lateral plasma membrane (Fig. 3a). 30 min after shifting to the permissive temperature, focal labeling of the lateral membrane was conspicuous (Fig. 3, a and b), particularly in regions where the two adjacent lateral membranes were well separated, as frequently happened just below the tight junctions (Fig. 3a). However, the initial appearance of gold particles was not restricted to these regions and labeling seemed to take place at any point on the lateral plasma membrane (Fig. 3b). At these early times (up to 30 min after the shift), virtually no labeling of apical or basal surfaces was detected. During the first hour after transfer to 32.5°C, the G
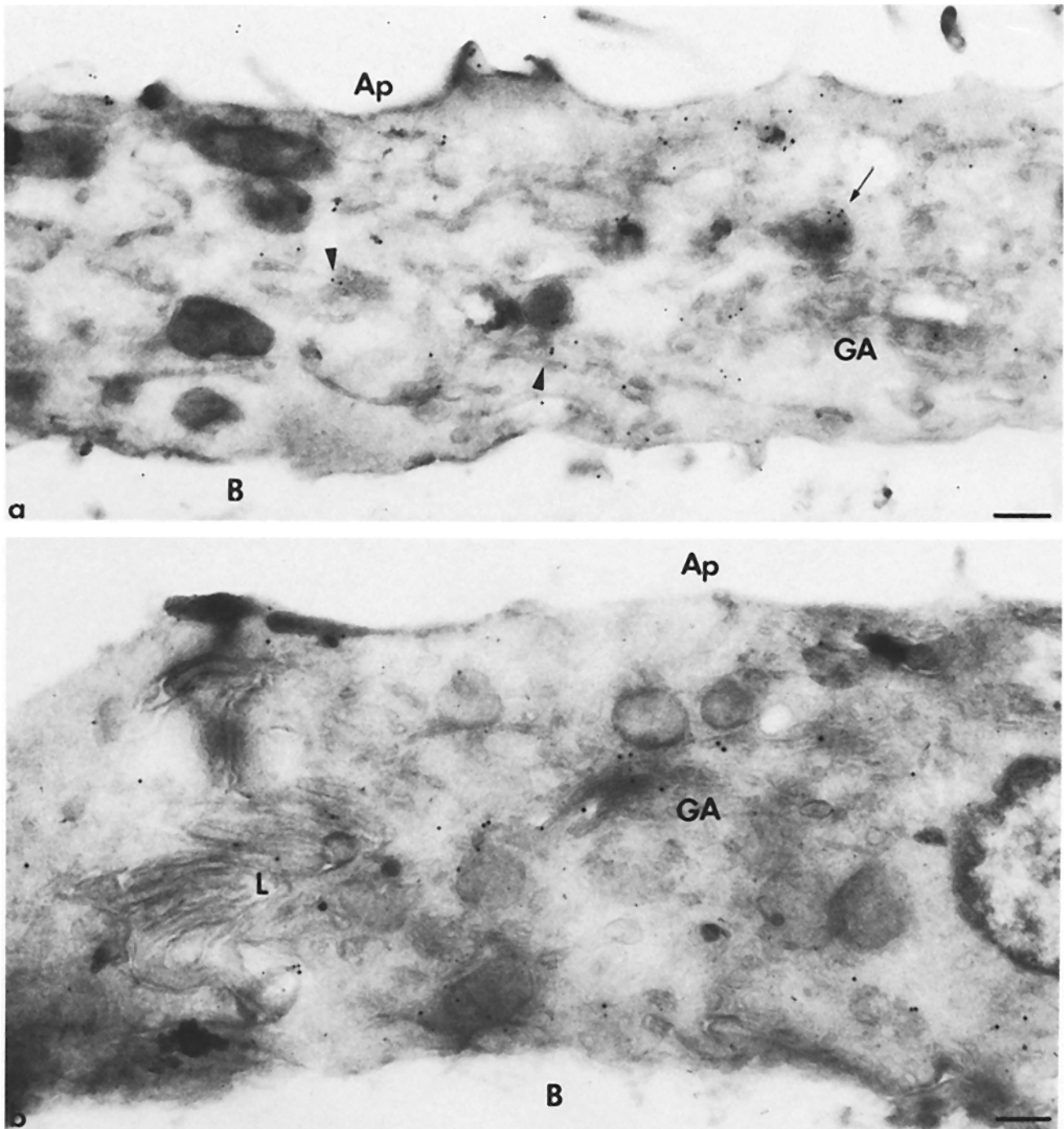
FIGURE 2   Intracellular distribution of the G protein at the nonpermissive temperature and shortly after shift to the permissive one in MDCK cells infected with the ts045 mutant of VSV. Monolayers infected as described in the legend to Fig. 1 were fixed in glutaraldehyde and cryosectioned according to the procedures detailed in Materials and Methods. Sections were treated with rabbit anti-G protein antibodies and goat anti-rabbit second antibodies complexed to colloidal gold particles (18 nm). (a) In monolayers kept at 39.5°C, the G protein is present primarily in ER cisternae (arrowheads) and multivesicular bodies (arrow). Stacks of Golgi cisternae (GA) were generally free of label, but frequently gold particles were found in vesicular structures at the Golgi periphery. Very few gold particles were associated with the apical (Ap) and basal (B) plasma membranes. (b) 15 min after a temperature shift to 32.5°C, labeling for G protein is prominent in the Golgi apparatus (GA), but only very small amounts of G were detected in the lateral (L) plasma membrane. Apical (Ap) and basal (B) surfaces are virtually unlabeled. Bar, 0.2 µm. × 45,000.

protein continued to accumulate over the lateral plasma membrane, and after this period, heavy labeling of this area was striking in many cells (Fig. 4a). The basal surface also contained substantial amounts of G protein, and in a small fraction of the infected cells (not shown), the appearance of

G protein on the apical surface was first noted at this time. However, 2 h after the temperature shift—when the first budding of VSV particles was observed on lateral aspects of the cells (Fig. 4b)—gold particles could be found on all aspects of the cell surface in many cells. In a number of instances it
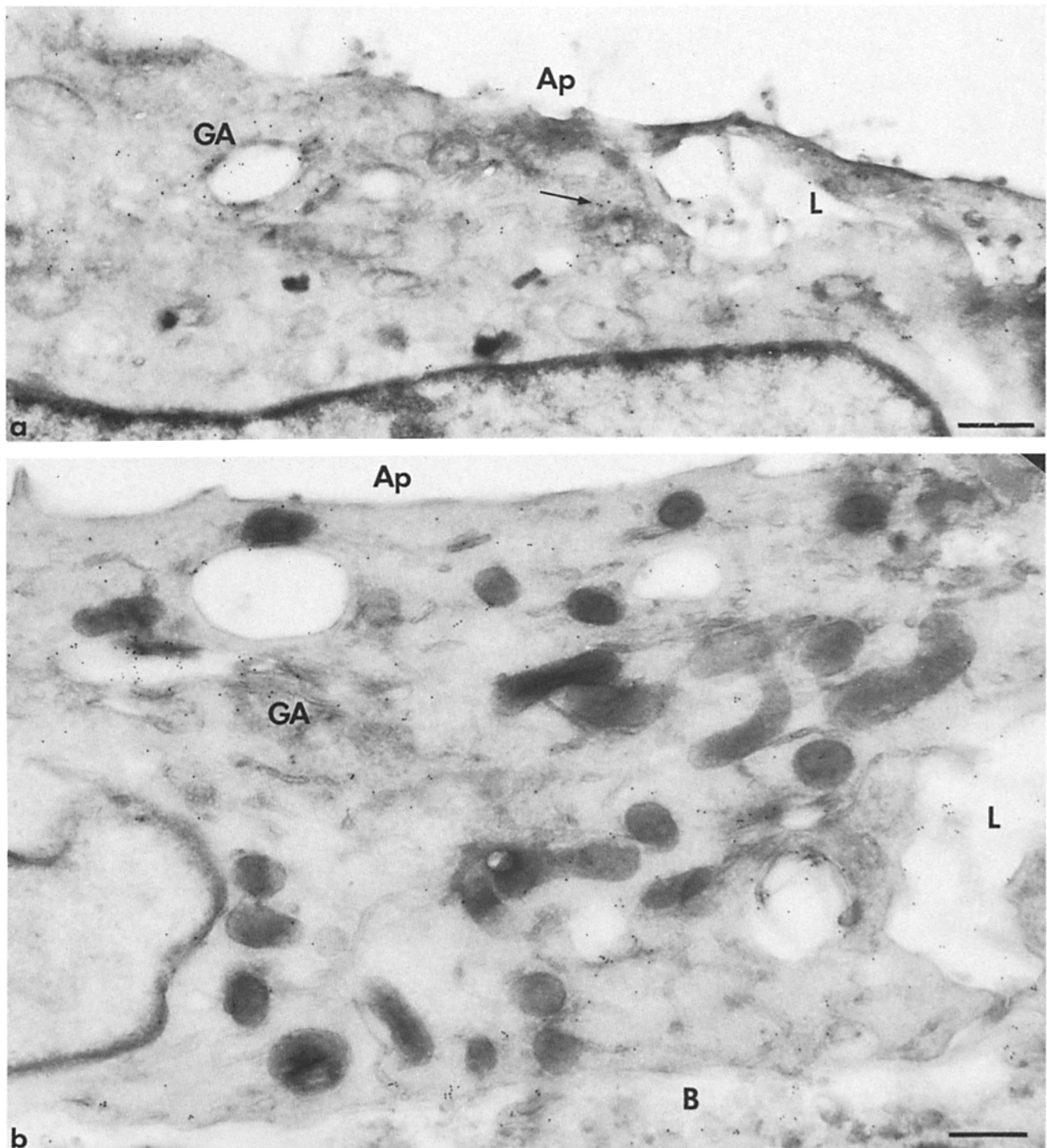
FIGURE 3 First appearance of the G protein on the lateral surface 30 min after a temperature shift to 32.5°C. (a and b) By 30 min after a shift to 32.5°C, the G protein began to accumulate in regions of the lateral plasma membrane (L) facing dilated intercellular spaces, either proximal to the tight junction (a) or closer to the base of the cell (b). A group of vesicular elements (a, arrow) near to the lateral surface is also labeled. The apical (Ap) and basal (B) surfaces remain very sparsely labeled, in contrast to stacks of the Golgi cisternae (GA) where gold particles are now abundant. Monolayers were infected and sections prepared as described in Fig. 2. Bar, 0.5 μm. × 25,000.

was apparent that initial labeling of the apical membrane domain was restricted to regions closest to the intercellular junction (Fig. 5, a and b), as if G protein, which had accumulated in the lateral region, had now diffused through the junctions. Even though subsequently the distribution of the G protein was not limited to basolateral domains, lateral regions of the plasma membrane always remained the most intensely labeled and, as was the case with the wild-type virus (28), even at this and later times budding of VSV particles occurred only on lateral and basal surfaces.
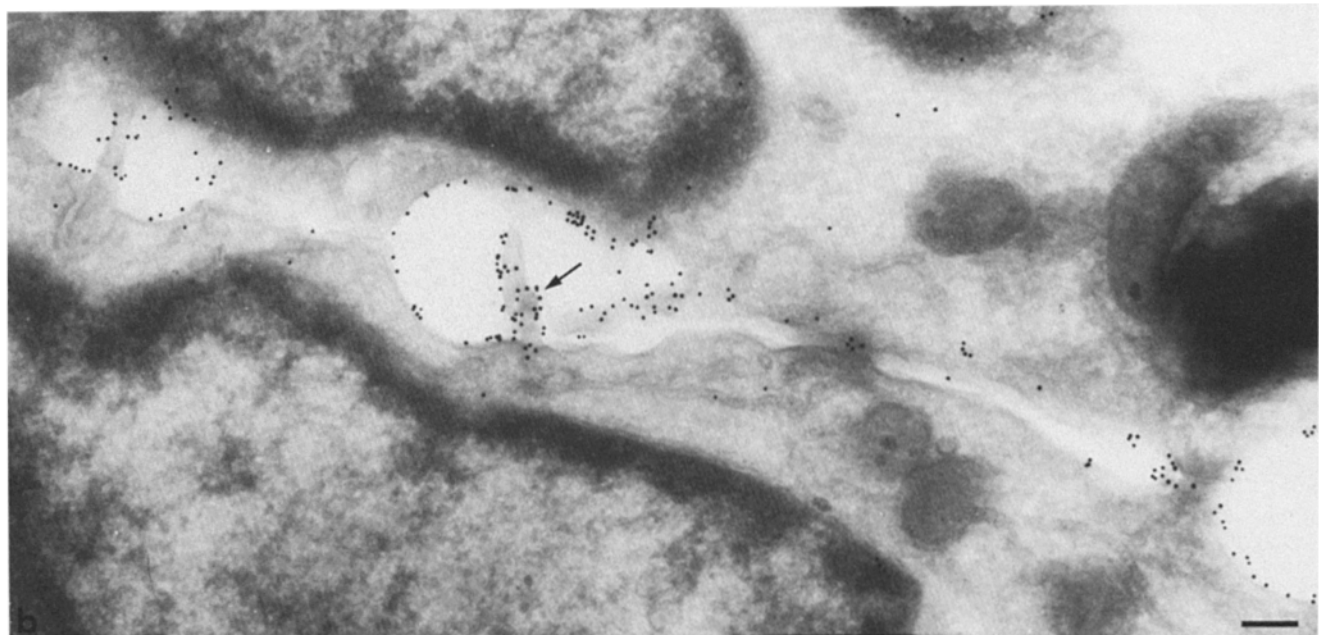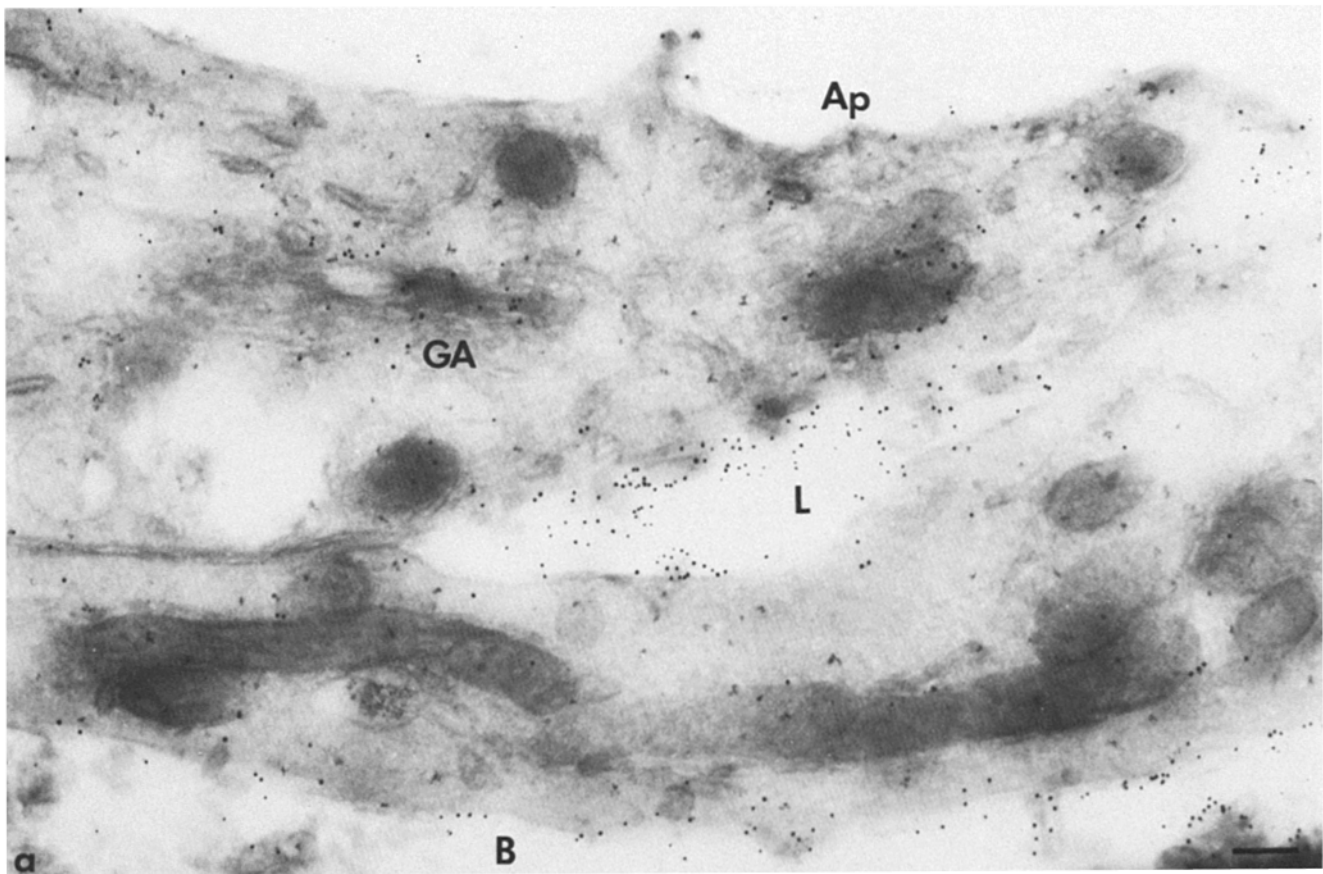
FIGURE 4 Accumulation of the G protein in the lateral and basal surfaces 1–2 h after the temperature shift. (a) By 60 min after the temperature shift to 32.5°C, the lateral aspect of the plasma membrane (L) is heavily labeled. Gold particles are also found on the basal surface (B) of the cell. In contrast, the apical domain of the plasma membrane (Ap) is essentially free of label, while labeling of the Golgi complex (GA) continues to be prominent. Bar, 0.2 μm. × 42,000. (b) 2 h after the temperature shift, virions (arrow) are frequently seen budding into the lateral intercellular spaces. Bar, 0.2 μm. × 38,000. Cells were infected and frozen thin sections were labeled as outlined in Fig. 2.

Localization of the HA Glycoprotein in MDCK Cells Infected with the ts61s Mutant of Influenza Virus

Electrophoretic analysis of products synthesized in infected cells incubated with [^{35}S]methionine demonstrated that a fast-migrating form of HA was synthesized at 39.5°C and that, upon temperature shift to either 32.5 or 18.5°C, this was replaced by a slower-migrating form (not shown) of the protein shown. This result was essentially the same as that reported for wild-type influenza HA as the protein matures during its passage through the Golgi apparatus from an en-
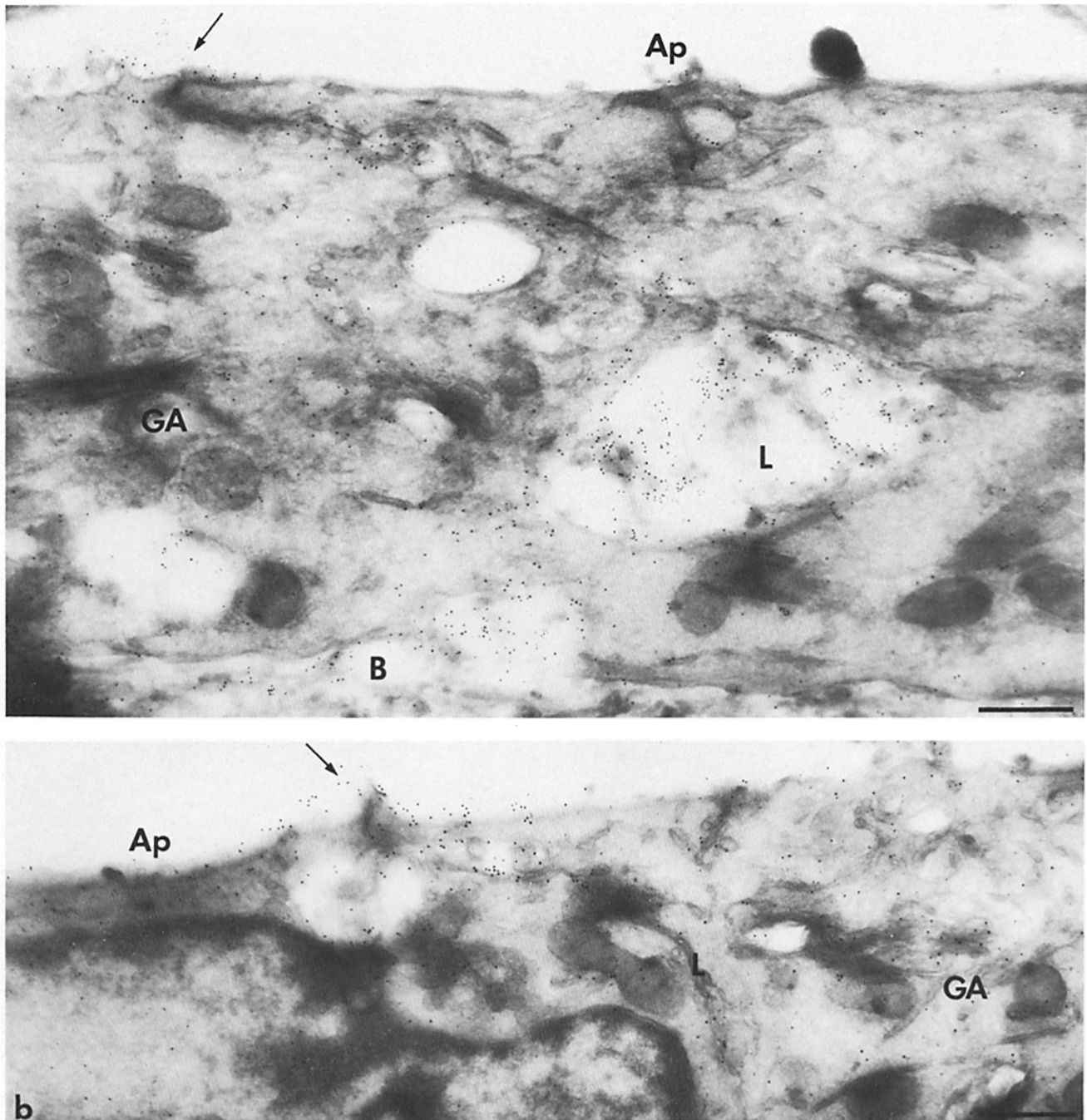
FIGURE 5 Appearance of the G protein on the apical surface late after the temperature shift. (a and b) 180 min after the shift to 32.5°C, when labeling was prominent on the lateral and basal surfaces (L, B) and in the Golgi apparatus (GA), gold particles were also found on the apical surfaces, often concentrated near the junctional areas (arrows). Specimens were prepared as indicated in Fig. 2. Bar, 0.5 μm. × 30,000.

doglycosidase H-sensitive form to a terminally glycosylated glycoprotein (1, 22, 31).

IMMUNOFLUORESCENT LABELING: No significant labeling of the apical plasma membrane was observed when anti-HA antibody was applied to MDCK cells that, after infection with the ts61s mutant, were kept at 39.5°C. When these cells were permeabilized with detergent, however, a weak and diffuse cytoplasmic labeling was detectable, consistent with the presence of the protein in the ER (Fig. 6a). 10–15 min after the shift to the permissive temperature (32.5°C), fluorescence in the juxtanuclear region presumed to contain

the Golgi apparatus was apparent (Fig. 6b). In addition, after 20–30 min, labeling of the plasma membrane began to be prominent (Fig. 6, c–f). An even better synchrony in transport of HA to the plasma membrane was obtained when, before transfer to 32.5°C, MDCK cells infected with the ts61s mutant were maintained for 90 min at 18.5°C. It has previously been shown, by using the wild-type virus (22), that incubation at 18.5°C prevents release of HA at the cell surface and leads to its accumulation in the Golgi region of the infected cells. A temperature shift to 18.5°C had the same effect in cells infected with the ts61s mutant: HA fluorescence became striking
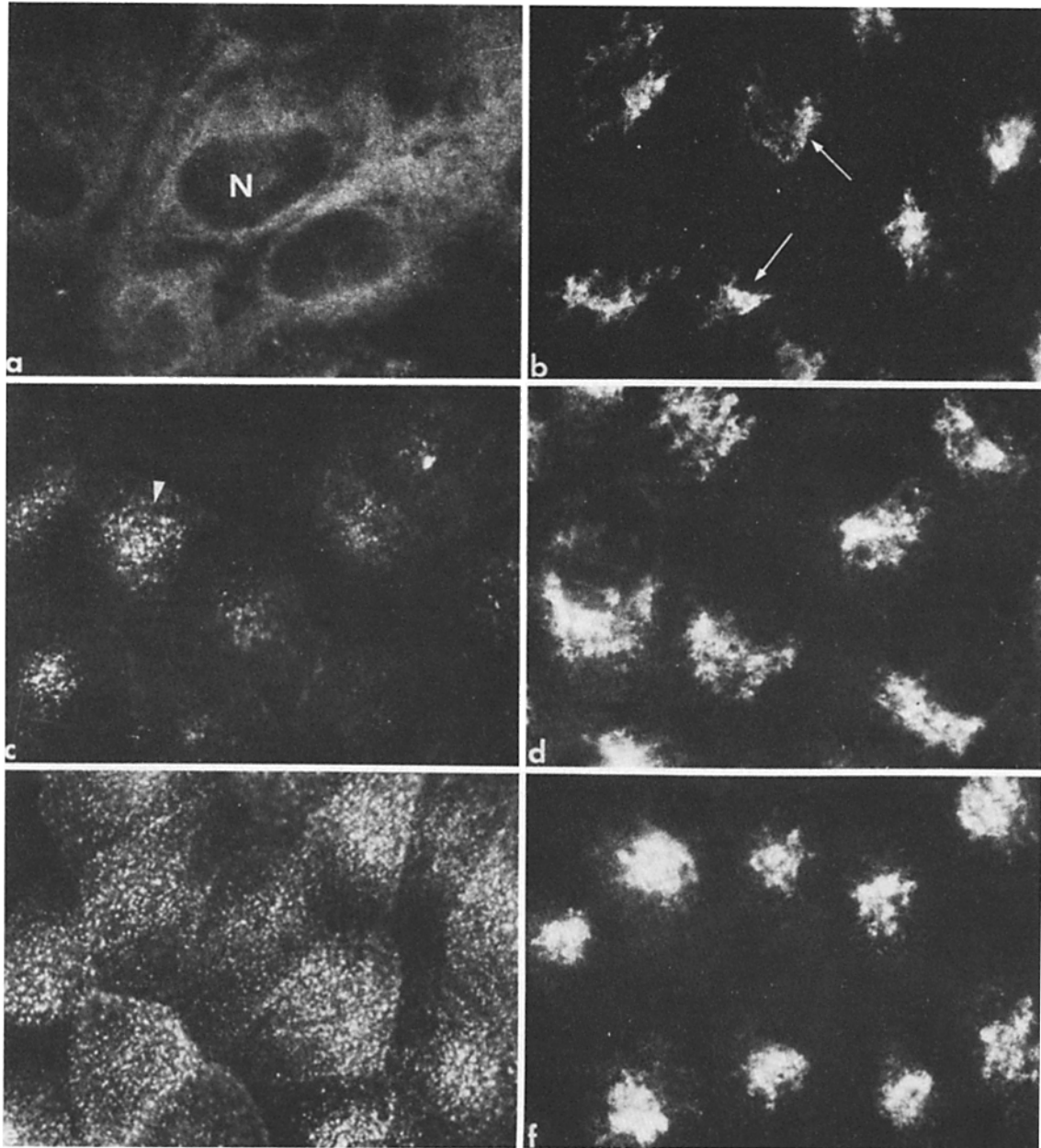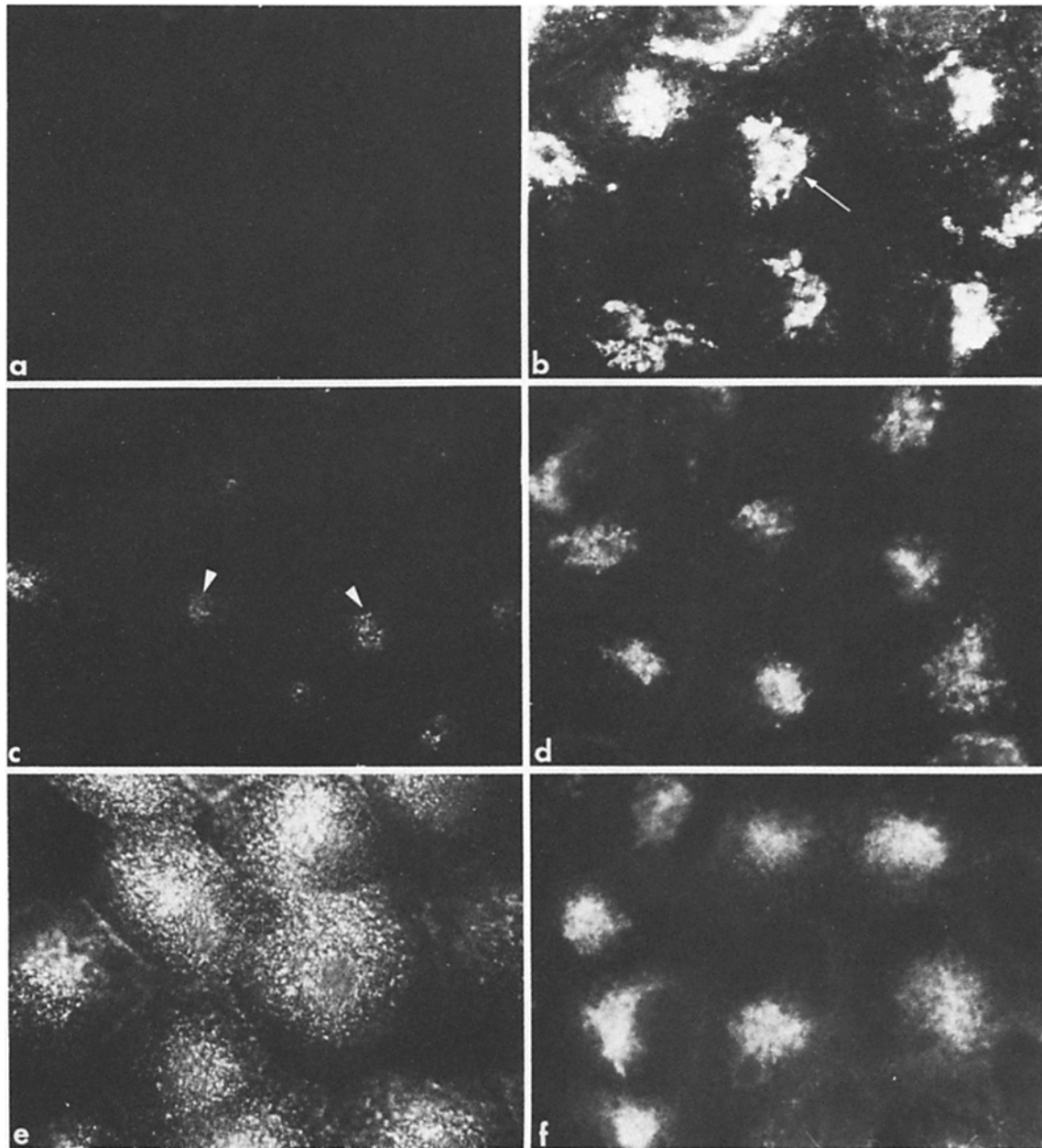
FIGURE 6 Immunofluorescent localization of HA in cells infected with the ts61 mutant of influenza. MDCK cells were infected by incubation with the virus for 1.5 h at 32.5°C, and then incubated for 5 h at 39.5°C, before a temperature shift to 32.5°C (the permissive temperature). At different times, formaldehyde-fixed monolayers were treated either directly with monoclonal anti-HA, to label the apical plasma membrane (c, e), or after permeabilization with 0.2% TX-100, to label cytoplasmic structures now accessible to the antibodies (a, b, d, f). Rhodamine-conjugated goat anti-mouse IgG was applied in a second step. (a) In cells maintained at the nonpermissive temperature (39.5°C), a low level of cytoplasmic staining is detectable. The nucleus (N) is unlabeled. (b) 15 min after a temperature shift to 32.5°C, the HA is concentrated in the Golgi region (arrows). No labeling of the apical surface was detectable at this time. (c) 30 min after the shift the HA protein is found on the apical surface (arrowhead). (d) At this time the Golgi region remains heavily labeled but there is little detectable label in the lateral membrane. (e) After 60 min the apical plasma membrane is uniformly fluorescent. (f) At this time the Golgi is still a major site of accumulation but the lateral membrane contains little detectable HA. Photo exposure times were (a) 2 min, (c, e) 30 s, and (b, d, f) 15 s. × 2,900.

in a crescent-shaped region located towards one side of the nucleus, thought to represent the Golgi apparatus (Fig. 7b), but no label could be detected on the apical surface of nonpermeabilized cells (Fig. 7a). After the temperature was raised to 32.5°C, the HA that had accumulated intracellularly was rapidly transferred to the cell surface. Some cells manifested apical fluorescence 5 min after the shift, but only after 10–20 min was surface staining readily observed on most cells (Fig. 7c). A single fluorescent spot was found on the apical

membrane in an off-center position reminiscent of the asymmetric location of the Golgi apparatus within the cell (Fig. 7d). By 30–40 min, however, the initially concentrated molecules had diffused extensively over the entire apical surface, and by 45 min the brighter spot, which indicated the initial site of appearance of HA, was all but unrecognizable (Fig. 7e). At no time during the first 45 min was a significant amount of fluorescent label detectable on the lateral plasma membrane (Fig. 7, d and f).

FIGURE 7 Immunofluorescent localization of HA in cells infected with the ts61 mutant of influenza and subjected to an 18.5°C block to synchronize transfer to the cell surface. MDCK cells were infected by incubation with the virus for 1.5 h at 32.5°C, maintained for 5 h at 39.5°C, and then for 1.5 h at 18.5°C before a temperature shift to 32.5°C. Individual coverslips of formaldehyde-fixed cells were labeled as described in the legend to Fig. 1, either directly (a, c, e) or after permeabilization with detergent (b, d, f). (a and b) At 18.5°C, HA has not reached the plasma membrane (a) but accumulates in the Golgi apparatus (b) (arrow), which is heavily labeled. (c and d) 10 min after the temperature shift to 32.5°C, the HA is initially detected on the apical surface (c) (arrowheads), in off-center focal regions. Little or no fluorescence is found on the lateral surfaces at this time, but the Golgi region remains intensely labeled (d). (e and f) 45 min after the shift the HA is found over the entire apical surface (e) and in the Golgi apparatus (f). Labeling of the lateral surface is extremely low. Photo exposure times were (a, c, e) 30 s and (b, d, f) 15 s. × 2,900.

IMMUNOELECTRON MICROSCOPIC OBSERVATIONS: In ts61s-infected cells maintained at the nonpermissive temperature (39.5°C), the level of immunolabeling was quite low (not shown), but occasional grains that resembled ER cisternal elements were seen on cytoplasmic vesicles and structures. Upon temperature shift to 18.5°C, when fluorescence of the juxtanuclear region was striking, the gold particles were more frequently found in the Golgi region. However, they were not particularly concentrated over the stacked cisternae but were mainly found on their dilated ends and over associated vesicular elements (Fig. 8 a). In agreement with the observations using fluorescence, virtually no label was observed at this time on the plasma membrane. Only after the temperature was raised did the HA begin to appear on the apical membrane. In some cells it was detected as soon as 5 min after the shift (Fig. 8 b), but after 20 min the surface localization was apparent in most cells. Interestingly, HA first appeared in discrete regions, near and often directly above Golgi elements, sug-
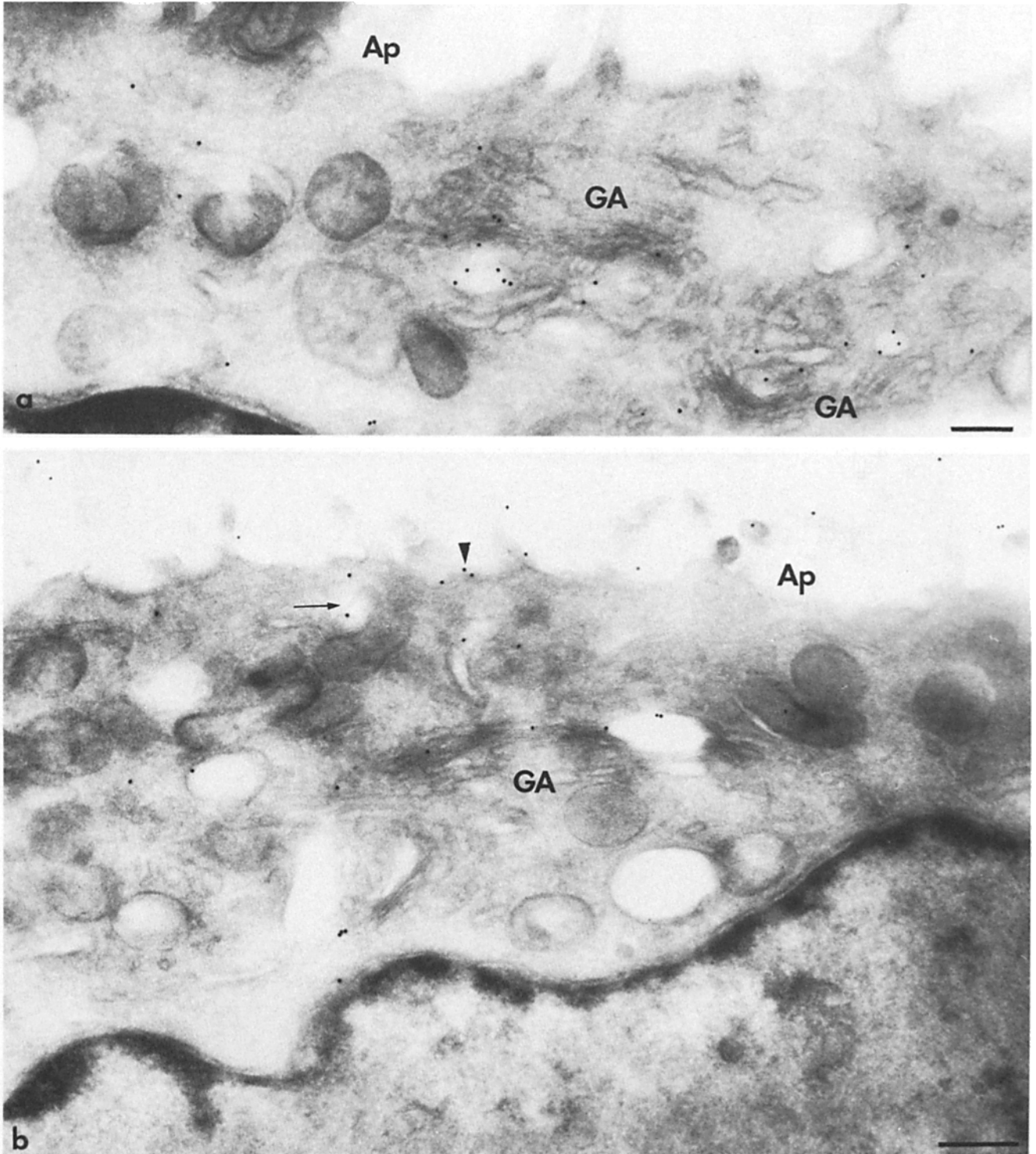
FIGURE 8 Intracellular distribution of HA during the cold-temperature block and shortly after shift to the permissive temperature in MDCK cells infected with the ts61 mutant of influenza. Cells were infected by incubation with the virus for 1.5 h at 32.5°C and then maintained for 5 h at 39.5°C and 1.5 h at 18.5°C before shift to 32.5°C. Cryosections of glutaraldehyde-fixed monolayers were treated with monoclonal anti-HA antibodies followed by colloidal gold–goat anti-mouse IgG complexes. (a) In cells maintained at 18.5°C HA is found in Golgi apparatus (GA). Two sets of Golgi cisternae, possibly interconnected, are presented, each with a concentration of gold particles over vesicular elements toward one side of the stack. Apical plasma membranes (Ap) are unlabeled. Bar, 0.2 μm. × 50,000. (b) 5 min after the shift to 32.5°C HA initially appears on the apical plasma membrane (Ap), in focal regions (arrowhead) that are not far from the Golgi apparatus (GA). Labeled vesicles are also found near the apical surfce (arrow). Very little labeling was seen on lateral or basal surfaces (not shown). Bar, 0.5 μm. × 30,000.

gesting that its transport from the Golgi apparatus to these sites was direct (Figs. 8b and 9). At these and later times, labeled vesicles with an apparently smooth surface and an often oblong shape with an average diameter varying between 60 and 120 nm (Figs. 8b, 9, and 10a) were also visible underlying the apical membrane. It may be presumed that
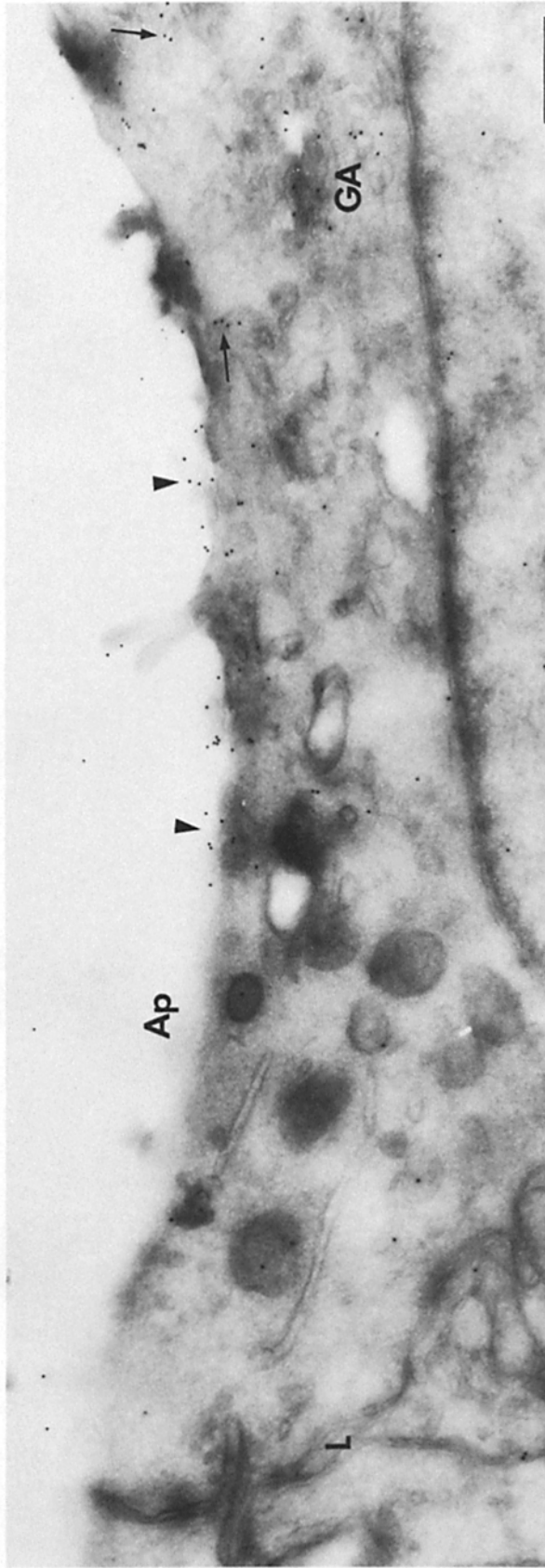
FIGURE 9   Focal appearance of HA in the apical plasma membrane after release from the cold temperature block. 20 min after transfer from 18.5°C an off-center focal region of the apical plasma membrane (Ap) shows a high concentration of HA (arrowheads) whereas areas of apical membrane near the intracellular junction are devoid of label. A labeled Golgi apparatus (GA) is found near the site of accumulation of HA in the plasma membrane, and labeled vesicles are present in the cytoplasm near the cell surface (arrows). At this time the lateral plasma membrane (L) is free of label. Specimens were infected and labeled as in Fig. 8. Bar, 0.5 μm. × 30,000.
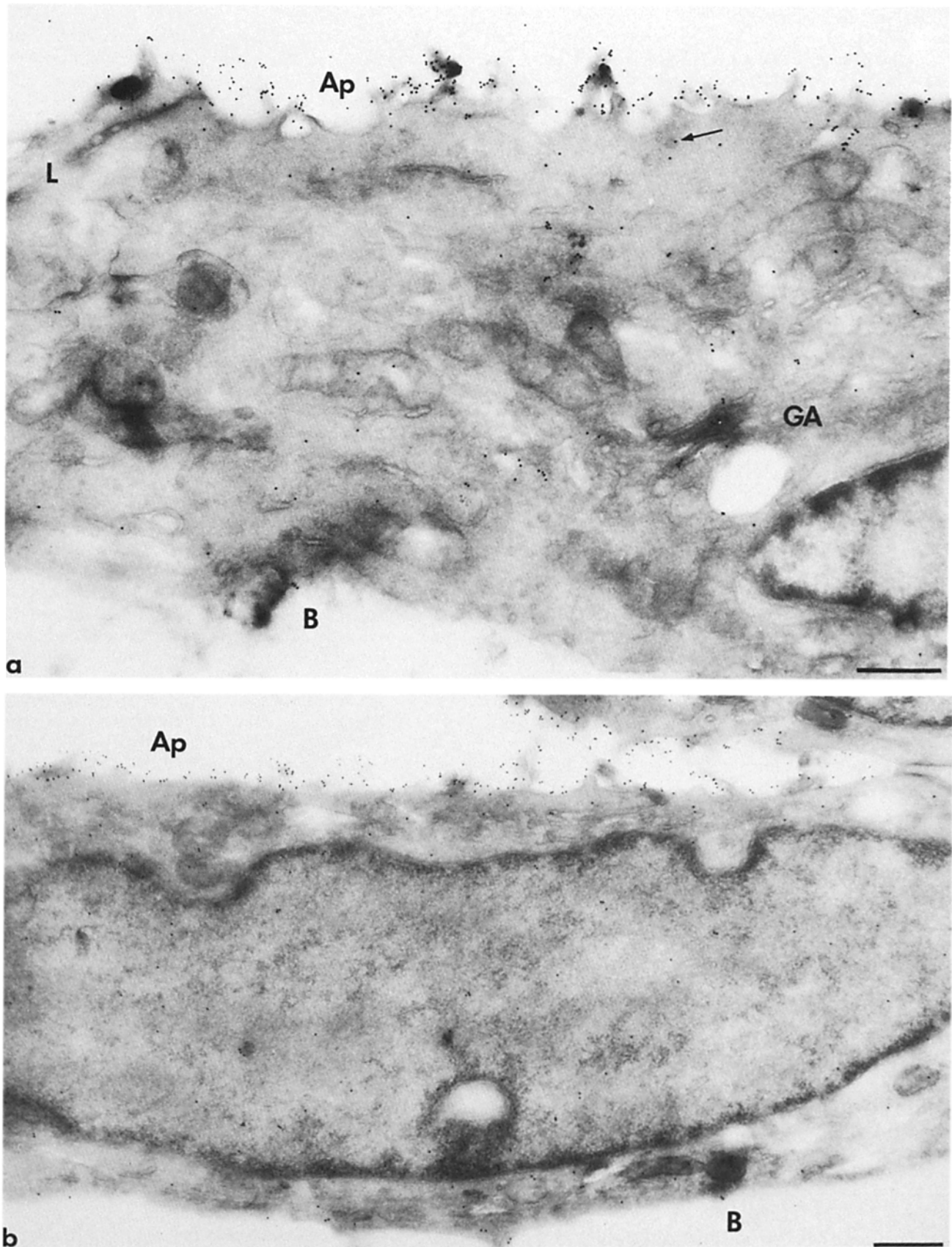
FIGURE 10 Distribution of the HA in the apical membrane 1–2 h after the shift to 32.5°C. (a) 60 min after the temperature shift from 18.5 to 32.5°C, the HA that has reached the cell surface is uniformly distributed on the apical domain (Ap). HA is also present in the Golgi apparatus (GA) and some cytoplasmic vesicular elements (arrow). Virtually no label is associated with the lateral (L) or basal (B) plasma membrane domains. Bar, 0.5 μm. × 30,000. (b) Even 2 h after the temperature shift HA remains concentrated on the apical membrane and only occasional gold particles are present on the basolateral domain (B). Cells were infected and sections prepared as described in Fig. 8. Bar, 0.5 μm. × 25,000.

these vesicles are involved in the transport of HA to the plasma membrane. Subsequently, the level of labeling increased significantly and HA was found over the entire apical surface of many cells (Fig. 10). Throughout the first 2 h at 32.5°C, however, there was no accumulation of HA over the lateral or basal plasma membrane, although a few cells had a very low level of labeling on their lateral surfaces.

IMMUNOLABELING OF CELLS FIXED IN A HYPERTONIC BUFFER: Tokuyasu (40) and Chen and Singer (8) have cautioned that the intensity of cross-linking during fixation can limit the ability of antibodies to penetrate the frozen sections. To determine whether the cross-linking of tightly apposed lateral surfaces of adjacent cells within MDCK monolayers creates a significant barrier to antibody penetration, we carried out immunolabeling of ts61s-infected monolayers, which were fixed in a hypertonic solution (1.5 osM) (Fig. 11). Under these conditions the cells shrink considerably and the lateral membranes of adjacent cells separate. Although intracellular details are difficult to discern in the highly condensed cytoplasm of these specimens, the lateral surfaces of many cells should become accessible to the antibodies. Background surface labeling measured in cells maintained at 18.5°C was 0.2–0.3 particles/μm. At very early times (10–15 min) after the temperature shift to 32.5°C, when surface labeling was becoming apparent (0.7–1 particles/μm), some cells were found that had as much label on the lateral surfaces as on their apical ones (Fig. 11a). Subsequently, however (at 40 min and later), many cells showed the clear and often overwhelming labeling of the apical surface already detected in conventionally fixed specimens. In most cells examined at 40 min or later, the density of labeling of the lateral membrane was either at background levels or slightly elevated but not higher than 1 particle/μm (Fig. 11b). These results suggest that a small flux of HA to the lateral surface occurred at early times after the temperature shift.

DISCUSSION

In previous work, using doubly infected MDCK cells (28), we demonstrated the simultaneous presence of the G protein of VSV and the HA of influenza in the same cisternae of the Golgi apparatus. We therefore concluded that sorting of these glycoproteins cannot take place before their arrival at this organelle. The observations presented in this paper, with cells infected with temperature-sensitive mutants of the same viruses, demonstrate that the G protein is transferred directly from the Golgi apparatus to the lateral aspect of the plasma membrane, whereas the bulk of HA is transferred to the apical domain. Sorting of these proteins must, therefore, be intracellular and may occur in the Golgi apparatus, upon exit from this organelle, or along the route to the cell surface.

As expected from previous observations with Vero (16) and Chinese hamster ovary (4) cells, at the nonpermissive temperature G protein was distributed throughout the cytoplasm. Labeling was observed on tubular and vesicular elements that, although without the typical cisternal configuration, are likely to belong to the ER. Occasionally, labeling was also found on lysosomes, probably reflecting some degradation of the abnormal polypeptides synthesized at the nonpermissive temperature or the presence of residual material phagocytosed during the initial infection. Golgi cisternae became labeled only after the shift to 32.5°C, and this was followed by the rapid deployment of G at the lateral cell surface. The sequence of events and the presence of labeled vesicles between the

Golgi and the lateral cell surface left no doubt as to the directness of the pathway, which is schematically represented in Fig. 12.

It has been proposed (38, 39) that coated vesicles are involved in the transfer of the G protein from the ER to the Golgi apparatus and from this organelle to the plasma membrane. However, neither the labeled vesicles found at 39°C nor those we detected near the cell surface after the shift to the permissive temperature appeared to be of the clathrin-coated variety. Their size and shape varied more widely than those of coated vesicles (which are uniformly spherical and do not generally exceed 50 nm in diameter as measured on sections of Epon-embedded MDCK cells). In most cases, the thickness of the frozen sections obscured the actual visualization of a clathrin coat; however, other investigators, using immunocytochemistry in detergent permeabilized specimens, were equally unsuccessful in establishing the presence of G protein in coated structures of VSV-infected 3T3 cells (43).

Our work clearly indicates that newly synthesized G protein is delivered to the lateral and not the basal aspects of the plasma membrane, even though these two regions of the cell surface are not separated by recognizable barriers that could maintain an heterogeneous distribution of membrane components. In an autoradiographic study, Bennett et al. (2) observed that labeled glycoproteins first appeared at the lateral surface of intestinal cells. A regionalized insertion of viral glycoproteins in the apparently uniform plasma membrane of nonpolarized cells was first recognized in cells infected with Newcastle disease virus (20) and later in cells chronically infected with measles virus (9). More recently, Bergmann et al. (3) observed that in migrating fibroblasts the G protein of VSV is inserted in the plasma membrane at the leading edge of the cell, proximally to the Golgi region and its associated microtubule-organizing center. This led these authors to propose the existence of a cytoskeletally directed vectorial traffic of Golgi-derived vesicles to the plasma membrane. Whether in MDCK cells the existence of a directed route of this kind accounts for the localized insertion in one aspect of the basolateral domain cannot be established at this moment. Alternatively, specialized sites may exist in the lateral membrane for the insertion of newly synthesized proteins, or simply preferential delivery to this region may result from proximity to the Golgi apparatus.

We have previously noted (28) that, in the course of VSV infection, significant amounts of G protein eventually accumulate in the apical surface of the cell, before a measurable disruption of the junctional complexes. We have now observed that the appearance of G protein in the apical surface can take place near the intercellular junctions, where the protein was sometimes concentrated before other regions of the apical surface showed any labeling. This suggests that, after it accumulates in the basolateral domain, G protein may diffuse across the tight junctions to the apical domain. The diffusion of G protein could reflect a localized loss in junctional integrity, which was not detected by electrical resistance measurements. It is also possible that junctions undergo local disassembly and reassembly reactions that in a dynamic fashion may control the lateral diffusion of membrane components. A role of junctions in controlling the passage of leukocytes and horseradish peroxidase across intact monolayers of MDCK monolayers has recently been suggested (23).

The results with the ts61s mutant of influenza, and in particular the observations after the release of the low-tem-
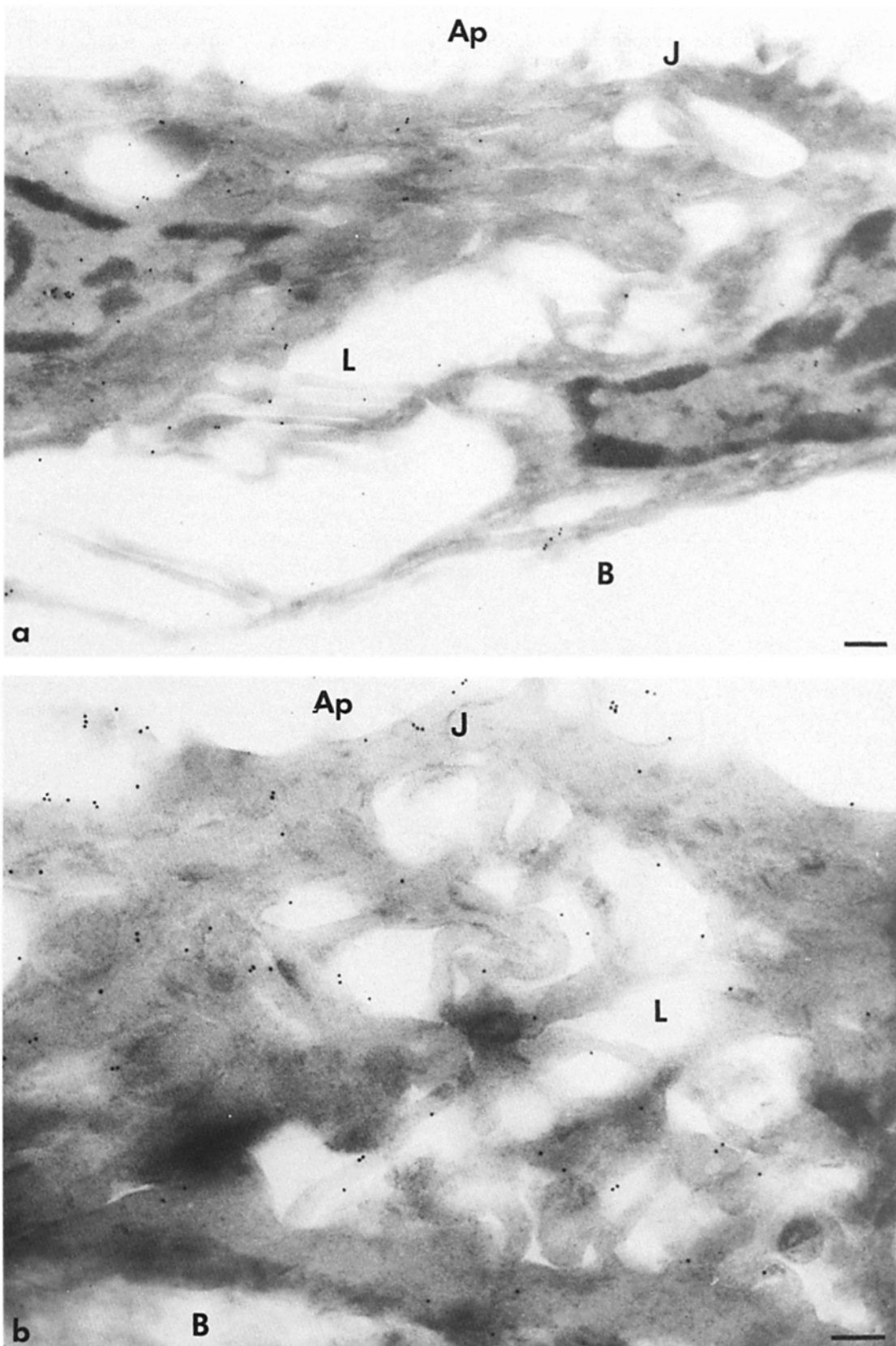
Figure 11 Immunolabeling of cells infected with ts61 and fixed in hypertonic buffer. MDCK monolayers infected and labeled as in Fig. 8 were fixed in a glutaraldehyde solution containing PBS at five times the normal concentration. This causes a shrinkage of the cells and a dilation of the intercellular spaces that should facilitate access of labeling reagents to the lateral membranes after cryosectioning. (a) 15 min after the temperature shift from 18.5 to 32.5°C, a low level of labeling (0.7 grains/μm) is observed below the junctional complex (J) on the lateral membrane (L). Bar, 0.2 μm. × 34,000. (b) At 40 min after the temperature shift, labeling of the lateral surface (L) remains low (1 grain/μm), but as expected, labeling of the apical surface (Ap) has increased considerably. The basal membrane remains virtually unlabeled (B). Bar, 0.2 μm. × 45,000.
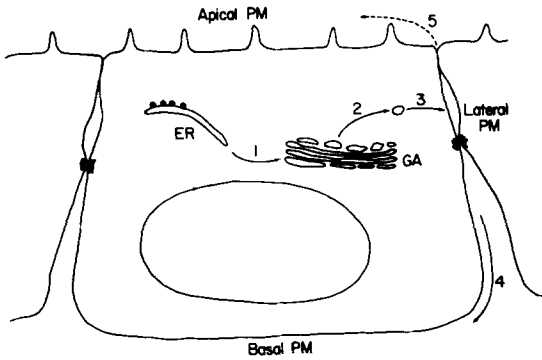
FIGURE 12 Stages in the transport pathway for the VSV G protein from the ER to the plasma membrane of MDCK cells. After synthesis in the rough ER, G protein is transferred to the Golgi apparatus (1), whence vesicles emerge (2) that will carry it to the lateral plasma membrane (3). Subsequently, the glycoprotein may migrate to the basal surface (4) and eventually to the apical domain, most likely after diffusion through the junctional complexes (5).

perature block led us to conclude that the major pathway followed by HA after it leaves the Golgi apparatus is directed to the apical plasma membrane (Fig. 13). Initially, at 18.5°C, the HA accumulated in Golgi cisternae and in vesicles located towards one side of Golgi stacks. This suggests that the low temperature impairs exit from the Golgi apparatus or imposes a post-Golgi arrest in the delivery to the plasma membrane. This is consistent with the original observation of Matlin and Simons (22), confirmed by our own data, that under these conditions HA may undergo terminal glycosylation, a process that is thought to take place in *trans*-Golgi cisternae (12, 34). Histochemical staining will be necessary to unequivocally prove the site of accumulation of HA at 18.5°C.

Soon after the cold-temperature block was relieved, the appearance of a brightly fluorescent spot revealed the focal insertion of HA in the apical surface. Immunoelectron microscopy also showed a high concentration of gold particles in an apical region, almost directly above the Golgi apparatus, as well as the presence of vesicles containing HA in the neighboring cytoplasm. These vesicles were detected soon after the shift to the permissive temperature, before any substantial accumulation of HA in the plasma membrane had taken place and therefore are not likely to be endocytic. As was the case with the vesicles carrying the G protein of VSV, and as was noted recently by Rodriguez-Boulan et al. (31), vesicular elements labeled with anti-HA antibodies were irregularly shaped and larger than typical coated vesicles.

Our observations on the appearance of HA in the central region of the apical membrane are in contrast to those of Louvard (19), who reported that, after its internalization was induced by antibody cross-linking, the apical enzyme leucyl aminopeptidase reappeared in regions of the plasma membrane near the intercellular junctions. This was also the site where leucyl aminopeptidase from an internal pool inaccessible to the antibodies was brought to the surface. The differences between our observations and those of Louvard (19) may be explained by the different experimental conditions. The massive amount of membrane retrieval induced by antibody cross-linking may, indeed, have altered the number of distribution of sites for insertion of membrane proteins in the apical surface. A recent study (31) on the transport of HA to the apical surface also reported the initial appearance of HA in the central region of the apical surface.

It has been proposed that in polarized intestinal cells the basolateral domain represents an intermediate stage in the pathway followed by apical plasma membrane components from the Golgi apparatus to their final destination (26, 27, 44). Evidence to support this notion was derived from cell fractionation studies on the biosynthesis of sucrase-isomaltase, an enzyme of the intestinal microvillus that is synthesized as a precursor which is cleaved upon arrival to the apical surface (13, 14). The kinetic analysis carried out by these authors showed that, soon after synthesis the uncleaved precursor of sucrase-isomaltase appeared in a cell fraction containing basolateral membranes, whence it disappeared when the mature protein was detected in a fraction rich in microvillar membranes. However, the same results would have been expected if the basolateral membrane fraction had been contaminated with Golgi membranes, a possibility that was not considered in this work. In other experiments that use enzyme histochemistry, it was reported that a small amount of aminopeptidase N (an apical membrane enzyme) was present in the lateral membrane of enterocytes below the tight junctions, but this could not be confirmed immunocytochemically (10).

We noted, in specimens labeled in hypertonic medium that distended the lateral spaces, the rapid appearance of a small number of HA molecules in the lateral membrane. This was not, however, consistently observed in all cells, nor did the extent of the labeling rise with time. It, therefore, seems unlikely that passage to the lateral membrane represents an obligatory intermediary stage in transport to the apical surface that would have to be extremely fast. Most probably this pathway to the cell surface reflects the saturation of the intracellular sorting and transport system, which takes place when the backlog of accumulated HA in the Golgi complex is released over a short period of time. It is not clear what the fate of the misdirected HA would be. The continuous recycling of plasma membrane components through the cytoplasm, which in epithelial cells is the basis for transcytosis (6, 29, 30), could provide a mechanism for the transport of HA to the apical surface. A similar mechanism has been postulated to account for the transport to the lateral surface of
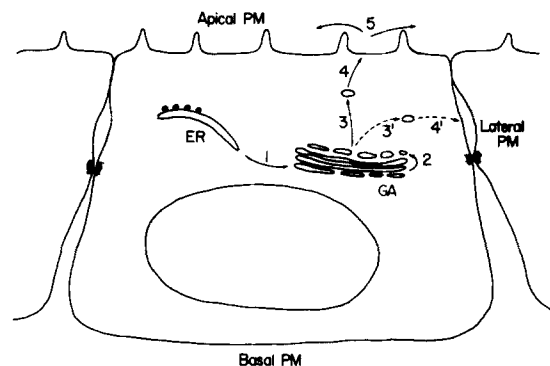

FIGURE 13 Stages in the transport pathway of the HA of influenza from the ER to the plasma membrane of MDCK cells. After synthesis in the rough ER, HA is transferred to the Golgi apparatus (1). At 18.5° (2) transport through the GA and processing of oligosaccharide side chains may occur and some HA is packaged into vesicles (3). Upon release of the cold temperature block, post-Golgi vesicles convey the glycoprotein to a region of the apical plasma membrane proximal to the Golgi apparatus (4). After insertion in this region, HA can then diffuse throughout the entire apical surface (5). A secondary pathway can direct small amounts of HA to the lateral plasma membrane (3', 4') where, however, the glycoprotein does not accumulate.

proteins inserted heterotopically in the apical domains (21, 25). Alternatively the lateral HA may be degraded as is the case with some misdirected G protein, which in the wild-type infections appeared at the apical surface (28).

It would be important to determine what features of the post-Golgi vesicles carrying the envelope proteins direct them to either the lateral or apical plasma membrane, or promote their selective fusion with one or the other membrane domain. In principle, vesicles that deliver proteins to opposite domains could differ only in the glycoprotein that they carry, in which case the Golgi apparatus would not contribute to the sorting. It is also possible that two types of vesicles of intrinsically different membrane composition could be derived from the Golgi apparatus. In any case, sorting of carrier vesicles could be effected by cytoskeletal elements that guide them to the appropriate cell surface domain. Alternatively or additionally, a restriction in the delivery of the glycoprotein to the proper domain could be imposed by specific plasma membrane proteins that determine the site of vesicle fusion. Recent work in our laboratory (Rindler, M. J., I. E. Ivanov, and D. D. Sabatini, manuscript in preparation) points to a role of the cytoskeleton in the sorting process. We have observed that treatment of influenza-infected cells with colchicine or other drugs that affect the structure of microtubules leads to the appearance of large amounts of HA in the basolateral domain of cells that still maintain intact intercellular junctions. Thus, by itself the presence of the glycoprotein appears insufficient to ensure that the vesicle be delivered to the proper domain, even though transport of the vesicle to the cell surface and its fusion with the plasma membrane seemed to proceed unaffected. A characterization of the vesicles transporting HA and G protein and a study of their interaction with the cytoskeleton and the plasma membrane should shed more light on the nature of post-Golgi sorting events that control the accumulation of proteins on each surface.

REFERENCES

1. Alonso-Caplen, F. V., and R. Compans. 1983. Modulation of glycosylation and transport of viral membrane glycoproteins by a sodium ionophore. *J. Cell Biol.* 97:659–668.
2. Bennett, G., C. P. Leblond, and A. Haddad. 1974. Migration of glycoprotein from the Gogli apparatus to the surface of various cell types as shown by radioautography after labeled fucose injection into rats. *J. Cell Biol.* 60:258–284.
3. Bergmann, J. E., A. Kupfer, and S. J. Singer. 1983. Membrane insertion at the leading edge of motile fibroblasts. *Proc. Natl. Acad. Sci. USA.* 80:1367–1371.
4. Bergmann, J. E., K. T. Tokuyasu, and S. J. Singer. 1981. Passage of an integral membrane protein, the vesicular stomatitis virus glycoprotein through the Golgi apparatus en route to the plasma membrane. *Proc. Natl. Acad. Sci. USA.* 78:1746–1750.
5. Bonner, W. M., and R. A. Laskey. 1984. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
6. Brown, W. R., Y. Isobe, and P. K. Nakane. 1976. Studies on the translocation of immunoglobulins across epithelium. II. Immunoelectron-microscopic localization of immunoglobulins and secretory component in human intestinal mucosa. *Gastroenterology.* 71:985.
7. Cereijido, M., E. S. Robbins, W. J. Dolan, C. A. Rotunno, and D. D. Sabatini. 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* 77:853–880.
8. Chen, W.-T., and S. J. Singer. 1982. Immunoelectron microscopic studies of the sites of cell-substratum and cell-cell contacts in cultured fibroblasts. *J. Cell Biol.* 95:205–222.

9. Ehrnst, A., and K. G. Lundquist. 1975. Polar appearance and nonligand induced spreading of measles virus hemagglutinin at the surface of chronically infected cells. *Cell.* 5:351–359.
10. Feracci, H., A. Bernadac, J. P. Gorvel, and S. Maroux. 1982. Localization by immunofluorescence and histochemical labeling of aminopeptidase N in relation to its biosynthesis in rabbit and pig enterocytes. *Gastroenterology.* 82:317–324.
11. Geuze, H. J., J. W. Slot, P. A. van der Ley, and R. C. T. Scheffer. 1981. Use of colloidal gold particles in double-labeling immunoelectron microscopy of ultrathin frozen section. *J. Cell Biol.* 89:653–666.
12. Griffiths, G., R. Brands, B. Burke, D. Louvard, and G. Warren. 1982. Viral membrane proteins acquire galactose in *trans*-Golgi cisternae during intracellular transport. *J. Cell Biol.* 95:781–792.
13. Hauri, H. P. 1983. Biosynthesis and transport of plasma membrane glycoproteins in the rat intestinal epithelial cell: studies with sucrase-isomaltase. *Ciba Found. Symp.* 95:132–149.
14. Hauri, H. P., A. Quaroni, and K. J. Isselbacher. 1979. Biosynthesis of intestinal plasma membrane: post-translational route and cleavage of sucrase-isomaltase. *Proc. Natl. Acad. Sci. USA.* 76:5183–5186.
15. Ivanov, I. E., H. Plesken, D. D. Sabatini, and M. Rindler. 1984. Immunolabelling of frozen thin sections and its application to the study of the biogenesis of epithelial cell plasma membranes. *Curr. Top. Membr. Transp.* 20:199–212.
16. Knipe, D. M., D. Baltimore, and H. F. Lodish. 1977. Maturation of viral proteins in cells infected with temperature-sensitive mutants of vesicular stomatitis virus. *J. Virol.* 21:1149–1158.
17. Leighton, J., Z. Brada, L. W. Estes, and G. Justh. 1969. Secretory activity and oncogenicity of a cell line derived from canine kidney. *Science (Wash. DC).* 163:472–473.
18. Lingappa, V. R., F. N. Katz, H. F. Lodish, and G. Blobel. 1978. A signal sequence for the insertion of a transmembrane glycoprotein. *J. Biol. Chem.* 253:8667–8670.
19. Louvard, D. 1980. Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. USA.* 77:4132–4136.
20. Marcus, P. 1961. Dynamics of surface modification in myxovirus infected cells. *Cold Spring Harbor Symp. Quant. Biol.* 27:351–365.
21. Matlin, K., D. F. Bainton, M. Pesonen, D. Louvard, N. Genty, and K. Simons. 1983. Transepithelial transport of a viral membrane glycoprotein implanted into the apical plasma membrane of Madin-Darby canine kidney cells. I. Morphological evidence. *J. Cell Biol.* 97:627–637.
22. Matlin, K. S., and K. Simons. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell.* 34:233–243.
23. Milks, L. C., M. J. Brontoli, and E. B. Cramer. 1983. Epithelial permeability and the transepithelial migration of human neutrophils. *J. Cell Biol.* 96:1241–1247.
24. Misfeldt, D. S., S. T. Hamamoto, and D. R. Pitelka. 1976. Transepithelial transport in cell culture. *Proc. Natl. Acad. Sci. USA.* 73:1212–1216.
25. Pesonen, M., and K. Simons. 1983. Transepithelial transport of a viral membrane glycoprotein implanted into the apical plasma membrane of Madin-Darby canine kidney cells. II. Immunological quantitation. *J. Cell Biol.* 97:638–643.
26. Quaroni, A., K. Kirsch, and M. M. Weiser. 1979. Synthesis of membrane glycoproteins in rat small intestinal villus cells. Redistribution of L-(1,5,6-³H)fucose-labelled membrane glycoproteins among Golgi, lateral basal and microvillus membranes in vivo. *Biochem. J.* 182:203–212.
27. Quaroni, A., K. Kirsch, and M. M. Weiser. 1979. Synthesis of membrane glycoproteins in rat small intestinal villus cells. Effect of colchicine on the redistribution of L-(1,5,6-³H)fucose-labelled membrane glycoproteins among Golgi, lateral basal and microvillus membrane in vivo. *Biochem. J.* 182:213–221.
28. Rindler, M. J., I. E. Ivanov, H. Plesken, E. J. Rodriguez-Boulan, and D. D. Sabatini. 1984. Viral glycoproteins destined for apical or basolateral plasma membrane domains traverse the same Golgi apparatus during their intracellular transport in Madin-Darby canine kidney cells. *J. Cell Biol.* 98:1304–1319.
29. Rodewald, R. 1973. Intestinal transport of antibodies in the newborn rat. *J. Cell Biol.* 58:189–211.
30. Rodewald, R. 1976. Intestinal transport of peroxidase-conjugated IgG fragments in the neonatal rats. *In* Maternofoetal Transmission of Immunoglobulins. W. A. Hemmings, editor. Cambridge University Press, New York. 137–153.
31. Rodriguez-Boulan, E., K. T. Paskiet, P. J. I. Salas, and E. Bard. 1984. Intracellular transport of influenza virus hemagglutinin to the apical surface of Madin-Darby canine kidney cells. *J. Cell Biol.* 98:308–319.
32. Rodriguez-Boulan, E. J., and M. Pendergast. 1980. Polarized distribution of viral envelope protein in the plasma membrane of infected epithelial cells. *Cell.* 20:45–54.
33. Rodriguez-Boulan, E. J., and D. D. Sabatini. 1978. Asymmetric budding of viruses in epithelial monolayers: a model for the study of epithelial polarity. *Proc. Natl. Acad. Sci. USA.* 75:5071–5075.
34. Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyl transferase in HeLa cells: codistribution with thiamine pyrophosphatase in trans Golgi cisternae. *J. Cell Biol.* 92:223–229.
35. Roth, M. G., and R. W. Compans. 1981. Delayed appearance of pseudotypes between vesicular stomatitis virus and influenza virus during mixed infection of MDCK cells. *J. Virol.* 40:848–860.
36. Roth, M. G., R. W. Compans, L. Giusti, A. R. Davis, D. P. Nayak, M. J. Gething, and J. S. Sambrook. 1983. Influenza virus hemagglutinin expression is polarized in cells infected with recombinant SV40 viruses carrying cloned hemagglutinin DNA. *Cell.* 33:435–443.
37. Roth, M. G., R. V. Srinivas, and R. W. Compans. 1983. Basolateral maturation of retroviruses in polarized epithelial cells. *J. Virol.* 45:1065–1073.
38. Rothman, J. E., H. Bursztyn-Pettegrew, and R. E. Fine. 1980. Transport of the membrane glycoprotein of vesicular stomatitis virus to the cell surface in two successive stages. *J. Cell Biol.* 86:162–171.
39. Rothman, J. E., and R. E. Fine. 1980. Coated vesicles transport newly synthesized membrane glycoproteins from endoplasmic reticulum to plasma membrane in two successive stages. *Proc. Natl. Acad. Sci. USA.* 77:780–784.
40. Tokuyasu, K. T. 1978. A study of positive staining of ultrathin frozen sections. *J. Ultrastruct. Res.* 63:287–307.
41. Tokuyasu, K. T., and S. J. Singer. 1976. Improved procedures for immunoferritin labeling of ultrathin frozen sections. *J. Cell Biol.* 71:894–906.
42. Ueda, M., and E. D. Kilbourne. 1976. Temperature sensitive mutants of influenza virus: a mutation in the hemagglutinin gene. *Virology.* 70:425–431.
43. Wehland, J., M. C. Willingham, M. G. Gallo, and I. Pastan. 1982. The morphological pathway of exocytosis of the vesicular stomatitis virus G protein in cultured fibroblasts. *Cell.* 28:831–841.
44. Weiser, M. M., M. M. Neumeier, A. Quaroni, and K. Kirsch. 1978. Synthesis of plasmalemma glycoproteins in intestinal epithelial cells. *J. Cell Biol.* 77:722–734.