Intracellular Transport of Influenza Virus Hemagglutinin to the Apical Surface of Madin-Darby Canine Kidney Cells

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ABSTRACT   The intracellular pathway followed by the influenza virus hemagglutinin (HA) to the apical surface of Madin-Darby canine kidney cells was studied by radioimmunoassay, immunofluorescence, and immunoelectron microscopy. To synchronize the migration, we used a temperature-sensitive mutant of influenza WSN, ts61, which, at the nonpermissive temperature, 39.5°C, exhibits a defect in the HA that prevents its exit from the endoplasmic reticulum. Upon transfer to permissive temperature, 32°C, the HA appeared in the Golgi apparatus after 10 min, and on the apical surface after 30–40 min. In the presence of cycloheximide, the expression was not inhibited, indicating that the ts defect is reversible; a wave of HA migrated to the cell surface, where it accumulated with a half time of 60 min. After passage through the Golgi apparatus the HA was detected in a population of smooth vesicles, about twice the size of coated vesicles, located in the apical half of the cytoplasm. These HA-containing vesicles did not react with anti-clathrin antibodies. Monensin (10 μM) delayed the surface appearance of HA by 2 h, but not the transport to the Golgi apparatus. Incubation at 20°C retarded the migration to the Golgi apparatus by ~30 min and blocked the surface appearance by acting at a late stage in the intracellular pathway, presumably at the level of the post-Golgi vesicles. The initial appearance of HA on the apical surface was in the center; no preference was observed for the tight-junctional regions.

Work in recent years has shown that plasma membrane glycoproteins resemble secretory proteins in their synthesis and processing (43, 54, 68, 73). They are (a) produced by membrane-bound polysomes, inserted cotranslationally via signal sequences into the membrane of the ER (10, 14, 30, 37, 42), and anchored by additional hydrophobic sequences (15, 16, 31, 63, 75); (b) cotranslationally glycosylated by transfer from dolichol phosphate of an oligosaccharide, GluNAc2-Man9-Glc3, later trimmed and further processed by endoplasmic reticulum (ER)[1] and Golgi enzymes to the simple and complex carbohydrate residues of mature glycoproteins (24, 40, 41, 56, 58, 70, 71, 76); and (c) subject to

proteolytic cleavage (39), sulfation (52), and covalent linking of lipids (71). Cell fractionation (2, 8, 23, 34, 35) and ultrastructural studies (3, 4, 17, 81) have documented the pathway ER—Golgi apparatus—plasmalemma; biochemical evidence suggests that the intermediate structures are coated vesicles (66, 67). The nature of the intracellular distribution mechanisms that determine their final surface localization is, however, completely unknown.

Epithelial cells infected with enveloped RNA viruses provide a convenient system to study the sorting of plasma membrane proteins (59). In cultured epithelial lines, such as Madin-Darby canine kidney (MDCK) cells (7, 51), these viruses assemble with a striking polarity: influenza (a myxovirus), Sendai and simian virus 5 (two paramyxoviruses) bud from the apical surface while vesicular stomatitis virus (VSV) (a rhabdovirus) obtains its envelope from the basolateral plasmalemma (62). Polarized viral budding is preceded, and presumably determined, by the asymmetric surface distribution of viral envelope glycoproteins (61), a property shared

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[1] Abbreviations used in this paper: DME, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; gam, goat anti-mouse; gar, goat anti-rabbit; PBS, phosphate-buffered saline containing 1 mM MgCl$_2$ and 0.1 mM CaCl$_2$; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; VSV, vesicular stomatitis virus; ts, temperature-sensitive; RIA, radioimmunoassay.

with intrinsic plasma membrane proteins of MDCK cells (25, 46). Carbohydrates do not appear to be responsible for this asymmetry (18, 65). Recently, it was shown that, in cells doubly infected with influenza and VSV, polarized budding is preserved and the respective viral glycoproteins hemagglutinin (HA) and G protein can be localized within the same Golgi apparatus, suggesting that the sorting of apical and basolateral proteins may be a post-Golgi event (57).

In this work, we followed by radioimmunoassay, immunofluorescence, and immunoelectronmicroscopy the intracellular pathway and the surface insertion of an apical glycoprotein, the HA of influenza virus. A temperature sensitive (ts) mutant of influenza WSN virus, ts61, with a defect in the HA that prevents its exit from the ER at the non permissive temperature (55, 80), was used to synchronize the migration. Our results characterize the kinetics of the HA passage through the Golgi apparatus and its insertion into the cell surface, describe vesicles presumably involved in the migration from Golgi apparatus to plasma membrane, and show the inhibitory effects of both the carboxylic ionophore monensin (78, 79) and low temperature (50) on the migration of HA.

MATERIALS AND METHODS

Growth of Cells and Viruses, Infection of MDCK Cells: MDCK cells were grown as described elsewhere (60). The ts61 mutant of influenza (WSN strain) was obtained from Dr. Peter Palese (Mount Sinai School of Medicine). Stocks of ts61 were grown in MDCK cells from viruses obtained from single plaques, and titered by plaque assay in the same cells. Titration by plaque and HA assay were carried out as described elsewhere (60). Only stocks that gave ratios greater than 100 between the infectivity titers at 32°C and 39.5°C (permissive and nonpermissive temperatures, respectively) were used. Titers at 32°C oscillated between 5×10^7 and 5×10^8 plaque-forming units/ml. In all the experiments described in this paper, infection of MDCK cells was carried out at a multiplicity of infection of 10 in Dulbecco's minimum essential medium (DME) containing 0.2% bovine serum albumin at 39.5°C, in a 5% CO_2/air incubator. In temperature shift experiments, transfer from the nonpermissive to the permissive temperature was carried out by quickly replacing medium at 39.5°C by medium prewarmed at 32°C, followed by incubation in a water-jacketed incubator set at this temperature. Incubations at 20°C were carried out in a water-bath; the pH was kept constant at 7.3 by the addition of 20 mM HEPES.

Radioisotopic Labeling of Monolayers: MDCK monolayers were infected with ts61 for 5.5 h at 39.5°C, incubated in either methionine- or leucine-free medium for 15 min and then, for 30 min, in the same medium containing 20 μCi/ml of [35S]methionine (Amersham, Arlington Heights, IL) or 50 μCi/ml [3H]leucine (New England Nuclear, Boston, MA). They were then transferred to normal DME prewarmed at 32°C and, at various time points, washed with cold phosphate-buffered saline, dissolved in 0.4% Triton X-100, 0.1% Na desoxycholate in water, and processed for SDS PAGE as described by Maizel (48). Samples were usually run on 10% acrylamide gels; these were fixed, processed with Enhance[R] (New England Nuclear) for fluorography, dried, and exposed to Kodak XAR-5 film during 3-7 d at -70°C.

Radioimmunoassay (RIA) of Influenza's HA: The appearance of HA on the surface of infected cells was followed by an indirect radioimmunoassay, using a mouse monoclonal anti-HA antibody (kindly provided by Dr. Webster, St. Jude's Children Research Hospital, Memphis, TN) in the first step, and a goat anti-mouse IgG (gam-IgG; Cappel laboratories Inc., Cochranville, PA) coupled to 125I (New England Nuclear) according to the procedure of Dr. Thomas Easton (Downstate Medical Center). Briefly, the gam-IgG was immobilized on a mouse IgG-Sepharose 4B column, iodinated with the chloramine T procedure (19), eluted with 0.2 M glycine-HCl buffer, pH 2.3, neutralized with 0.2 M Tris-HCl buffer, pH 8.6, and filtered through Sephacryl G-300 to remove aggregates. Specific activities were usually in the range of $2-4 \times 10^6$ dpm/μg protein. For RIA, cells were grown on 13-mm glass coverslips in 24-well tissue culture chambers or on 50-well (6-mm diameter) multichambers (Flow laboratories, McLean, VA). For each experimental point, cells on three coverslips or five microwells were fixed with 2% paraformaldehyde in phosphate-buffered saline containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS) at 4°C, usually overnight, and the following day were washed with PBS, the aldehyde

groups were quenched with 50 mM NH_4Cl, and the preparations were incubated with the first and second antibodies. The amounts of antibody used were ~0.02-0.1 μg/10^5 cells. To detect intracellular HA, we permeabilized the fixed monolayers with 0.1% Triton X-100 in PBS for 15 min. The radioactivity of individual coverslips or microwells was measured at 50% efficiency in a Packard gamma counter.

Immunofluorescence: The procedures for immunofluorescence are described in detail elsewhere (60). Briefly, cells on 13-mm glass coverslips were fixed with 2% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M Na phosphate, pH 7.4, at 4°C, quenched with 50 mM NH_4Cl, and processed as described for RIA, except for the use of affinity-purified fluoresceinated or rhodaminated gam-IgG as the second antibody (Cappel Laboratories). For intracellular fluorescence, cells were incubated with 0.1% (wt/vol) saponin in 0.1 M Na phosphate for 30 min and maintained in the same concentration of detergent for the rest of the procedure. In some experiments, PBS and 0.1% Triton X-100 were used as the buffer and the detergent, respectively, with approximately the same permeabilization efficiency. When rabbit anti-influenza virus IgG (prepared by one subcutaneous and two intradermal injections of purified virus, spaced every 2 wk) or rabbit anticlathrin serum (a gift of Dr. Saul Puszkin, Mount Sinai School of Medicine) were used as first antibodies, the second antibodies were affinity-purified goat anti-rabbit (gar) coupled to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) (Cappel laboratories). Coverslips were mounted on glass slides using a 15% polyvinylalcohol/30% glycerol mix in PBS and examined with a Leitz Ortholux epifluorescence microscope. Photography was done on Kodak Tri-X film (400 ASA); exposure times were 8-15 s.

Immunoelectron Microscopy: The procedure used was essentially similar to the one described by Louvard et al. (47; see reference 60 for a more detailed description). MDCK monolayers, infected with ts61, were fixed as described for immunofluorescence, except for the addition of 50 mM sucrose to the P buffer. A three-step procedure was used for all experiments reported in this paper: (a) monoclonal anti-HA; (b) affinity-purified rabbit anti-mouse (ram-IgG); (c) affinity-purified F(ab')2 fragments from gar-IgG coupled to peroxidase (Cappel laboratories). The monolayers were processed and embedded directly in the petri dish and reembedded after removal from the dish; sections stained in block with uranyl acetate were examined in a Zeiss EM 10 electron microscope set at the high contrast mode.

RESULTS

Mutants of enveloped RNA viruses with temperature-sensitive defects in the surface expression of the membrane glycoproteins provide convenient tools to study the mechanisms and intracellular pathways utilized in their migration to the cell surface. Monolayers of MDCK cells inoculated with ts61 and incubated for 7 h at 32°C (permissive temperature) express large amounts of the HA on the apical cell surface (Fig. 1 a), easily recognized by the typical spotty pattern given by the fluorescent staining of the microvilli (61). Infected monolayers incubated for 7 h at 39.5°C (nonpermissive temperature) do not express the HA on the surface (Fig. 1 b) but, after 1-h shift to permissive temperature, exhibit large amounts of the glycoprotein (Fig. 1 c).

The basic experiment used throughout this paper was the following: monolayers of MDCK cells (usually 1 d after reaching confluence, or ~1.5 × 10^5 cells/cm^2) were inoculated with ts61 for 1 h at 39.5°C, and then incubated at the same temperature for an additional 5 h. At this time, the monolayers were transferred to 32°C, fixed at various times of incubation at this temperature, and the presence of HA was detected with specific monoclonal antibodies.

Insertion of HA into the Apical Surface Measured by RIA

Using the experimental protocol described above, the kinetics of the insertion of HA into the apical surface after shift to permissive temperature were studied by RIA, using 125I-gam IgG as the second antibody (Fig. 2). The HA was first detected after a lag of ~30 min and continued to be inserted
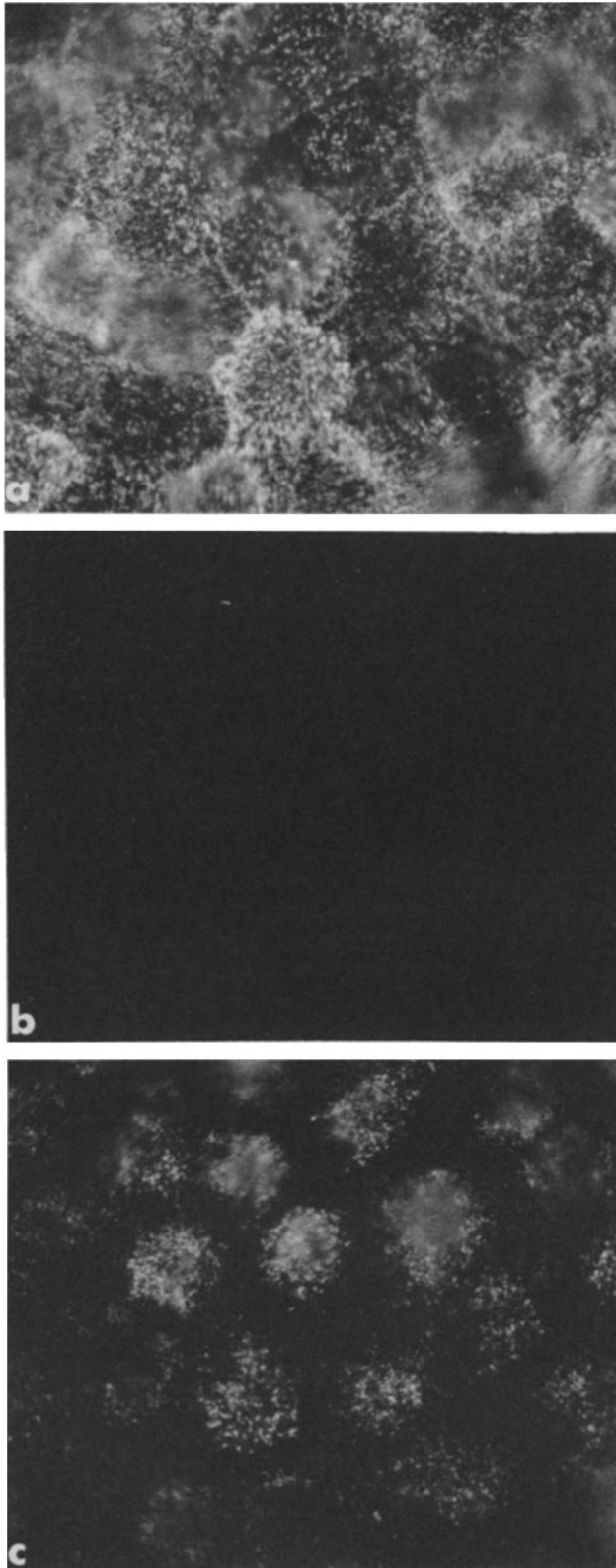
Figure 1 Infection of MDCK cells with ts61 at permissive and nonpermissive temperature. Confluent monolayers of MDCK cells, grown on glass coverslips, were inoculated with ts61 at 4°C for 1 h and then incubated for an additional 7 h at 32°C (a), at 39.5°C (b), or for 6 h at 39.5°C and 1 h at 32°C (c). The monolayers were fixed as described in Materials and Methods and processed for immunofluorescence using a mouse monoclonal anti-HA antibody in the first step, and affinity-purified TRITC-gam in the second step. × 2,900.

for at least 100 min (Fig. 2, empty circles). Addition of cycloheximide 15 min before the temperature shift did not block the raise in binding, but resulted in its saturation after ~90 min (Fig. 2, filled circles). Thus, the ts defect that prevents the migration of HA is reversible at permissive temperature. This resembles the situation with similar mutants of Semliki Forest virus (29), but is different from reported observations with the G protein of the ts045 mutant of VSV, which cannot exit from the ER after shift to permissive temperature (36, 38). From the data in Fig. 2, a half time of 60 min was calculated for the transport of HA to the apical surface.

Only a very slight increase in binding (<5%) was detected at any point during the 90-min shift to permissive temperature when the monolayers were treated with 10 mM EGTA in Ca, Mg-free PBS for 5 min before fixation, a condition that partially disrupts the tight junctions and allows access to the basolateral region of the plasma membrane (7, 61). These results (data not shown) indicate either that little HA is present in the basolateral surface at any given time during its migration to the apical surface or, alternatively, that the EGTA-permeabilization procedure does not allow complete access to the basolateral membrane. Low amounts of HA are usually detected in the basolateral membrane by immunoelectron microscopy during infection of MDCK cells by influenza WSN (57).

Intracellular Transport of HA: Pulse Chase Analysis and Immunofluorescence

To study the modifications undergone by the HA during its intracellular transport, we incubated monolayers of
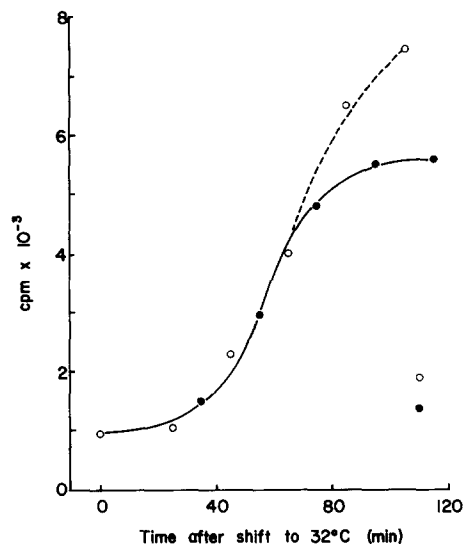

Time after shift to 32°C (min)

Figure 2 Appearance of HA in the apical surface of MDCK cells infected with ts61 after shift to permissive temperature. Confluent monolayers of MDCK cells grown on glass coverslips were inoculated with ts61 at 4°C, incubated at 39.5°C for 6 h, and transferred to 32°C in the presence (full circles) or in the absence (empty circles) of cycloheximide (100 μg/ml), added 15 min before the shift. At each time point, triplicate monolayers were fixed with 2% paraformaldehyde and processed for RIA with the monoclonal anti-HA antibody and ¹²⁵I-gam. The two points at the lower right correspond to monolayers that were not transferred to the permissive temperature. All standard deviations were less than 18%. The background binding to uninfected monolayers (520 cpm) was not substracted.
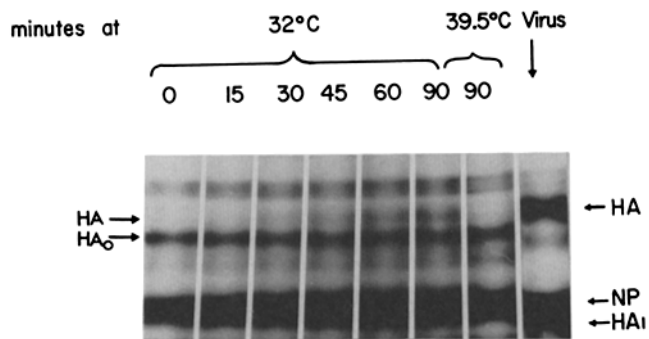
FIGURE 3 Acquisition of higher molecular weight by the HA during the shift to permissive temperature. Confluent monolayers of MDCK cells grown on 35-mm petri dishes were infected with ts61 as described in the legend to Fig. 2, incubated for 5.5 h at 39.5°C, pulsed with [^{3}H]leucine for 30 min, and transferred to 32°C in chase medium for various times before processing for SDS PAGE. Note that at $t = 0$ only HA$_1$ is present. A band with the same mobility of the viral HA is faint at 15 min of chase, but becomes progressively more intense with time, while the intensity of HA$_0$ decreases. Monolayers kept at 39.5°C during the chase display only HA$_1$. Note that purified virions (Virus) exhibit both cleaved (HA$_1$) and uncleaved forms of HA.

MDCK cells infected with ts61 for 5.5 h at 39.5°C, labeled with [^{3}H]leucine for 30 min, and processed for SDS PAGE at different times of chase at 32°C in the presence of cycloheximide (Fig. 3). As previously shown by Palese (55), only the ER form of the glycoprotein, HA$_1$, was detected after 6 h of incubation at nonpermissive temperature (Fig. 3, 0 min). After 15–30-min incubation at 32°C, a second band, with a higher apparent molecular weight and the same electrophoretic mobility as the mature uncleaved HA found in released virions, became apparent (Fig. 3, 15 and 30 min). (ts61 virions grown in MDCK cells contain approximately equal amounts of uncleaved and cleaved forms of HA.) This HA band, the higher mobility of which apparently reflects the addition of carbohydrates during the passage through the Golgi complex (50), increased in intensity with time (Fig. 3, 30–90 min) and became the major form of HA after 2 h (see below). Monolayers kept at 39°C after the radioactive pulse did not exhibit the mature form of HA (Fig. 3, 39.5°C). Since the temperature shift was carried out in the presence of cycloheximide, this experiment also shows the reversibility of the defect preventing the migration of HA.

To identify the structures involved in the intracellular migration of HA and its initial site of insertion into the apical surface, we carried out immunofluorescence experiments (Fig. 4). After 6 h of infection with ts61 at 39.5°C, no specific fluorescence was detected on the cell surface (Fig. 4a, see also Fig. 1b). Little intracellular fluorescence was present in monolayers permeabilized with saponin (Fig. 4a′), a surprising finding since HA is synthesized in large amounts at 39.5°C (see Fig. 3). Possible explanations could be that the ER form of HA (a) is not recognized by the monoclonal antibody, (2) is not accessible to it, and (c) is present at such small concentrations that it fails to originate a sufficiently strong signal. After 10 min of incubation at 32°C, however, a juxtanuclear mass with the typical shape of the Golgi apparatus became clearly labeled (Fig. 4b′). The fluorescence intensity in the Golgi complex increased with time (Fig. 4, b′–f′), but no significant labeling of the surface was observed until 40–50 min (Fig. 4, b–e). The initial appearance of HA in the apical

surface was diffuse or, sometimes, concentrated in the center (Fig. 4e). No preference for the tight-junctional regions was noticed, as has been described for the reinsertion of recycling leucine aminopeptidase-antibody complexes (46). After 60 min, the apical surface became brightly stained, with its characteristic punctate pattern (Fig. 4f). At the 40- and 50-min time points, most of the cells in permeabilized monolayers displayed, in addition to an enlarged Golgi mass, brightly fluorescent dots observable in at least two to three distinct focal planes with the 63X lens, which suggested a cytoplasmic localization (Fig. 4, d′ and e′). At later time points, this image was confused by the brightly-stained apical surface. At no time was the typical ring-like image given by the staining of the basolateral surface (46, 61) observed, suggesting that the amount of HA in that surface was always low, or that the access of the antibodies to that surface was restricted, even in the presence of detergent.

To determine whether the fluorescent "dots" described above, presumably vesicles involved in post-Golgi transport, were, indeed, intracellular, i.e., located in a focal plane below that of the surface microvilli, we carried out a double-label immunofluorescence experiment (Fig. 5). Monolayers infected with ts61 and fixed 50 min after the shift to permissive temperature were treated first with rabbit antibodies against HA, then permeabilized with saponin, incubated with the monoclonal anti-HA antibody and, in a third step, treated with a mixture of FITC-gar and TRITC-gam. Thus, FITC fluorescence originated exclusively from the apical surface and TRITC fluorescence was given mostly by intracellular structures. Using the 63X lens and the fluorescein filter, the focus was placed on the apical microvilli (Fig. 5a); change to the rhodamine filter showed that the Golgi mass and the tiny fluorescent dots were in a completely different focal plane (Fig. 5b). These structures became clearly focused upon displacement to a focal plane closer to the basal surface (Fig. 5c), which resulted in defocusing of the apical microvilli (not shown). The number of these "vesicles" was relatively small, approximately 30–50/cell, and most of them appeared to be located in the apical half of the cytoplasm, above the nucleus. Double immunofluorescence experiments in which a 20-min pulse of FITC-dextran was applied to monolayers infected with ts61, 30 min after shift to permissive temperature (not shown), suggest that the tiny "vesicles" were not of endocytic origin: the fluorescent dextran was seen only in very large vacuoles in the cytoplasm, and did not co-distribute with the tiny HA-carrying structures. At the electron microscope level, similar giant vacuoles are observed labeled with cationized ferritin in cells fixed 10 min after addition of the probe to the apical medium (D. Misek, unpublished results).

Intracellular Migration of HA: Immunoelectron Microscopic Studies

The intracellular migration of HA was studied by immunoelectron microscopy on monolayers permeabilized with saponin, using a three-step procedure: monoclonal anti-HA antibody, affinity-purified ram-IgG, and affinity-purified gam (Fab′)$_2$ coupled to peroxidase (Fig. 6). These experiments confirmed the immunofluorescence results described above (Fig. 4). Little specific labeling was observed throughout the cytoplasm 6 h after infection with ts61 at 39.5°C; the Golgi apparatus, typically located between the nucleus and the apical surface, was essentially free from reaction product (Fig.
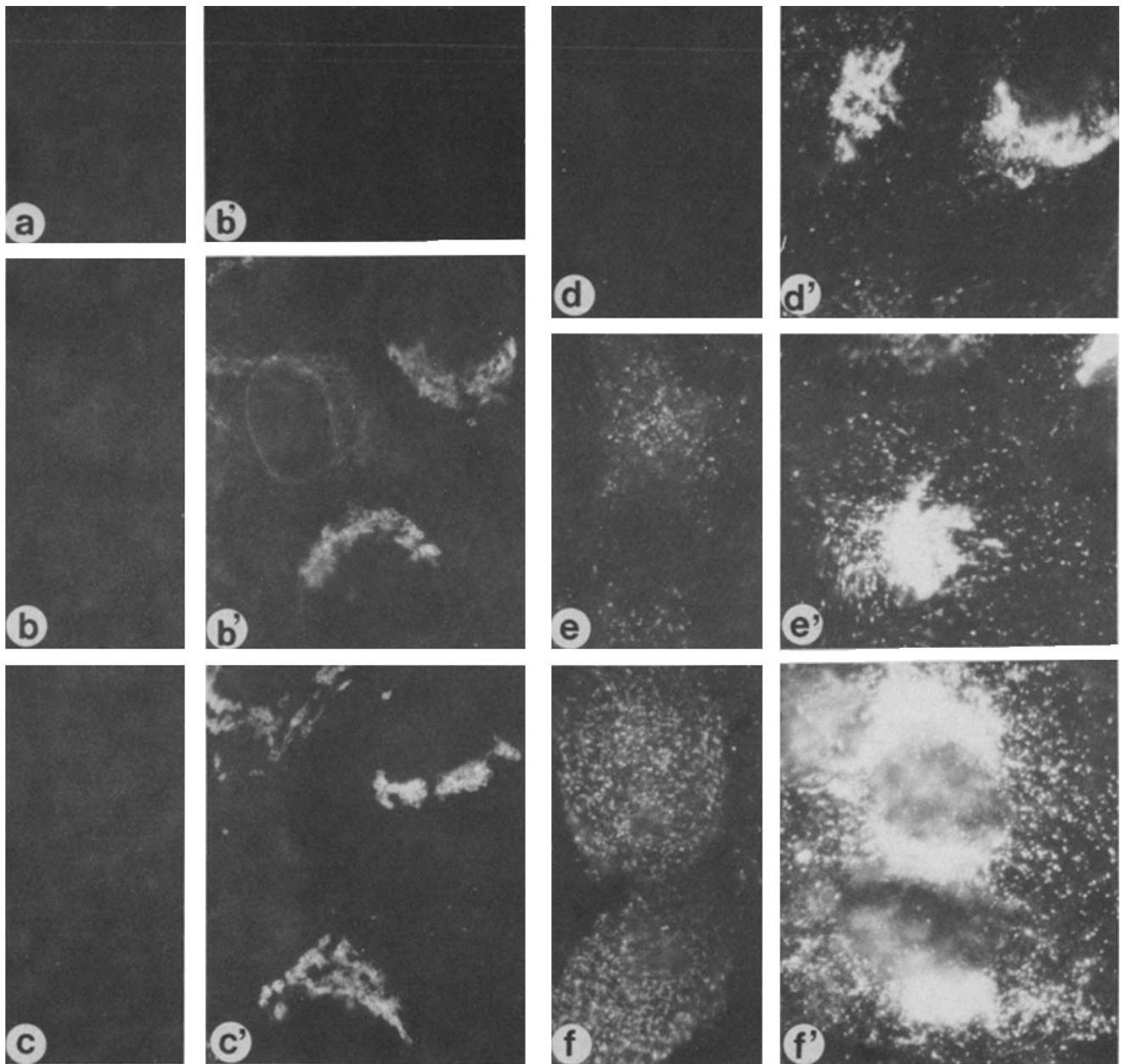
FIGURE 4   Passage of HA through the Golgi apparatus and insertion into the apical surface of MDCK cells. Confluent monolayers of MDCK cells, grown on glass coverslips, were infected with ts61, incubated at 39.5°C for 6 h, transferred to 32°C, and fixed with 2% PFA 0.05% GA in P (see Materials and Methods) at 0 min (a and a'), 10 min (b and b'), 20 min (c and c'), 40 min (d and d'), 50 min (e and e'), and 60 min (f and f'). Samples a–f were processed for surface immunofluorescence as indicated in the legend to Fig. 1. Samples a'–f' were treated with 0.05% saponin for intracellular immunofluorescence. All exposure times were the same during photography and during printing. Note the early labeling of structures with the typical crescent shape of the Golgi apparatus (b', 10 min), the first detection of surface fluorescence at 50 min (e), and the staining of intracellular "dots" at intermediate time points (d', 40 min). × 3,900.

6a). After shift to 32°C, labeling of the stack was evident at 10 min (Fig. 6b), and intense at 20 min (Fig. 6c). The label was distributed in a nonhomogeneous fashion; usually about half of the cisternae had reaction product (Fig. 6c); these labeled cisternae were facing either the nucleus or the apical surface. Analysis of well-oriented sections, where transitional elements of the rough endoplasmic permitted the tentative identification of proximal Golgi apparatus (77), suggested that the positive cisternae were the distal ones. These results suggested that either the HA was concentrated in the distal Golgi cisternae or, alternatively, that these cisternae were preferen-

tially permeabilized by the saponin treatment. Since in this experiment the temperature shift was carried out in the absence of cycloheximide, little change in the amount of precipitate in the Golgi apparatus was seen at later time points. After 40–50 min, tiny smooth vesicles displaying reaction product, larger than coated vesicles (~200–300 nm in diameter), were observed in the vicinity of the Golgi apparatus and in the apical half of the cytoplasm (Fig. 6d, large arrowheads, and inset), at a relatively low frequency (an average of one or less per cell and per section). Considering the time when these vesicles are observed and the fact that 50 or more ultra-thin
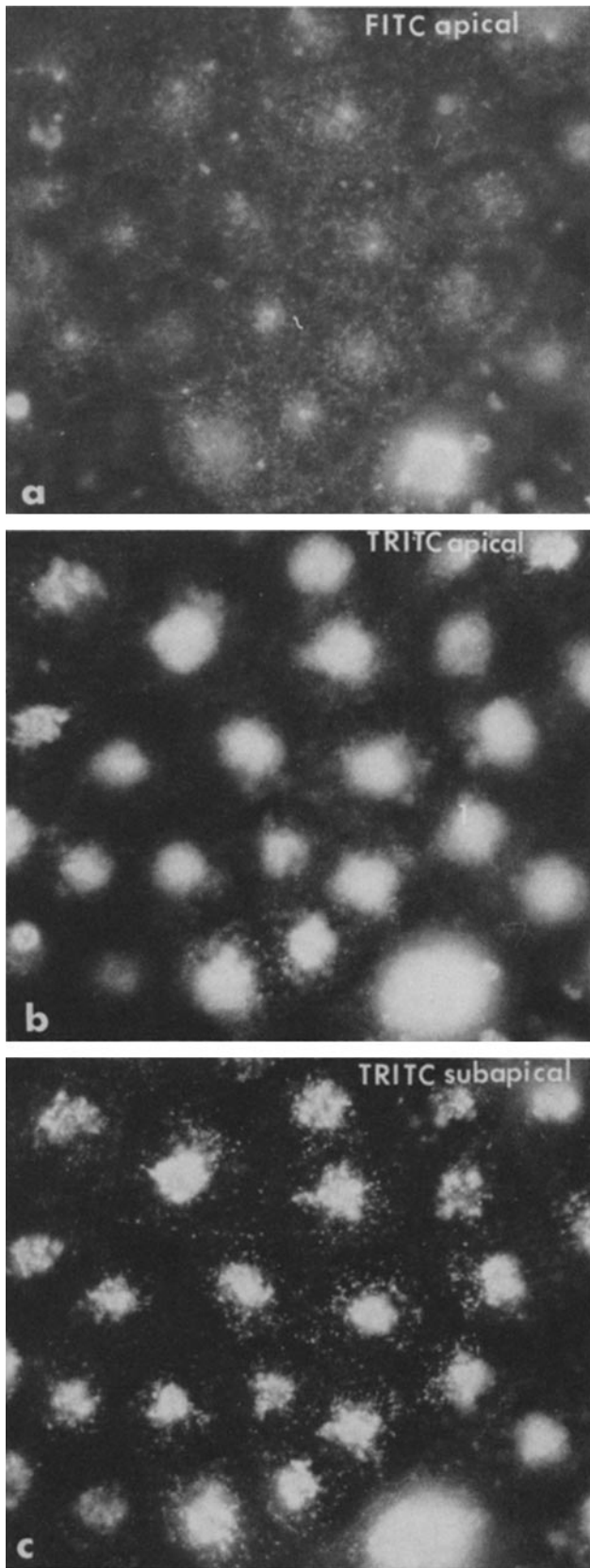
sections are needed to span a whole MDCK cell in a confluent monolayer, these vesicles may correspond to the tiny dots observed by immunofluorescence (see Figs. 4 and 5). No reaction product was detected in the basolateral membrane at any time point, in agreement with the RIA results described above.

To follow a wave of HA migrating to the cell surface, the same experiment was carried out with cycloheximide added 15 min before the shift to permissive temperature (data not shown). 20 min after shift to 32°C, the only cytoplasmic structure displaying intense peroxidase reaction was the Golgi apparatus. By 60 min, the amount of reaction product in this organelle had decreased to levels close to those observed at time 0; instead, the label was observed in groups of vesicles, often of irregular shape, frequently located very close to or fusing with the apical surface. By 90–120 min, all the HA had moved to the apical surface, which displayed intense reaction product on the membrane itself and on multiple budding virions. The level of staining of the cytoplasm was very low, similar to the one observed at time 0, suggesting that little HA was recycled intracellularly after insertion in the cell surface.

The immunoperoxidase experiments indicated that the vesicles becoming labeled 40–50 min after the shift to permissive temperature were of the uncoated type. To gain additional information on the presence or absence of clathrin in these structures, we carried out double-label immunofluorescence experiments. Monolayers of MDCK cells were infected with ts61, fixed 50 min after shift to 32°C, and incubated with monoclonal anti-HA and rabbit anti-clathrin antibodies in the first step, and FITC-gar and TRITC-gam in the second step (Fig. 7). Immunofluorescence with anti-clathrin antibodies resulted in bright staining of the Golgi apparatus and of multiple tiny dots, some of them concentrated in basolateral membrane regions, and in diffuse staining of the cytoplasm. Comparison of anti-HA and anti-clathrin fluorescence patterns in photographs of the same cell demonstrated (a) coincidence of the Golgi image for both antibodies and (b) a much smaller number of fluorescent dots with the anti-HA antibody. Careful analysis of both patterns in the vicinity of the Golgi apparatus showed that many vesicles reacting with the anti-HA antibody were clearly negative with the anti-clathrin antibody (Fig. 7, arrowheads). All the experiments described above suggested that the putative post-Golgi vesicles involved in HA transport were not of the coated type.

Effect of Monensin and Low Temperature on the Migration of HA

Previous studies have demonstrated an inhibitory effect of the cationic ionophore monensin on the secretion of proteins, the production of enveloped RNA viruses, and the expression of viral glycoproteins on the cell surface (27, 29, 78, 79). Alonso and Compans (1) have shown that monensin (10-μM)

Figure 5 Intracellular location of the immunofluorescent "dots" that stain with the anti-HA monoclonal antibody. Confluent monolayers of MDCK cells, grown on glass coverslips, were infected with ts61, incubated at 39.5°C for 6 h, transferred to 32°C, and fixed with 2% PFA 0.05% GA 50 min later. The monolayers were incubated with rabbit antibody anti-HA in the first step, treated with 0.05% saponin, then incubated with monoclonal anti-HA (second step), and finally with a mixture of FITC-gar and TRITC-gam (third step). The monolayers were photographed with the focus placed on the apical surface, identified by the FITC-gar fluorescence, with the fluorescein filter (a). Upon change to the rhodamine filter, without modification of the focal plane, the Golgi apparatus and the intracellular "vesicles" were completely out of focus (b), but became distinctly focused when the objective was displaced towards the bottom of the cell (c). × 2,900.
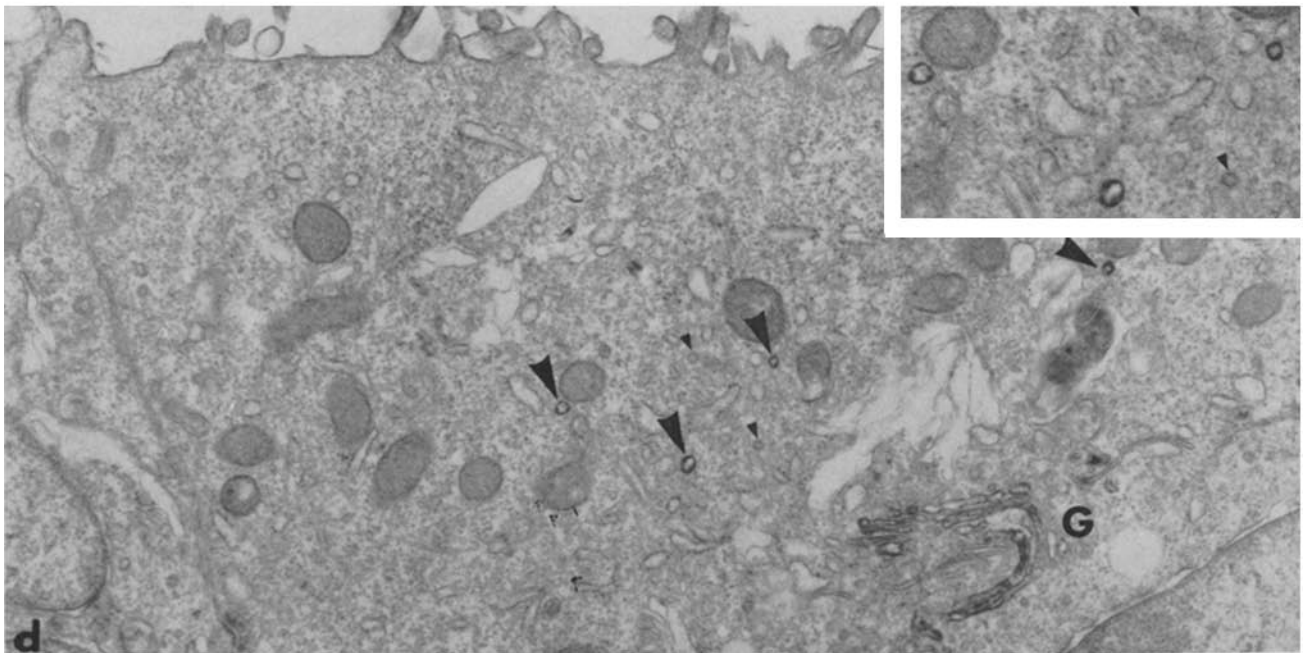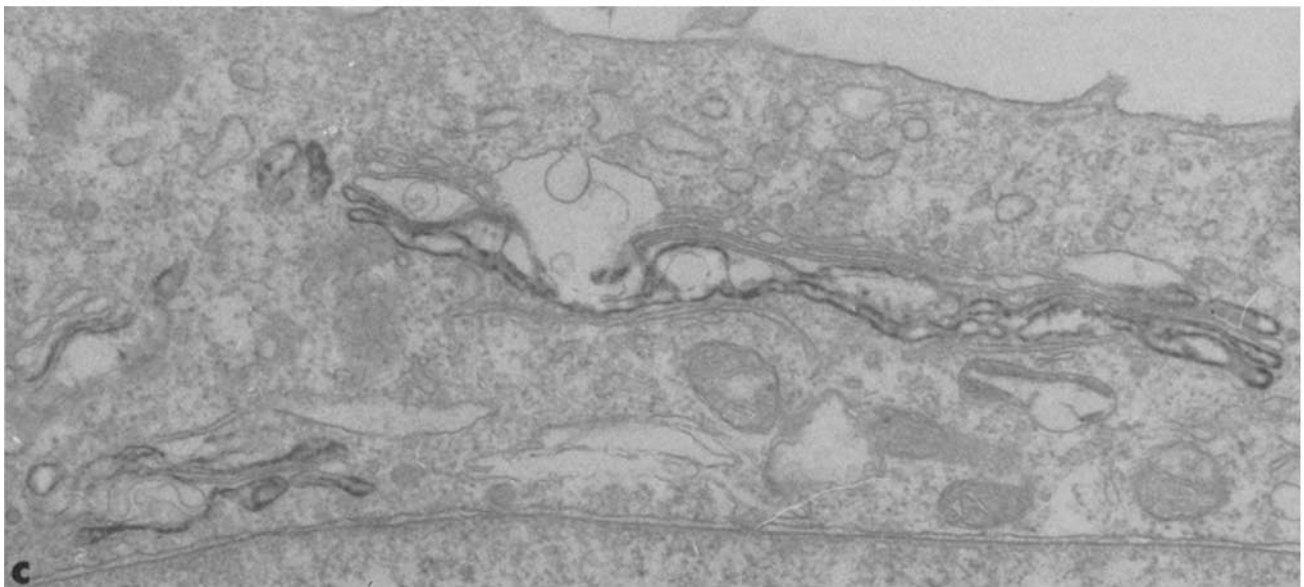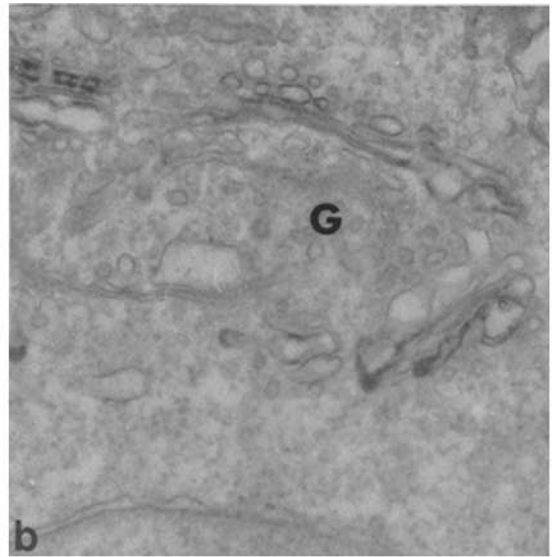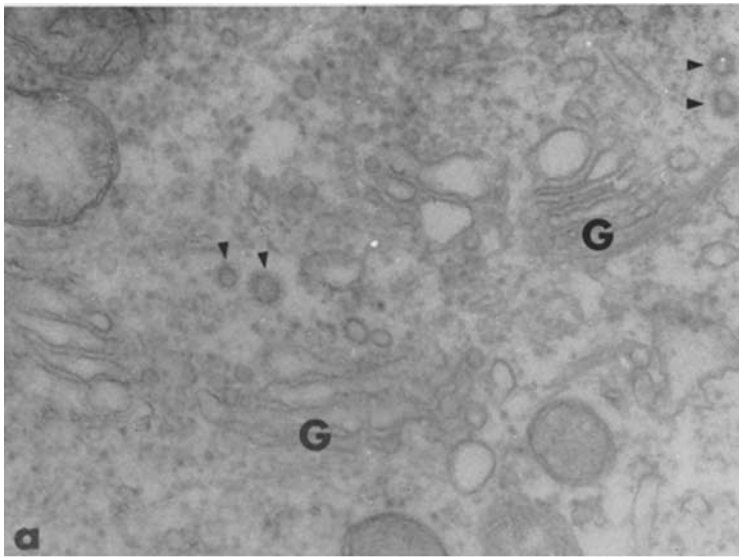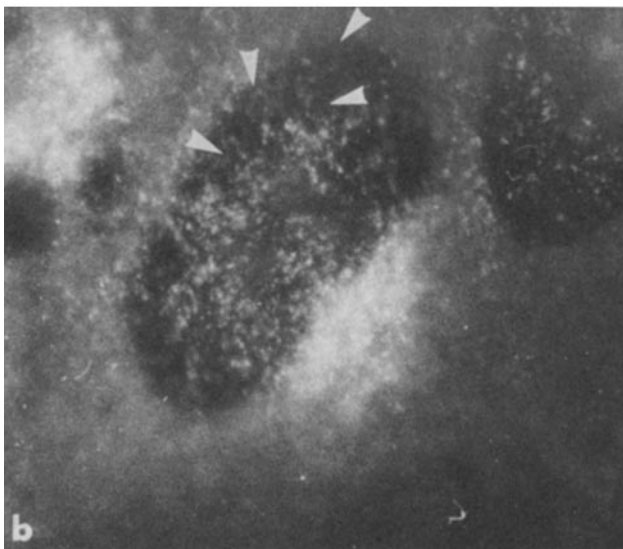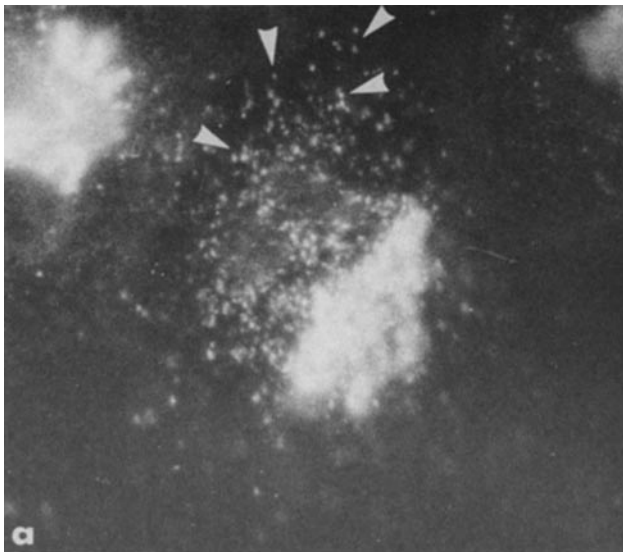
FIGURE 7 Double immunofluorescence staining with monoclonal anti-HA and rabbit anti-clathrin antibody of MDCK cells infected with ts61. Monolayers of MDCK cells were infected with ts61, incubated at 39.5°C for 6 h, transferred to 32°C, and fixed 50 min later, exactly as in Fig. 5. The staining for intracellular immunofluorescence, in the presence of saponin, was carried out in two steps. In the first step, the monolayers were incubated in the presence of monoclonal anti-HA and rabbit anticlathrin antibodies. In the second step, they were treated with TRITC-gam and FITC-gar. (a and b) Photographs of the same cells taken with the rhodamine filter (for HA) and the fluorescein filter (for clathrin), at exactly the same focal plane. The arrowheads point at regions where vesicles stained with anti-HA antibody (a) show no crossreaction with anticlathrin antibodies (b). × 4,900.
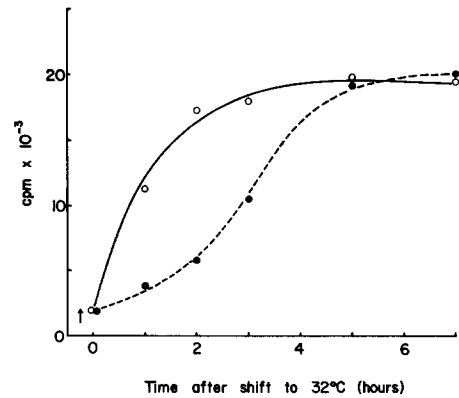

FIGURE 8 Effect of monensin on the migration of HA to the apical surface. Monolayers of MDCK cells grown in 50-well tissue culture dishes were infected with ts61, incubated at 39.5°C for 6 h and transferred to 32°C medium in the presence of cycloheximide (100 μg/ml), added 15 min before the shift. The empty circles are control monolayers; the full circles correspond to monolayers that received 10 μM monensin 15 min before the shift (arrow); in these samples the drug was kept throughout the incubation at 32°C. For each control or experimental time point, five monolayers were fixed with 2% paraformaldehyde and processed for RIA with the monoclonal anti-HA antibody and ^{125}I-gam. Standard deviations were <20%.

blocks the production of vesicular stomatitis virus and the migration of its glycoprotein, but does not affect the production of influenza virus, in MDCK cells. We studied the effect of monensin on the migration of HA to the surface of MDCK cells infected with ts61, by RIA, immunofluorescence, and immunoelectron microscopy. Addition of 10 μM monensin 15 min before the temperature shift and during the incubation at 32°C delayed the appearance of HA in the apical surface by about 2 h (Fig. 8, closed circles); by 4 h, the levels of HA had reached control values. A similar delay was found in the release of viral HA to the medium (not shown). The production of virus after 12 or 24 h was not inhibited by monensin, in agreement with a previous report (1). The arrival of HA to the Golgi apparatus, as detected by immunofluorescence, was not delayed; the HA was first observed there 15 min after shift to permissive temperature (not shown); the labeled Golgi cisternae appeared swollen, both by immunofluorescence (Fig. 9) and by immunoelectron microscopy (not shown). A study of the monensin effect by pulse-chase experiments analyzed by SDS PAGE demonstrated, however, a delay in the maturation of HA. It also showed that the mature form of HA in the presence of monensin had a higher electrophoretic mobility than the mature HA in control cells, apparently as a consequence of defective glycosylation (F. V. Alonso and R. W. Compans, manuscript submitted for publication). The results of these experiments are in agreement with previous

FIGURE 6 Intracellular migration of HA in MDCK cells infected with ts61: immunoelectron microscopy. Confluent monolayers grown on plastic petri dishes were infected with ts61, incubated at 39.5°C, transferred to 32°C, fixed with 2% paraformaldehyde 0.05% glutaraldehyde at 0 min (a), 10 min (b), 20 min (c), and 40 min (d), and processed for the immunoperoxidase procedure as described in Materials and Methods. Note the absence of labeling in the Golgi apparatus and coated vesicles (arrowheads) at time 0. The label can be detected at 10 min and becomes intense at 20 min in some of the cisternae, apparently the distal ones. At 40 min, label can be detected in four smooth vesicles (large arrowheads in d), considerably larger than coated vesicles (small arrowheads in d); no label is observed yet on the apical or basolateral membrane. (a) × 50,000; (b) × 50,000; (c) × 32,000; (d) × 19,000; (inset) × 32,000.
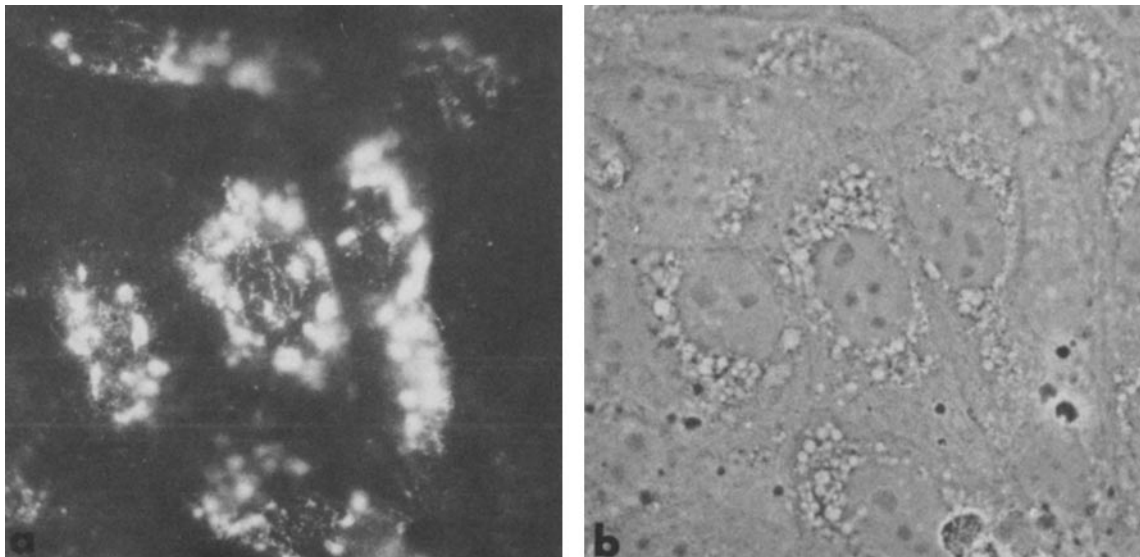
FIGURE 9 Localization of HA in dilated Golgi vesicles in MDCK cells infected with ts61 after treatment with monensin. Fluorescence (a) and phase-contrast (b) photographs of confluent monolayers of infected MDCK cells fixed and processed for intracellular immunofluorescence 60 min after transfer to permissive temperature in the presence of monensin (10 μM) added 15 min before the shift. Infection with ts61 and incubation at nonpermissive temperature were exactly as in Fig. 5. The same monolayers, processed for surface immunofluorescence, showed only background staining (not shown). × 2,900.
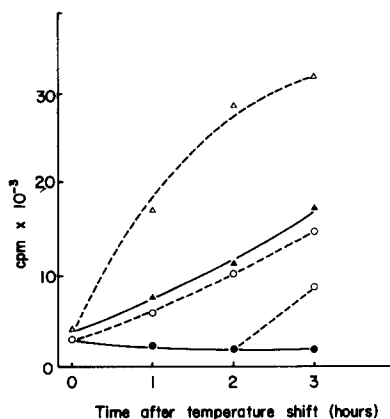

FIGURE 10 Effect of incubation at 20°C on the intracellular migration of HA. Confluent monolayers of MDCK, grown in 50-well tissue culture dishes, were infected with ts61 and incubated at 39.5° for 6 h before shift to 32°C (---) or 20°C (——), fixed at various times, and processed for RIA in the presence (△, ▲) or absence (○, ●) of 0.1% Triton X-100. Five monolayers were incubated for 2 h at 20°C and then transferred to 32°C for 1 h (●---○), before fixation. Each point represents the average of five determinations. The standard deviations were <20%.

work by other laboratories indicating that the monensin block is at the level of the Golgi apparatus, and may or may not affect the terminal glycosylation of the transported glycoproteins (20, 27, 74).

Matlin and Simons (50) have observed that incubation at low temperature inhibits the transport of fowl plague virus HA to the cell surface after its glycosylation has been completed. We tested the effect of low temperature in our system by RIA. In monolayers infected with ts61 and incubated for 6 h at 39.5°C, transfer to 20°C blocked the arrival of HA to the apical surface for at least 3 h (Fig. 10a, solid line, filled circles), but accumulation in the cytoplasm still continued (Fig. 10, solid line, full triangles), at a lower rate than in

control monolayers incubated at 32°C (dashed line, empty triangles). Reversion of the temperature from 20°C to 32°C resulted in the appearance of HA on the surface (Fig. 10). Immunofluorescence studies (data not shown) showed that, different from the monensin block, incubation at 20°C caused a 1-h delay in the arrival at the Golgi apparatus. After 3 h at this temperature, however, the cells displayed large Golgi masses and bright fluorescent dots, similar to those shown in Fig. 5c. No evidence was seen in these experiments of accumulation of HA in the basolateral membrane during the incubation at 20°C. These results suggested that low temperature blocks a relatively late step in the transport of HA, perhaps the fusion of post-Golgi vesicles with the target membrane.

DISCUSSION

The results of this report show that, in MDCK cells infected with influenza virus, the HA is transported to the Golgi apparatus in 10–15 min and to the apical surface in ~30–40 min, with a half time of 60 min. Approximately similar kinetics have been observed for other viral glycoproteins, such as the G protein of VSV (2, 4, 35), and the spike glycoproteins of SFV (17), as well as for cell membrane glycoproteins, such as erythrocyte's glycophorin (28) and band III (5, 69), H2 and HLA antigens (10, 37), the liver plasma membrane enzymes nucleotide pyrophosphatase and dipeptidyl peptidase IV (13), and the sucrase-isomaltase of intestinal epithelia (22, 32). These rates of intracellular transport are very similar to those of secretory proteins (26, 54, 74). Some membrane glycoproteins, however, like influenza's neuraminidase (23), the acetylcholine receptor (9), and the HLA-DR antigens (53), are transported at much slower rates through the cell.

The migration of HA through the Golgi apparatus correlated with the acquisition of a higher molecular weight in SDS PAGE, which apparently reflected the addition of complex

carbohydrates, as deduced from the development of resistance to the action of endoglycosidase H (50). Previous immuno-cytochemical work has shown that terminal carbohydrates are added in distal Golgi cisternae (20, 64). Our immunoper-oxidase experiments revealed a higher concentration of reaction product in the distal Golgi cisternae, suggesting that the HA is concentrated here. Alternative explanations could be that the monoclonal antibody used in these experiments binds with higher affinity to the mature HA, or that some of the Golgi cisternae are more susceptible to permeabilization by saponin. Further studies are needed to clarify this point.

After passing through the Golgi apparatus, the HA was detected in a population of smooth vesicles, somewhat larger than coated vesicles, near the Golgi complex. At times when delivery to the apical surface was taking place, the HA was found in tubular elements and vesicles of irregular shape, some of which were very close to the apical surface and appeared to be fusing with it. Experiments with fluorescent dextran and cationized ferritin (D. Misek, unpublished results) indicate that these vesicles are much smaller than endosomal vacuoles in MDCK cells. 2 h after the shift to permissive temperature in the presence of cycloheximide, all HA was localized on the apical plasmalemma, which suggests that little HA recycles as endocytic vesicles, probably because of the interaction with nucleocapsids at the cell surface. Our results suggest that if coated vesicles are involved in transport between the Golgi apparatus and the apical surface, as has been proposed by Rothman and collaborators (66, 67), this step is relatively short-lived. Perhaps it involves just the budding of the vesicle from the Golgi complex. In a recent immunoelectron microscopic study on the intracellular migration of VSV G protein, Wehland et al. (81) have found no evidence of the participation of coated vesicles, which, they think, are only involved in endocytic processes. Higher resolution and structural preservation in immunocytological procedures, as well as methods to block transport at specific intracellular steps, are needed to give a definitive answer to this question.

Although our data suggest direct delivery of the HA from Golgi complex to the apical surface, a fast passage through the basolateral surface cannot be ruled out on the basis of the experiments reported here. A certain amount of HA (10–20%) was detected by colloidal gold labeling on ultrathin frozen sections of MDCK monolayers infected with influenza virus (57). Passage through the basolateral surface has been proposed by Hauri et al. (22) for sucrase-isomaltase, an apical enzyme of intestinal cells, on the basis of pulse chase experiments on subcellular fractions. In these experiments, pulse-labeled sucrase-isomaltase was detected at 0, 15, 30, and 45 min, respectively, in ER, Golgi, basolateral, and apical brush border cell fractions. Although our results do not invalidate the hypothesis of Hauri et al. (22), they suggest an alternative explanation. If the basolateral membrane fraction were contaminated with the putative post-Golgi vesicles mentioned above, this would explain the detection of sucrase-isomaltase in this fraction at intermediate chase times between those of Golgi and apical brush-border fractions. Further evidence against passage of bulk amounts of HA through the basolateral membrane during its biogenesis comes from recent work from our laboratory (D. Misek, E. Bard, and E. Rodriguez-Boulan, unpublished results). When confluent monolayers of MDCK cells, grown on collagen gels containing anti-HA antibody, are infected with influenza virus, the development of plaques

is not inhibited, while anti-VSV antibody in the collagen gel blocks the development of VSV plaques. These results suggest that only a small proportion of HA may be exposed to antibody in the basolateral medium.

We found that the initial appearance of HA was in the central region or diffuse over the apical surface of confluent MDCK cells; no preference was observed for the tight-junctional areas, as Louvard (46) has reported for recycling leucine aminopeptidase-antibody complexes. These results suggest that the exocytic pathways for newly synthesized membrane proteins and for recycling surface proteins may not be the same. In subconfluent MDCK cells, however, some preference for the cell periphery was evident. This may reflect variations in the position of the Golgi complex and in cytoskeletal organization depending on the degree of confluence of the monolayer: in flat subconfluent cells the Golgi complex was usually located besides the nucleus, while in confluent monolayers it was clearly supranuclear. In his pioneering experiments, Marcus (49) demonstrated that the HA of Newcastle disease virus was first detected in the periphery of giant, isolated HeLa cells. The initial site of surface insertion was usually correlated with the intracellular position of the Golgi complex, which was intensely labeled with anti-NDV antibodies. A polar appearance of a viral envelope glycoprotein has also been described in fibroblasts infected with measles virus (12).

One important goal in the investigation of how membrane proteins are sorted into specific surface domains is to find ways to block or slow down transport at specific intracellular steps. This report illustrates the usefulness of mutants with ts defects in the glycoprotein migration. Similar mutants from viruses other than influenza are available (6, 33, 38, 44, 72); some of them have been previously used for studies on intracellular transport (4, 29, 35, 45, 82). Usually, the ts defect prevents the exit from the ER, but mutants defective in a post-Golgi step have also been described (82).

A different approach to dissect the intracellular pathway is to use pharmacologic inhibitors. Since we have previously shown (57) that both the apical HA and the basolateral G protein can be both detected throughout the Golgi apparatus, it is particularly important to find and characterize inhibitors that block at different Golgi or post-Golgi steps. Monensin appears to fulfill some of these requisites (20, 27, 74, 78, 79). We found that monensin did not affect the migration of HA to the Golgi apparatus but caused a delay of 2 h in the transport from the Golgi apparatus to the cell surface. This was accompanied by a delay in the maturation of HA, which never reached the full molecular weight of the mature glycoprotein, suggesting a defective glycosylation step in the presence of the drug. These results are in agreement with the hypothesis of Griffiths et al. (21) that monensin inhibits glycoprotein transport by acting at some intermediate step in the Golgi stack.

We have confirmed, in our system of MDCK cells infected with ts61, the findings of Matlin and Simons (50) on the effect of incubation at 20°C on the migration of fowl plague virus HA. Low temperature was more effective than monensin as an inhibitor of HA transport, since little glycoprotein was detected on the cell surface after 3–4 h. Because incubation at 20°C does not seem to inhibit the recycling of products taken by endocytosis (11), these data again suggest that the exocytic pathways of newly synthesized proteins and recycling surface proteins may be distinct. We hope that the pharma-

cologic and genetic dissection of the late stages in the exocytosis of apically and basolaterally directed viral glycoproteins will eventually provide a complete description of the mechanisms responsible for the sorting of plasma membrane proteins in epithelial cells.

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