Exit of Newly Synthesized Membrane Proteins from the *Trans* Cisterna of the Golgi Complex to the Plasma Membrane

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ABSTRACT The intracellular location at which the G protein of vesicular stomatitis virus accumulated when transport was blocked at 20°C has been studied by biochemical, cytochemical, and immunocytochemical methods. Our results indicated that the viral G protein was blocked in that cisterna of the Golgi stack which stained for acid phosphatase. At 20°C this *trans* cisterna became structurally altered by the accumulation of G protein. This alteration was characterized by extensive areas of membrane buds which were covered by a cytoplasmic coat. These coated structures were of two kinds—those that labeled with anti-clathrin antibodies and those that did not. The clathrin-coated pits consistently did not label with anti-G antibodies. Upon warming infected cells to 32°C, G protein appeared on the surface within minutes. Concomitantly, the *trans* cisterna lost its characteristic structural organization. Double-labeling experiments were performed in which G protein localization was combined with staining for horseradish peroxidase, which had been taken up from the extracellular medium by endocytosis. The results suggest that the *trans* cisterna was distinct from the endosome compartment and that the latter was not an obligatory station in the route taken by G protein to the cell surface.

Exocytosis of both secretory and membrane proteins is inhibited by reducing the temperature to 15–20°C (7, 20, 22, 26a, 31, 34). At 20°C, for example, both the hemagglutinin of influenza and the G protein of vesicular stomatitis virus (VSV)[1] accumulate in a Golgi compartment (7, 22). Most of the accumulated hemagglutinin is resistant to endoglycosidase H (endo H) (22) and most of the G protein had acquired sialic acid (7), indicating that the proteins had at least reached the *trans* Golgi cisternae where terminal glycosylation is known to occur (11, 32, 36). If the temperature is raised to 32°C, the accumulated hemagglutinin and G proteins are transported to the surface within minutes, (7, 22), indicating that the block is completely reversible.

Recently, Saraste and Kuismanen (35) used a temperature-sensitive mutant of Semliki Forest virus in which the spike proteins accumulate in the endoplasmic reticulum of baby hamster kidney (BHK) cells at the nonpermissive temperature (39°C). Upon shifting to the permissive temperature (28°C), the spikes are transported at normal rates to the plasma membrane. If however, after accumulation at 39°C the temperature is dropped to 20°C, transport proceeded to a stage where trimming of oligosaccharides and partial resistance to endo H has occurred. Using a pre-embedding immunoperoxidase technique, they localized the accumulated spike proteins to *trans* Golgi cisternae, "vacuoles and vesicular elements," although the evidence for the *trans* localization was equivocal. They suggested that a class of 200-nm vesicles which were immunoreactive for G protein at 20°C were the carrier vesicles from the Golgi complex to the plasma membrane.

The temperature block thus represents a potentially powerful tool for elucidating the pathway of newly synthesized plasma membrane proteins from the Golgi complex to the plasma membrane. The proteins can be accumulated in a compartment at low temperature and then synchronously released by warming. Large amounts of proteins will then be in transit, making it easier to identify the compartments of

[1] *Abbreviations used in this paper:* BHK, baby hamster kidney; endo H, endoglycosidase H; HRP, horseradish peroxidase; M, matrix; TPPase, thiamine pyrophosphatase; VSV, vesicular stomatitis virus.

the pathway beyond the site of the block.

Here we present a morphological and biochemical characterization of the intracellular site at which VSV G protein accumulates at 20°C. To obtain synchrony of transport we took advantage of a well-characterized, temperature-sensitive mutant, ts 045 (5), of VSV and a cell line, BHK-21, in which we have extensively studied intacellular transport of membrane proteins (9, 11, 12, 14, 28, 29, 39).

We have also compared the localization of newly synthesized G protein with the endocytic compartment defined by horseradish peroxidase (HRP) uptake (20a). When BHK cells are allowed to endocytose HRP at 20°C, the endosome compartment is selectively filled, and transport to the lysosomes is blocked (20a). In the present study, 20°C was used to block both the newly synthesized G protein of VSV during intracellular transport and to accumulate exogenous HRP into the endosome compartment. Both G protein and HRP were then visualized using the frozen thin section technique (38) which combines high resolution structural preservation with immunolabeling. This could be done both at 20°C, and after release of the G protein to the plasma membrane by raising the temperature to 32°C. Hence, we could ask directly whether the endosome compartment is an obligatory station during exocytosis of membrane proteins.

MATERIALS AND METHODS

Cells and Virus: Baby hamster kidney (BHK-21) cells were grown to confluency in 35-mm diameter culture dishes in Glasgow's minimum essential medium containing 10% tryptose phosphate broth and 5% fetal calf serum (18). Stocks of wild-type VSV were prepared as described before (21). The VSV ts 045 mutant was obtained from Dr. Nicole Genty, University of Orsay. It was plaque-purified in BHK cells. A plaque was selected that showed little appearance of G protein at the cell surface of infected BHK-cells 4 h after infection at the nonpermissive temperature (39.5°C) as estimated both by immunofluorescence and immunoprecipitation of surface G protein. The clone of ts 045 selected (ts 045-6) had a titer of 2.7×10^9 plaque-forming units per ml at 32°C and less than 10^4 units at 39.5°C. For experiments with ts 045-6, the cell monolayers were infected for 1 h at 37°C and then shifted to 39.5°C, usually for an additional 2.5–3 h. Temperature shifts were done by moving the dishes to water baths at the desired temperature, and, at the same time, medium containing 20 μg cycloheximide per ml, prewarmed to the appropriate temperature, was added. The medium was Eagle's minimum essential medium with 10 mM HEPES, pH 7.3, containing 10% fetal calf serum.

Surface Radioimmunoassay: The assay used to measure the appearance of VSV G protein at the cell surface has been described (26).

Immunoprecipitation and Gel Electrophoresis: BHK cells were infected with ts 045-6 for 1 h at 37°C and incubated at 39.5°C for 2.5 h. The cells were washed with the Eagle's medium referred to above (methionine-free) and labeled with 50 μCi [^{35}S]methionine (1,200 Ci/mmol; Amersham Corp., Arlington Heights, IL) in 500 μl methionine-free Eagle's medium per dish at 39.5°C. At the end of the 10-min pulse, the cells were shifted to prewarmed Eagle's medium containing a 10-fold excess of cold methionine and 20 μg cycloheximidide per ml, and incubated at the desired temperatures. The chase was terminated by washing with ice-cold phosphate-buffered saline containing 0.2% bovine serum albumin. Ice-cold Hanks' balanced salt solution with 10% fetal calf serum was added and the dishes were held on ice until they were ready for antibody labeling.

The appearance of pulse-labeled VSV G protein on the cell surface was quantitated using an adaptation of the immunoprecipitation method of Ploegh et al. (27). Endo H sensitivity of intracellular G protein was monitored on the same cells. The VSV G protein on the cell surface was labeled for 1 h at 4°C with 300 μl affinity-purified anti-VSV G protein per dish. At the end of the incubation period the cells were washed with phosphate-buffered saline in 0.1% bovine serum albumin. Next, 500 μl of 50 mM Tris-HCl, pH 7.6, 1% Triton X-100, 0.1% bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride were added. The cells were scraped from the dish into the lysis buffer with the aid of a metal spatula, passed through a 22G syringe needle into 1.5-ml Eppendorf centrifuge tubes and centrifuged at 4°C for 5 min at 10,000 *g*. The supernatant fractions were transferred to fresh tubes, frozen in liquid N_2, and stored at −80°C.

The supernatant fractions of the [^{35}S]methionine-labeled cell lysates were thawed and centrifuged for 5 min at 10,000 g. 20 μl of the washed *Staphylococcus* bacterial suspension were added to each supernatant. After vortex mixing, the tubes were incubated on ice for 5 min. The samples were centrifuged at room temperature for 1 min to give pellet and supernatant fractions. The bacterial pellet, containing the VSV G protein from the surface, was washed six times at 4°C, twice with wash buffer (50 mM Tris-HCl, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 1 mg/ml bovine serum albumin), twice with wash buffer containing 0.5 M NaCl, once with wash buffer minus bovine serum albumin, and, finally, once with distilled water. The final washed pellets were taken up into 25μl volume of pH 8.8 gel sample buffer and stored frozen at −20°C. The supernatant fractions from the *Staphylococcus* treatment, containing intracellular VSV G protein which had not reacted with the anti-G protein applied to the cell surface, were transferred to new tubes, centrifuged for 5 min at 4°C, and again transferred to new tubes. 2 μl of anti-G protein were added to 200-μl portions of each of the centrifuged supernatant fractions. The samples were incubated overnight with rotation at 4°C. *Staphylococcus* bacteria were then added to each sample and the samples were incubated and washed as described above. The washed bacterial pellets were suspended in 200 μl of autoclaved 0.2 M citrate buffer solution at pH 5.5 with 1 mM phenylmethylsulfonyl fluoride and 0.1% SDS. Each fraction was split into two 100-μl aliquots, to one of which was added 2.5 μl (25 mU/ml) endo H (Miles Laboratories, Naperville, IL). The samples were incubated at 37°C for 10 h with constant rotation. An equal volume of 40% trichloroacetic acid was added, and the samples were incubated overnight at 4°C. The tubes were centrifuged for 10 min at 10,000 *g*, the supernatant fractions were discarded, and the tubes were again briefly centrifuged and residual supernatant fluid removed. Finally, the pellets were resuspended in 25 μl sample buffer. Samples for electrophoresis and polyacrylamide gels (10%) were prepared as described (22). After running, the gels were fixed in 45% (vol/vol) methanol and 7% (vol/vol) acetic acid for 1 h, treated for fluorography with En^{3}Hance (Amersham Corp., England), vacuum dried, and exposed to XO Mat-AR film (Eastman Kodak Co., Rochester, NY).

Immunocytochemistry: Cells were removed with proteinase K (50 μg/ml) on ice (9), centrifuged, and fixed with either 0.5% glutaraldehyde in 200 mM PIPES buffer, pH 7.0, for 30 min or 8% formaldehyde in the same buffer for about 2 d. The pellets were rinsed with 200 mM PIPES (only if glutaraldehyde was used), infused with 2.1 M sucrose in phosphate-buffered saline (15 min) then prepared for cryo-sectioning (13, 15). Sections were labeled with two different antibodies to the G protein: a rabbit affinity-purified anti-spike (luminal domain) used at 8 μg/ml or a mouse monoclonal prepared against a synthetically prepared pentadecamer (15 amino acids) from the carboxy-terminal end of the G protein (a gift from Dr. Thomas Kreis). The specificity of this antibody is being documented elsewhere (Kreis, T., manuscript in preparation). Affinity-purified rabbit anti-clathrin antibodies (a gift of Dr. Daniel Louvard) were used at a concentration of 30 μg/ml. Labeling was carried out for 30–45 min at room temperature. The antibodies were visualized by protein A–gold of sizes ranging from 3 to 7 nm: these were prepared by a recently developed method of Slot and Geuze (35a). In the case of the mouse monoclonal, a sandwich antibody was used—an affinity-purified rabbit-anti mouse IgG (20 μg/ml). The sections were embedded in 25 centipoise methyl cellulose as described (15). (For a discussion of the factors affecting contrast of cryosections, see also references 11, 13, 15, and 38).

Cytochemistry: The procedure for acid phosphatase and thiamine pyrophosphate (TPPase) was as described previously (10, 12). A modification which improved the cytochemical localization was to add 10% dimethyl sulfoxide to the incubation media. When used for cryo-sections, pieces of cell pellet were incubated in the reaction mixture without dimethyl sulfoxide, rinsed with the same buffer, infused with sucrose, and prepared further for sectioning in the usual way.

Horseradish peroxidase (HRP) was added to the cell culture medium (5 mg/ml) of infected cells for 2 h at 20°C. After proteinase K treatment, the cells were centrifuged, fixed in 0.5% glutaraldehyde in 200 mM cacodylate buffer, pH 7.4, and then incubated for 1–2 min only in the diaminobenzidine reaction mixture consisting of 1 mg/ml diaminobenzidine, 0.01% hydrogen peroxide in 200 mM cacodylate buffer, pH 7.4 (20a). These cells were rinsed with cacodylate buffer and prepared for cryo-sectioning.

RESULTS

Immunological and Biochemical Assays for the Intracellular Transport of the VSV G Protein

We examined the appearance of VSV G protein at the plasma membrane of BHK-21 cells infected with ts 045-6

using a surface radioimmunoassay. Fig. 1 shows that at 39.5°C practically no G protein was detectable at the cell surface 3.5 h after infection. When the cultures were shifted to 32°C in the presence of cycloheximide, G protein was transported to the cell surface. If the cultures were shifted to 20°C instead, most of the G protein remained inside the cell. The block of transport of G protein to the cell surface at 20°C is not absolute; some surface G protein was detected at the plasma membrane after a 2-h chase and if the chase was continued for longer times more G protein appeared at the plasma membrane (data not shown). Shifting cells which had been held at 20°C for 2 h to 32°C resulted in the rapid appearance of the G protein at the cell surface (Fig. 1).

To examine the kinetics of newly synthesized G protein appearing at the cell surface, we used an immunoprecipitation assay in which anti-G protein antibody was allowed to bind to G protein on the cell surface at the end of the pulse-chase experiment. After a 60-min chase at 39.5°C, little G protein was detected at the plasma membrane (Fig. 2, lane *1*). If the chase was performed at 32°C after pulse-labeling for 10 min at 39.5°C, increasing amounts of ^{35}S-labeled G protein appeared at the cell surface (Fig. 2, lanes *2–4*). Transport to the cell surface was also found to be arrested with this method if the chase was performed at 20°C (Fig. 2, lanes *5–8*). By shifting the temperature from 20°C to 32°C, ^{35}S-labeled G protein was immunoprecipitated at the cell surface 5–30 min after the shift (Fig. 2, lanes *9–11*). We also examined the sensitivity of the VSV G protein to endo H, the enzyme that distinguishes between high mannose-type and complex-type glycans bound to glycoproteins (37). The mature G protein contains two N-linked oligosaccharide chains (4, 30). After the 10-min chase at 39.5°C, the G protein was completely sensitive to endo H, and this was also the case after chasing for 60 min at the restrictive temperature (data not shown). However, if the chase was performed at 20°C, an increasing proportion of the G protein pulse-labeled at 39.5°C became endo H–resistant; after 2 h about half of the G protein had acquired endo H resistance (Fig. 3).

These data show that the transport of the G protein of VSV ts 045-6 was arrested inside the cell at 39.5°C as is well known from previous studies (1, 5, 40). When the temperature is lowered to 32°C, the G protein made at 39.5°C moved to the cell surface. As shown previously for other proteins (7, 22, 26a, 31, 35), transport to the cell surface could be arrested by reducing the temperature to 20°C. As judged by the endo H resistance, G protein moved into the Golgi complex during the 2-h chase, and by shifting the temperature from 20 to 32°C, rapid and synchronous externalization of the intracellular G protein was achieved.

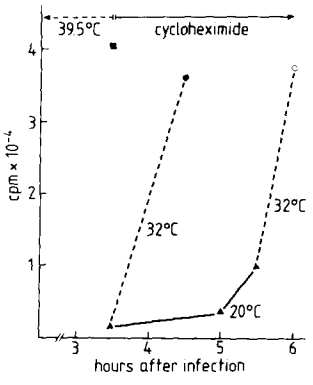


FIGURE 1 Surface appearance of VSV G protein. The cells were infected with either wild-type or mutant virus for 1 h at 37°C and were then incubated at 39.5°C for 2.5 h, after which they were shifted to 32°C (●) or to 20°C (▲). Cycloheximide (20 μg/ml) was added to the cells 3.5 h after infection. Cells which had been incubated for 2 h at 20°C were then shifted to 32°C (○). The relative amounts of G protein at the cell surface were measured by radioimmunoassay with anti-G protein antibody and iodinated protein A. Note that the amount of surface G protein after 3.5 h of infection with wild-type VSV (■) is only ~10% higher than after the shifts to 32°C during ts 045-6 infection.

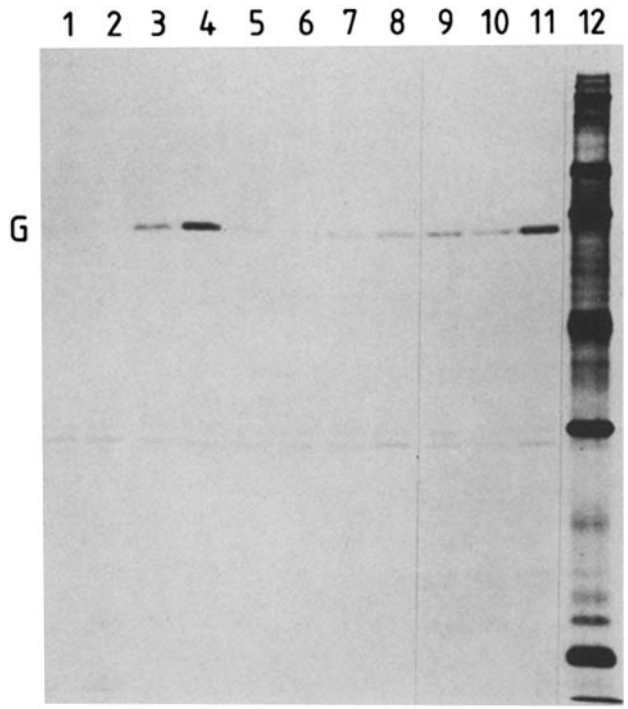


FIGURE 2 Transport of G protein to the cell surface. BHK cells were infected with ts 045-6 and held at 39.5°C for 2.5 h, pulse-labeled with [^{35}S]methionine for 10 min at 39.5°C, and thereafter chased at different temperatures. At each time point the cells were kept on ice until the end of the incubation. Then anti-G protein antibody was applied to the cells. The amount of G protein immunoprecipitated was analyzed by fluorography after SDS gel electrophoresis. Chase at 39.5°C for 60 min (lane *1*); chase at 32°C for 0 (lane *2*), 30 (lane *3*), and 60 min (lane *4*); chase at 20°C for 60 (lane *5*), 90 (lane *6*), 120 (lane *7*), and 150 min (lane *8*); chase at 20°C for 120 min and then shift to 32°C for 5 min (lane *9*), for 10 min (lane *10*), and for 30 min (lane *11*). Lane *12* represents molecular mass markers: lysozyme (14.3 kD), carbonic anhydrase (30 kD), ovalbumin (46 kD), phosphorylase b (99.5 kD), and myosin (200 kD).

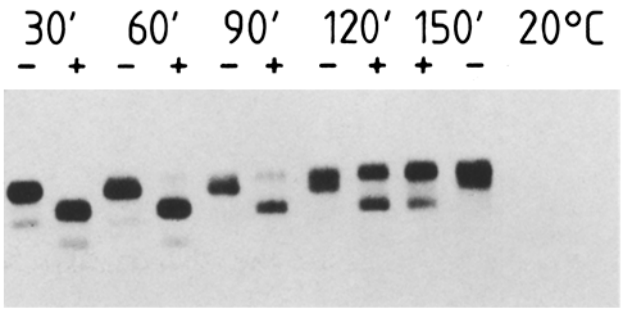
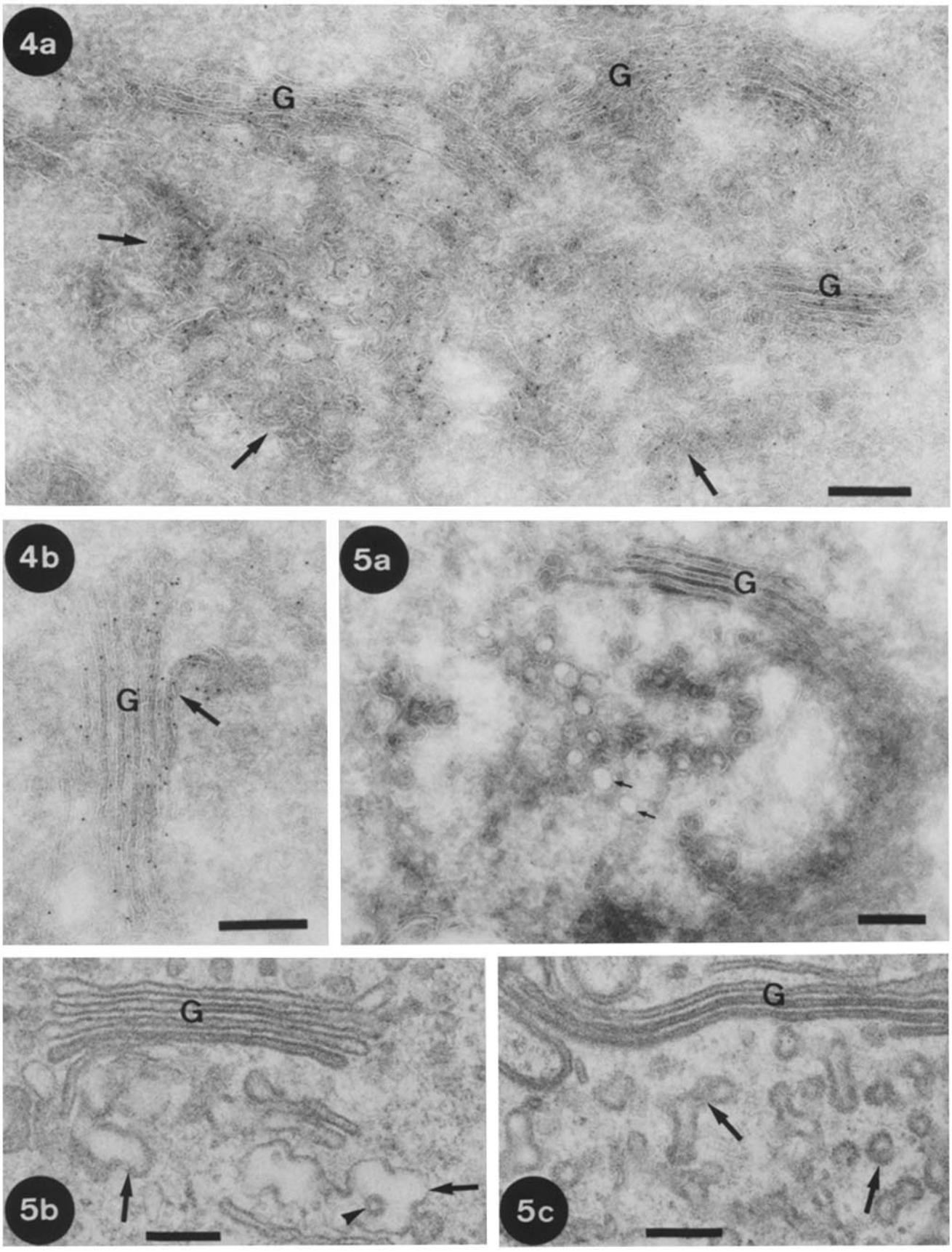


FIGURE 3 Conversion of VSV G protein from an endo H–sensitive to –resistant form after pulse labeling. BHK cells were infected with ts 045-6, and kept for 2.5 h at 39.5°C, pulse-labeled as in Fig. 2, and chased for different times at 20°C. One aliquot of the cell lysate was treated with endo H(+) and another was not (−). The G proteins were analyzed by fluorography after immunoprecipitation and SDS gel electrophoresis.


4a
G
G
G
4b
G
5a
G
5b
G
5c
G

Morphology

CHARACTERIZATION OF TS 045-6: When infected BHK cells were maintained at the nonpermissive temperature of 39.5°C, immunogold labeling of G protein was exclusively localized over the endoplasmic reticulum. Upon shifting to 32°C, in the presence of cycloheximide, the G protein moved through the Golgi stack (2–20 min after the shift) and appeared at the plasma membrane (30–45 min after the shift) (data not shown). The kinetics of transport agree with our biochemical data and with a previous morphological study by Bergmann et al. (1).

CHARACTERIZATION OF THE SITE OF ACCUMULATION OF G PROTEIN AT 20°C: BHK cells, infected with VSV ts 045-6 and kept at 39.5°C for 2.5 h, were switched to 20°C for 2 h in the presence of cycloheximide and then fixed and prepared for immunocytochemistry. Two different antibodies against the G protein of VSV were used; an affinity-purified anti-spike antibody and a mouse monoclonal antibody against the cytoplasmic (carboxy-terminal) tail of the G protein (which we shall refer to as the anti-tail antibody). In all these studies the two antibodies gave essentially the same labeling patterns but the anti-tail antibody generally gave a higher density of immunogold in the endoplasmic reticulum and Golgi membranes, whereas the anti-spike labeled more heavily at the plasma membrane. After 2 h at 20°C, G protein was predominantly localized in an extensive tubular reticulum close to the Golgi stacks (Fig. 4). There was also variable labeling of the Golgi stacks (cf. Figs. 4, 11, and 14). In some cases, one to three cisternae on the *cis* side of the stack (see below) was devoid of label; in most cases the whole stack was labeled (cf. Figs. 4*b* and 11). A small number of gold particles were observed on the endoplasmic reticulum (even in the presence of cycloheximide) and on the plasma membrane. No significant labeling was seen over other identifiable structures.

THE FINE STRUCTURE OF THE TUBULAR RETICULUM: The reticular membrane structure present in the Golgi region following the 20°C block had a very characteristic structure, especially when sections were heavily contrasted with uranyl acetate (Fig. 5*a*). Most of the structural details were lost in conventional epon sections (Fig. 5, *b* and *c*). Three features were particularly striking in the cryo sections. The first was the presence of a distinct periodicity in the lumen of the reticulum. The second was the existence of cylindrical structures in the reticulum. The third was extensive regions of budding (or fusing) vesicles with a morphological coat on the cytoplasmic side.

THE PERIODICITY WAS DUE TO ACCUMULATED G PROTEIN: The periodically arranged dense regions seen in oblique sections through the reticulum was evident, in cross-section, as protuberances or spikes attached to the membrane (Fig. 6).

Although we cannot give an overall three-dimensional description of this complex reticulum, it was evident that the G protein was assembled into two different kinds of periodic structures. The first were circular membrane structures in the lumen of the reticulum, which must represent cylindrical structures in three dimensions. These cylinders, which had the spikes on their outside (projecting into the lumen) were 39 ± 7 nm in diameter from membrane to membrane (excluding spikes). We cannot say whether their membranes were still continuous with that of the reticulum itself, but most likely they represent deep, finger-like invaginations into the lumen. The second type of cylindrical structures were made of buds that projected away from the reticulum with the spikes now on their inside (although still, of course, luminal). These appeared to be still continuous with the membrane of the reticulum (they are therefore "incomplete cylinders"). These structures were 69 ± 5 nm in diameter. In both cases the spikes were 7.8 ± 0.8 nm long, ~2 nm wide, and were spaced 8–9 nm apart (Figs. 6 and 7).

Normal budding of VSV virions at the plasma membrane is known to be dependent on the presence of the matrix (*M*) protein on the cytoplasmic side of the membrane (19). Preliminary labeling experiments with anti-M antibodies (a gift from Dr. Heinz Arnheiter) indicated that this reticulum was devoid of labeling at 20°C, whereas the plasma membrane labeled (results not shown).

THE G PROTEIN IS BLOCKED IN A MODIFIED *TRANS* GOLGI CISTERNA: In some sections direct continuities were evident between the reticular structure and a Golgi cisterna on one side of the stack (Fig. 4*b*). Cytochemical studies with the classical *trans* Golgi markers, acid phosphatase and thiamine pyrophosphatase (TPPase) indicated that this cisterna was the most distal or *trans* cisterna. Using both plastic and frozen sections (where cytochemistry and immunocytochemistry could be directly combined), TPPase was found to react with one or two cisternae on one side of the stack; there were variable amounts of reaction product in the reticular structures (Fig. 8, *a* and *b*). Acid phosphatase, in general, stained the reticular structure, plus the connecting (*trans*) cisterna (Fig. 8*c*). As expected, secondary lysosomes were also reactive (not shown). We have always observed some overlap in the activities of TPPase and acid phosphatase with respect to the penultimate (the "classical" TPPase-reactive) and the *trans* (the acid phosphatase–reactive) Golgi cisternae in BHK cells. A similar phenomenon has been described in other systems under certain conditions (17).

COATED STRUCTURES IN THE GOLGI REGION: TWO KINDS OF "COAT": As is evident in Fig. 6, there were many regions of the modified *trans* cisterna which had a

FIGURES 4 and 5 Frozen thin section of BHK cells infected with VSV ts 045-6, held at 39.5°C for 2.5 h, and shifted to 20°C for 2 h. Fig. 4, *a* and *b*, shows labeling with the anti-tail and anti-spike antibodies, respectively, followed by protein A–gold (5 nm). In 4*a* the arrows indicate the reticular structure on one side of the Golgi stacks (*G*). In 4*b* the arrow indicates the "peeling off" of the *trans* cisterna to form the reticulum (only a small part of which is evident in this micrograph). Fig. 5*a* shows a low power image of the *trans* reticulum as it appears in a well-contrasted frozen section. The arrows indicate the cylindrical structures which are prominent within the reticulum. Fig. 5, *b* and *c*, shows the appearance of the same structures in epon sections. The arrows indicate the *trans* reticulum. The periodicity evident in Fig. 6 is completely lost in these sections: the membrane of one "cylinder" is evident in cross-section within the lumen of the *trans* reticulum (arrowhead, Fig. 5*b*). Bars, 200 nm. (4*a*) × 75,000; (4*b*) × 79,000; (5*a*) × 59,000; (5, *b* and *c*) × 67,000.

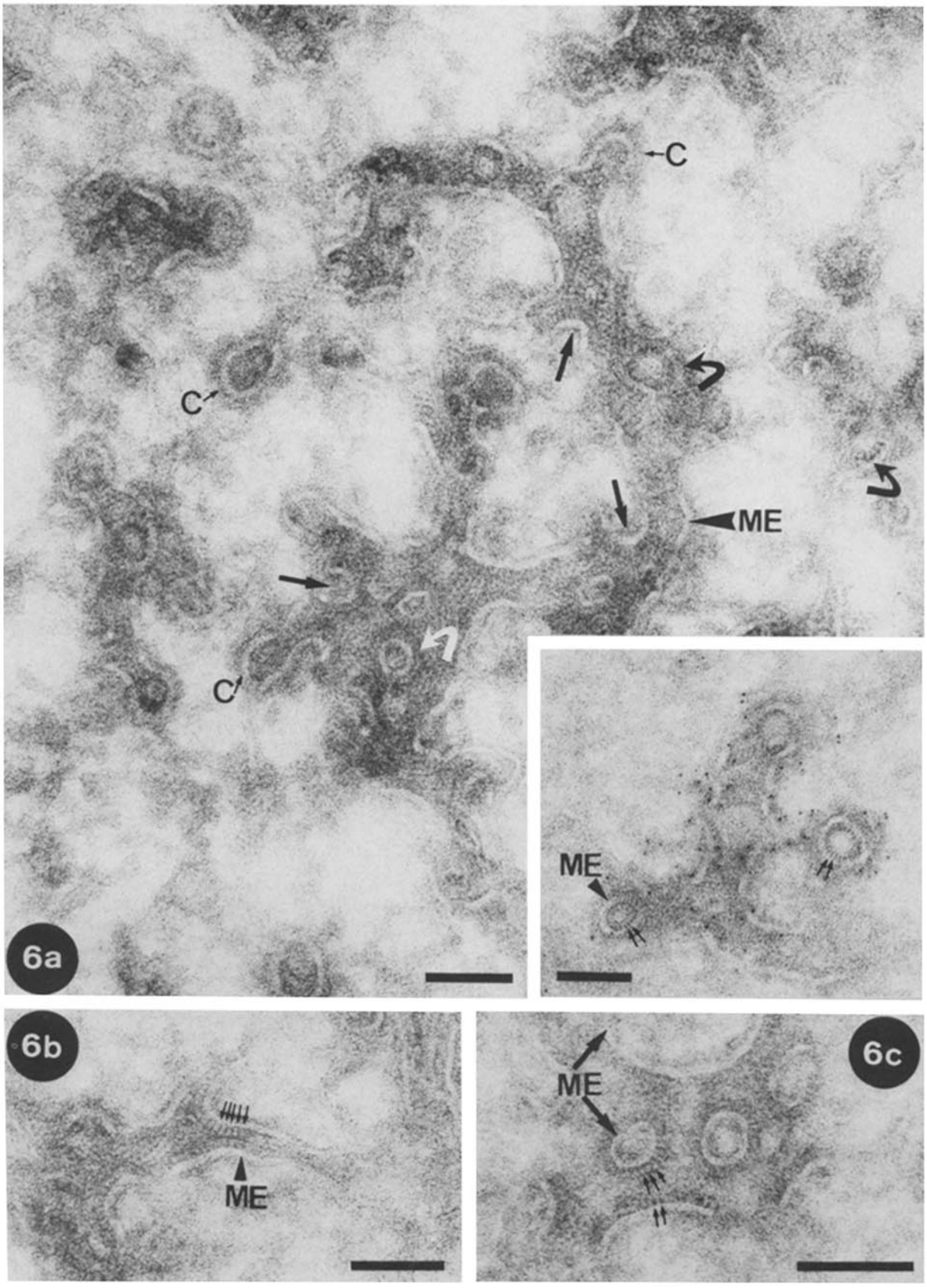


FIGURE 6 Appearance of the modified *trans* cisterna on cryosections heavily contrasted with uranyl acetate (*ME* is the negatively stained membrane). In *a*, the periodicity due to the accumulated G protein is apparent throughout the cisterna, as well as "cylinders" with spikes on their outside (white arrow) or on their inside (curved black arrows). The black, straight arrows indicate invaginations of the membrane with spikes on the lumenal side which may represent forming "cylinders." Note also the coated pit-like structures (*C*). The *inset* to Fig. 6*a* shows parts of three "cylinders" with spikes on their inside. This preparation is from cells that were warmed to 32°C for 5 min following the 20°C block: sections were labeled with the anti-tail antibody. In *b* and *c*, the structure of the spikes (arrows) in the modified *trans* cisterna is shown more distinctly. Bars, 100 nm. (*a*) × 150,000 (*inset*, × 133,000); (*b*) × 160,000; (*c*) × 200,000.

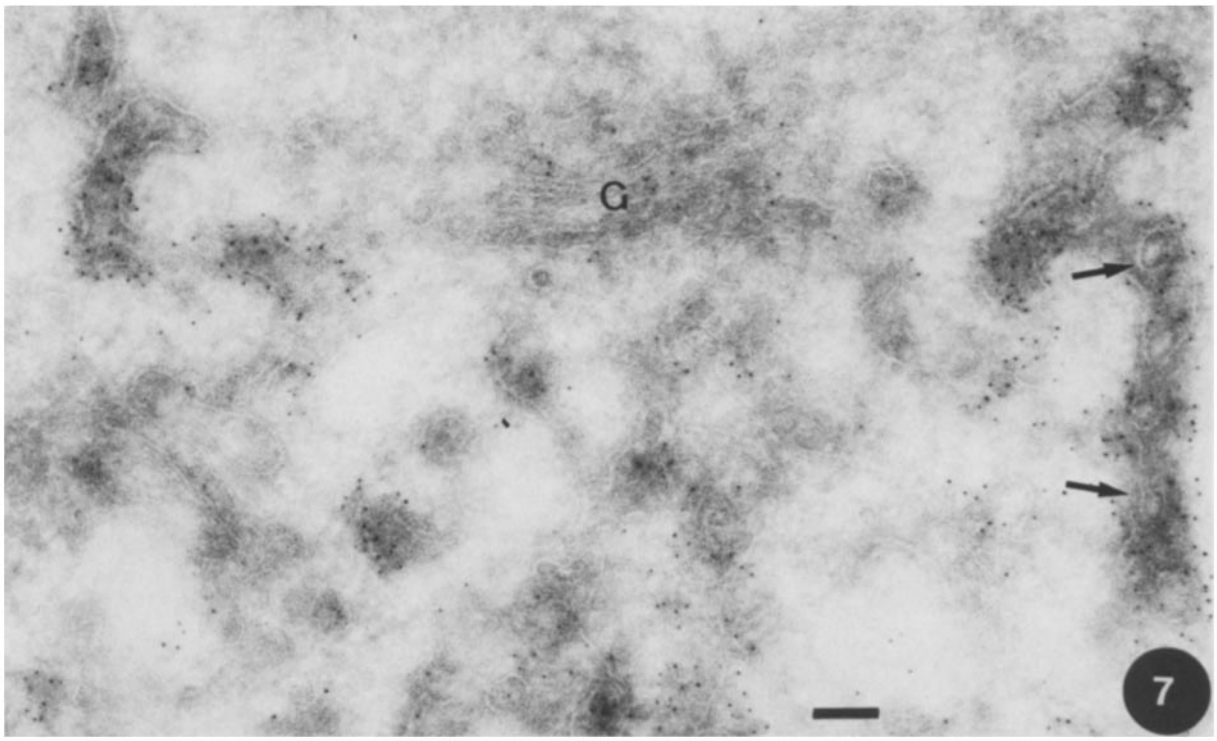


FIGURE 7 Appearance of the Golgi region labeled with the anti-tail antibody after a 5-min shift to 32°C (following 2 h at 20°C). The labeling of the Golgi stack (G) is greatly reduced compared to 20°C (cf., Figs. 4, 11, 14*a*). The *trans* reticulum is now less compact (cf., Figs. 4*a*, 5*a*) and, as a result, more accessible to the immunogold labeling. Note the row of labeled cylinders on the right of the micrograph (two are indicated by the arrows). Bar, 100 nm. × 94,000.

morphological coat reminiscent of clathrin. Clathrin-coated structures are, in fact, known to exist on the *trans*-most cisterna (2, 10, 25). When sections were labeled with an affinity-purified anti-clathrin antibody there was, indeed, extensive labeling of many of these vesicular profiles, which we assume to be in the process of budding (Figs. 9 and 10). The average diameter of these vesicles (membrane to membrane) was 72 ± 9 nm. The extent of this labeling was far greater than that seen in uninfected cells (at 37°C) or in infected cells maintained at the permissive temperature, 32°C. Significantly, there were also many budding profiles with morphological coats that consistently did not label with clathrin, although equal in size (average diameter 71 ± 10 nm) (Figs. 9 and 10). Similarly, the rims of other Golgi cisterna often appeared to be both budding and "coated" (Figs. 9 and 10). These were also invariably devoid of clathrin labeling. Coated pits at the plasma membrane were easily recognized morphologically: these labeled with clathrin as expected (results not shown).

The coated structures in the Golgi region, both on the rims of the cisternae, and on the *trans* reticulum, consistently did not label with either of the antibodies to the G protein (Fig. 11). A few budding profiles without a distinct coat structure did label with the anti-cytoplasmic tail antibody (Fig. 11). Preliminary double-label experiments with anti-clathrin and anti-G protein, using two different sizes of gold, have confirmed that the *trans* Golgi clathrin-coated pits are invariably devoid of G protein (results not shown—see Discussion).

THE EFFECT OF WARMING TO 32°C: Between 5 and 30 min after warming infected cells from 20 to 32°C there was a significant increase in the amount of G protein localized at the plasma membrane (Fig. 12), especially on microvilli. This was accompanied by a complete disappearance of label from the Golgi stack (cf. Figs. 7, 12, and 15*d*). Before warming, 257 ± 14 gold particles per μm^2 of Golgi stack were seen by labeling with the anti-tail antibody; this was reduced to 29 ± 6 after 15 min of warming. Concomitantly, a significant increase was seen in the amount of G protein detected both biochemically (Figs. 1 and 2) and immunocytochemically (Fig. 12) at the plasma membrane. The *trans* reticulum, recognizable by its structural characteristics, still showed significant labeling (Fig. 7), but progressively, the whole structure became more fragmented and with increasing time after the temperature shift, the "fragments" were less frequently found in proximity to the Golgi stack (e.g., Fig. 15*d*). During the fragmentation process, single rows of connected cylinders were often found (Fig. 7). These appeared to be more accessible to labeling than the more compact structure found at 20°C (cf. Fig. 4*a* with the inset of Fig. 6, and with Fig. 7). Even at 1 h after warming to 32°C, a few small fragments of labeled cylinders could be seen in the cytoplasm. Stereological measurements, now in progress, indicate that the total surface area of the *trans* reticulum is greatly reduced upon warming to 32°C.

Between 5 and 15 min of warming to 32°C, structures tentatively identified as transport vesicles between the *trans* Golgi cisterna and the plasma membrane could be visualized in the cytoplasm (see Figs. 12*c*, 15*d*). These were round structures, 50–100 nm in diameter, which were only recognizable due to their labeling with the anti-tail antibodies. Since their diameter is about half the average section thickness (see Discussion) and the spikes must be on their inside, most of these vesicles have probably not been sectioned and were therefore not accessible to anti-spike antibody. Membrane structure was only sometimes evident. This may be due to

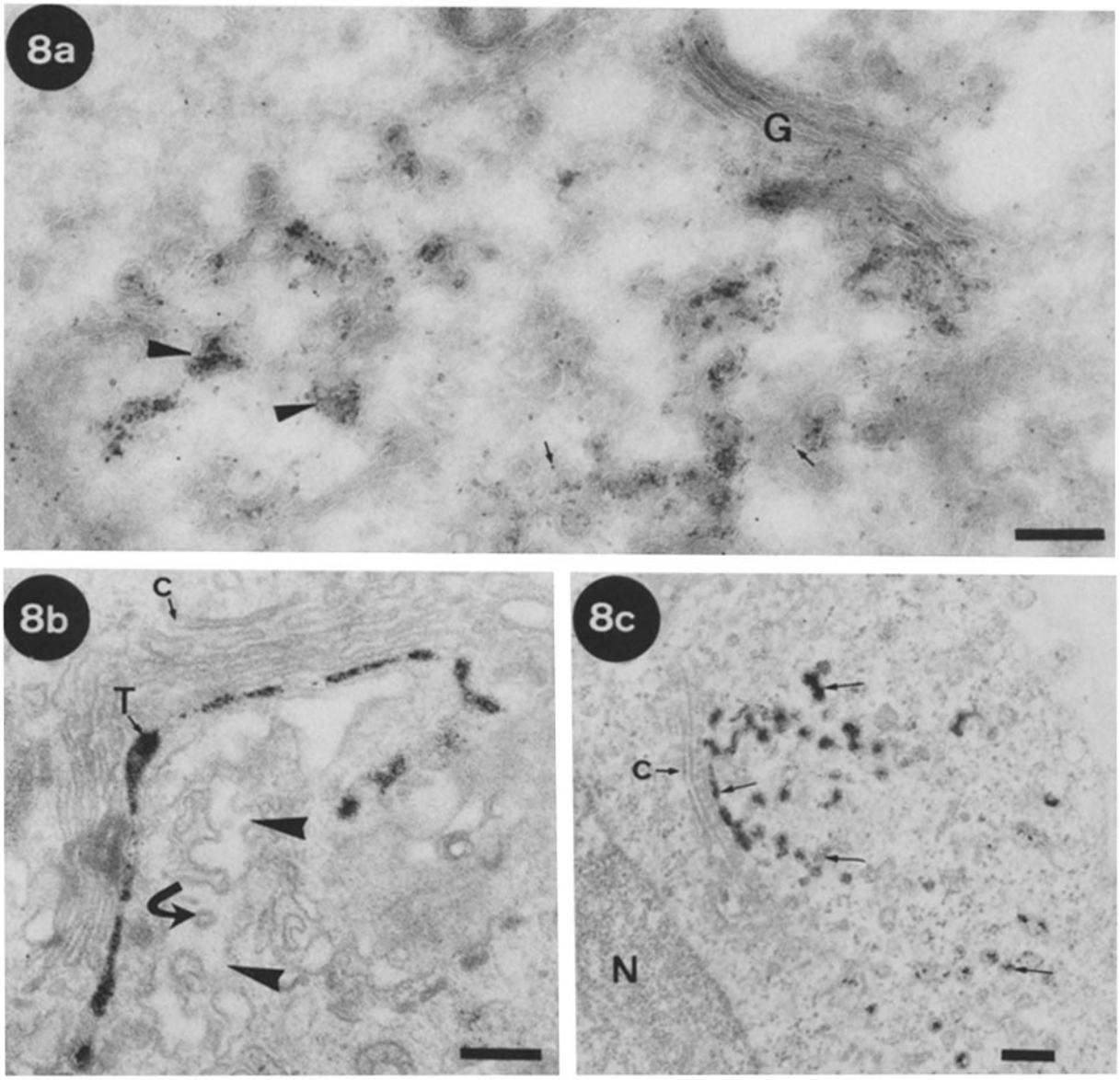


FIGURE 8 Cytochemistry of the *trans* Golgi cistern after 2 h at 20°C. (*a* and *b*) TPPase localizations; (*c*) acid phosphatase localizations. Fig. 8*a* is an image of a frozen section of a BHK cell infected with VSV ts 045-6 (2 h, 20°C) which had been incubated with TPPase prior to freezing. The thawed sections were labeled with anti-G-spike antibodies and protein A-gold. The gold particles (arrows) can easily be distinguished from the floculent precipitate of the reaction product (arrowheads). In this example, reaction product is evident in the *trans* cisterna. In Fig. 8*b*, a conventional epon section, the reaction product is exclusively localized in the penultimate cisterna (the "classical" TPPase cisterna). The *trans* cisterna, free of reaction product, can be recognized by its position and characteristic shape (arrowheads). Note the complete loss of fine structure within this cisterna after epon embedding (cf., Fig. 5, *b* and *c*): the membrane of a "cylinder" is apparent (curved arrow). Fig. 8*c* shows exclusive localization of acid phosphatase (epon section) over the modified *trans* cisterna (arrows). As for TPPase, localization of acid phosphatase is not always this clear cut; variable amount of reaction product is often observed in the penultimate cisterna also. *G*, Golgi stack; *c*, *cis* side of Golgi region; *N*, nucleus; *T*, TPPase cisterna. Bars, 200 nm. (*a*) × 63,000; (*b*) × 58,000; (*c*) × 38,000.

the inability of the methyl cellulose mixture, used to embed or dry the thawed frozen sections, to protect these labile structures upon drying (15, 38).

Preliminary studies were made to compare the labeling of the *trans* cisterna with anti-clathrin antibodies under different temperature conditions. The result indicated that the labeling of this structure was greatly diminished within 5 min of warming to the permissive temperature.

RELATIONSHIP OF THE *TRANS* GOLGI RETICULUM TO COMPARTMENTS INVOLVED IN ENDOCYTOSIS: The tubules of the endosome compartment are often near the Golgi stacks in BHK cells (20a). Gonatas et al. (8) and Essner and Haimes (3) have shown that some endocytosed ligands are localized, at least transiently, in the most distal cisterna of the Golgi stack that reacts for acid phosphatase. An obvious question, therefore, was whether the *trans* reticulum of the Golgi region is part of the endosome compartment?

We could test this directly, since the temperature at which the G protein of VSV accumulates in *trans* Golgi is the same as that required for exogenously administered HRP to be endocytosed into, and remain in, endosomes (20a). BHK cells infected with ts 045-6 were held for 2.5 h at 39.5°C and the

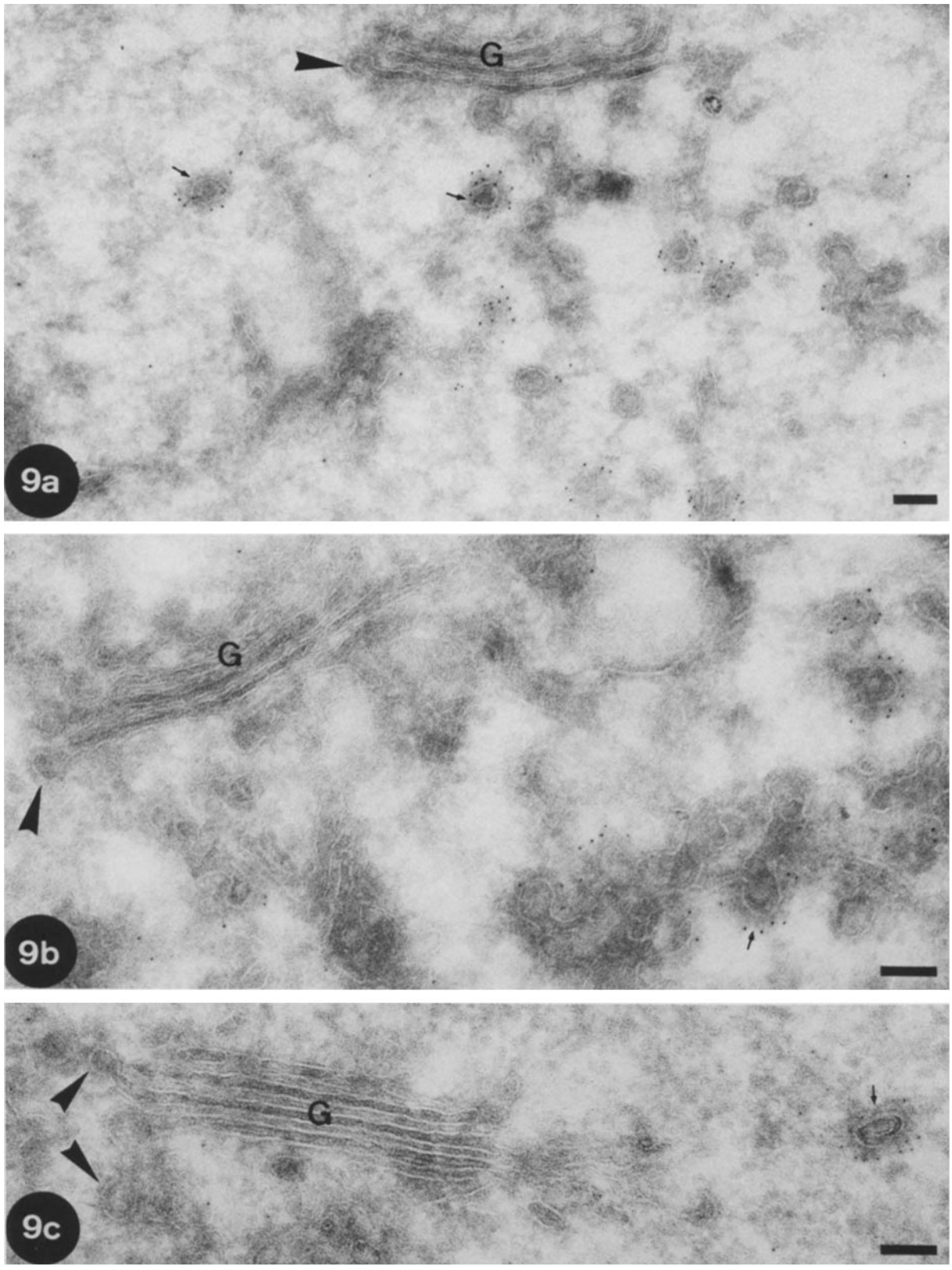
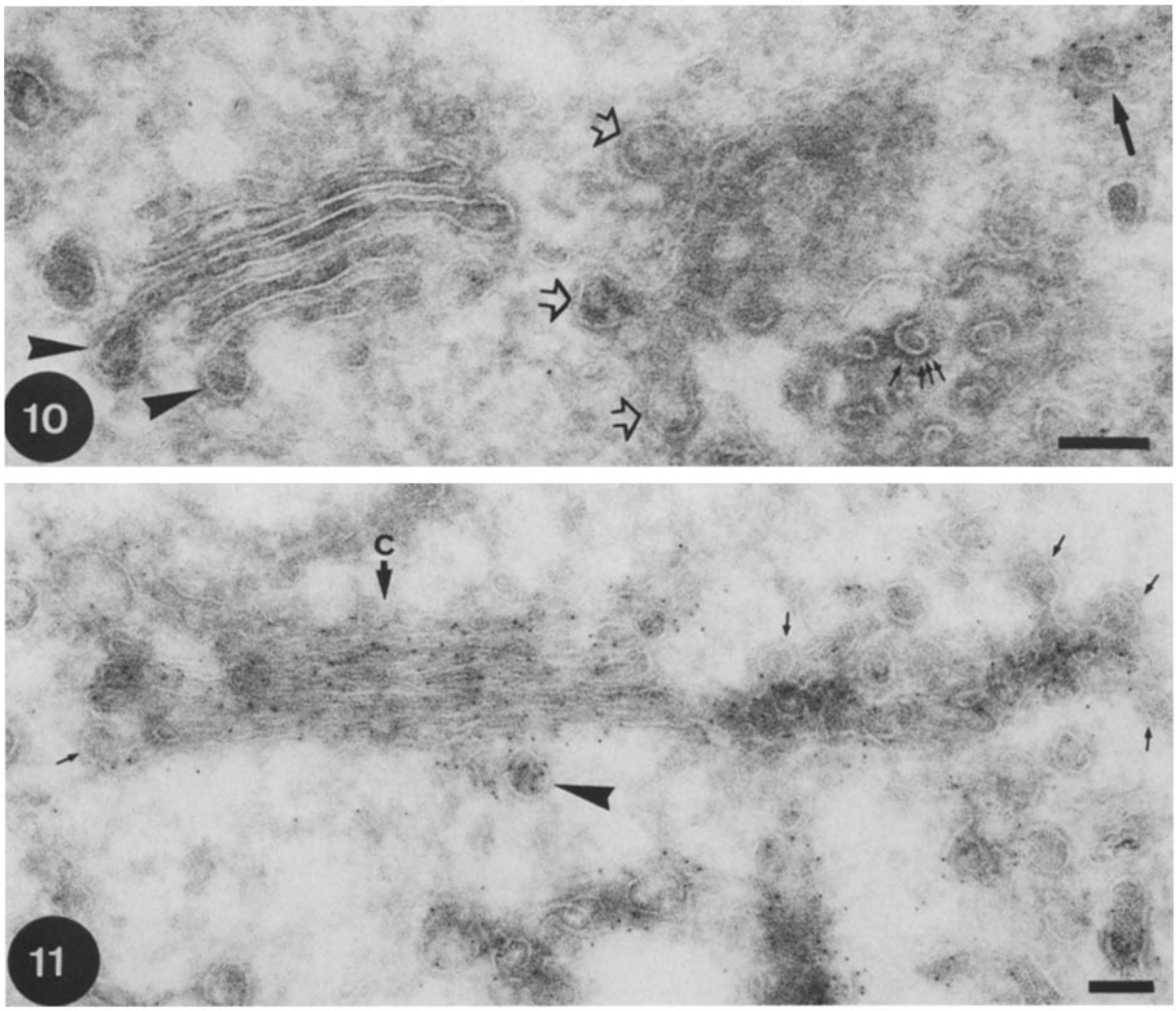


FIGURE 9 Examples of anti-clathrin labeling (arrows) of the *trans* cisterna after 2 h at 20°C. Various coated structures label (arrows). Coated rims of other Golgi cisternae are invariably devoid of label (arrowheads). Bars, 100 nm. (*a*) × 74,000; (*b*) × 92,000; (*c*) × 92,000.

temperature was then dropped to 20°C. At this time, 5 mg HRP was added per ml culture medium. After 2 h, the cells were fixed, treated cytochemically with diaminobenzidine and processed for frozen sectioning. The sections could also be labeled with anti-G protein antibodies.

Even without osmium tetroxide treatment, which is used



FIGURES 10 and 11 (Fig. 10) Anti-clathrin labeling of the *trans* Golgi region after 2 h at 20°C. Only one coated vesicle structure (arrow) labels in this field: coated rims of Golgi cisternae (arrowheads) as well as coated "buds" on the *trans*-most cisterna (open arrows) are free of label. The arrows indicate spikes on the lumenal side of a "cylinder." (Fig. 11) Labeling of the Golgi stack with the anti-tail monoclonal antibody after a 2-h VSV ts 045-6 infection at 20°C. Note the coated, budding profiles (arrows) on the *trans*-most cisterna that are devoid of label. c, *cis* side of the stack. The arrowhead indicates a possible budding vesicle on the *trans* cisterna that labels with the antibody. Bars, 100 nm. × 120,000 and × 90,000, respectively.

in plastic embedding to enhance the reaction product, the polymerized diaminobenzidine, indicating the site of HRP, was visible as highly electron-dense precipitate in frozen sections. The reaction product was present in vacuoles and tubules (Fig. 13) similar to those seen in our previous studies using plastic sections (20a). The structure of the tubular profiles found near the plasma membrane is shown in Fig. 15*a*. In the perinuclear region, vacuoles, which contain small vesicles in plastic sections, by definition multivesicular bodies, often appear to be filled with "strings" of small tubules in frozen sections (Fig. 15*b*). Other reactive vacuoles were seen that contained vesicles (Fig. 14*b*). These were structurally indistinguishable from secondary lysosomes. The latter could, however, be identified, since under these conditions, they were free of reaction product (Fig. 14*a*).

In these frozen section preparations there was no evidence of HRP reaction product in the Golgi stacks nor on the *trans* Golgi reticulum although close proximity of the two structures was often seen (Fig. 14). Since these structures are normally very electron dense due to uranyl deposits, uncontrasted or lightly contrasted sections were also examined. When the sections were also immunolabeled with antibodies against G protein there was no significant labeling of any HRP-positive structures. At low magnifications, the high electron density of the diaminobenzidine reaction product made visualization of gold particles difficult; at high magnifications, however, the gold, because of its higher density, could be identified (Fig. 15*e*). Under the electron beam, visualization of gold particles is unambiguous at high magnification.

Upon warming the cells to 32°C, the G protein appeared at the surface without being detected in any HRP-positive structures (Fig. 15, b–d). Only at 15 in or longer could a very small amount of significant labeling for G protein be seen over some HRP-positive profiles (data not shown). This is probably G protein that has gone to the surface and become endocytosed. The labeling of these profiles shows that the presence of the HRP reaction product is not the reason for the lack of detection of G protein in the endosome structures at 20°C. This was also confirmed by experiments in which HRP and VSV particles were endocytosed for 15 min at 37°C. In this

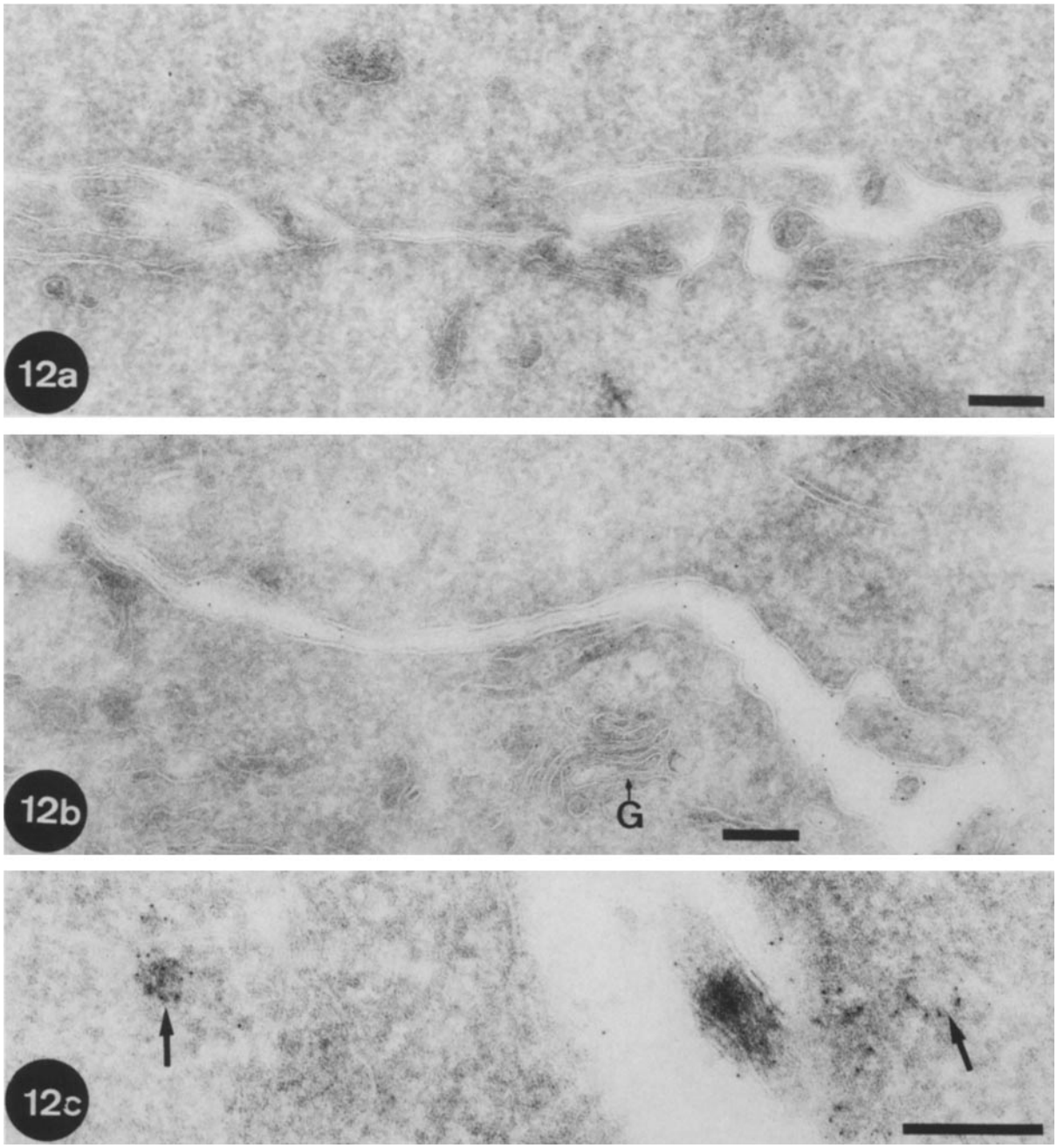


FIGURE 12 Surface labeling of G protein. *a* and *b* show the plasma membranes of two adjacent cells. *a* shows the absence of labeling with the anti-spike antibody after 2 h at 20°C. *b* shows a significant increase in labeling after 5 min warming to 32°C. Note also the absence of Golgi stack labeling (*G*). *c* shows labeling of two vesicular structures (arrows) in the peripheral cytoplasm of two adjacent cells with the anti-tail antibody after 15 min of warming to 32°C. Bars, 200 nm. (*a* and *b*) × 56,000; (*c*) × 107,000.

case, the membrane of HRP-positive structures with enclosed virions labeled extensively with G protein antibodies (Fig. 15*e*).

After 30 min of warming to 32°C, some of the G-protein "cylinders" were found in the HRP-positive structures. Since the HRP should be in both endosomes and lysosomes under these conditions (20a), we cannot say whether the HRP has been transported to the *trans* Golgi or, alternatively, residual *trans* Golgi fragments have been taken up by lysosomes.

DISCUSSION

We have shown that at 20°C, intracellular transport of G protein of VSV is blocked in that cisterna of the Golgi stack that stains for acid phosphatase. As a consequence of this block, the *trans* reticulum proliferates extensively. In addition to the G protein labeling of the *trans* cisterna, other cisternae of the stack are also significantly labeled, probably due to a back-up of protein proximal to the block. This in part explains the endo H sensitivity of a fraction of the G protein after incubation at 20°C. The *trans* cisterna is the most distal Golgi compartment as shown by the rapid disappearance of G protein from all the cisternae except the *trans* one, upon warming to the permissive temperature. It is from this cisterna that the G protein exits for the plasma membrane.

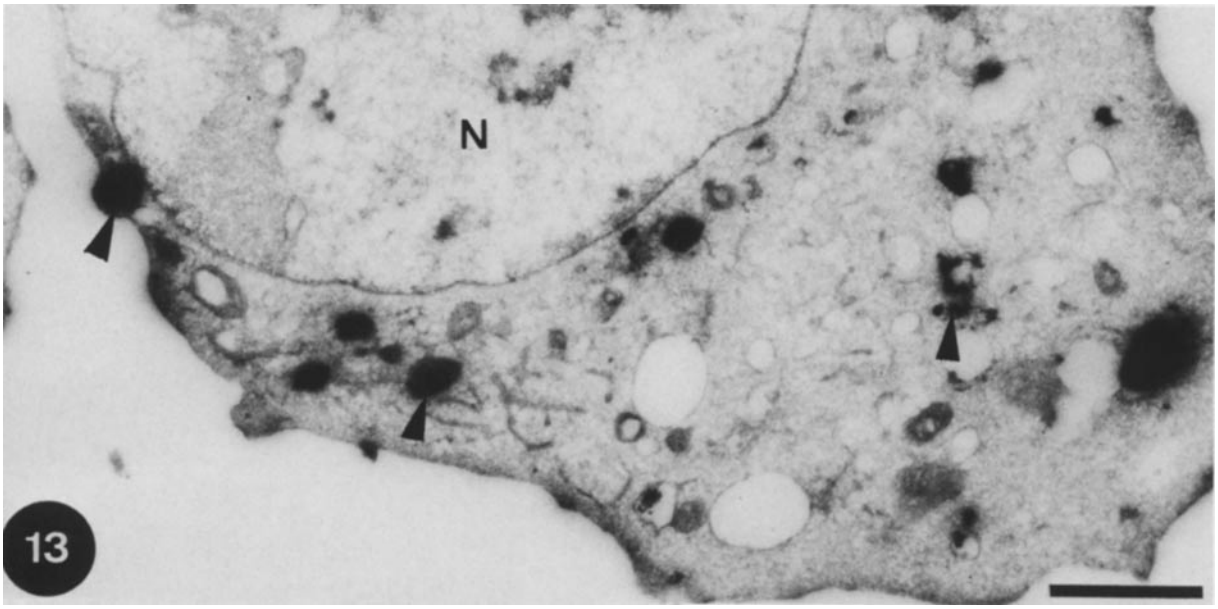


FIGURE 13 Frozen section of BHK cells infected with VSV ts 045-6 and kept for 2 h at 20°C with 5 mg/ml HRP in the culture medium. This is a low magnification image to show the HRP-reactive endosomes in the cytoplasm (arrowheads). *N*, nucleus. Bar, 2 μm. × 10,000.

Fuller et al. (7) have shown that when G protein is accumulated intracellularly at 20°C in Madin-Darby canine kidney cells, there is an increase in efficiency of sialation and galactosylation compared to G protein that is normally transported to the surface at 37°C. This effect was attributed to the fact that, at 20°C, G protein was exposed for a longer period to the compartment(s) where the relevant sugar transferases reside. Since G protein accumulates in significant amounts in at least three cisternae at 20°C, it is not possible from our data to pinpoint precisely the localization of these functions. Nevertheless, galactosyltransferase has been co-localized in HeLa cells to one or two cisternae on the *trans* side of the Golgi stack (36); it has also been directly co-localized with TPPase activity (32). It is therefore reasonable to suggest that this enzyme resides in the one or two penultimate cisternae on the *trans* side of the Golgi stack in BHK cells. Sialation, which is a later event, would then either reside in the same compartment or in the more distal compartment that reacts exclusively for acid phosphatase.

The G protein spikes which accumulate in the *trans* cisterna at 20°C are assembled into regular arrays. This assembly appears to form in the absence of the matrix protein. Further, there is no morphological evidence that nucleocapsids are bound in any way to this structure. Hence, it seems that the "budding" phenomena observed may be solely a consequence of the dense packing of the G protein itself in the plane of the membrane.

In addition to the unusual packing of the G protein in the *trans* reticulum, the other striking observation was the presence of large arrays of coated pits which are most likely in the process of budding from the reticulum. Many of these labeled with antibodies to clathrin. Clathrin-coated pits have been described on the *trans* Golgi cisterna in other systems (2, 10, 25). Even without quantitative studies, which are now in progress, it is clear that the number of these clathrin pits is greatly increased as compared to uninfected cells or to VSV-infected cells kept at the permissive temperature. Significantly, many of the coated profiles did not label with clathrin, nor did the coated rims of Golgi cisternae which also appear to be in the process of budding. The simplest explanation for these observations is that these coated structures are not made up of clathrin and have a different function than the clathrin-coated structures. The labeling experiments have consistently indicated that the clathrin-coated pits, as well as many of the non-clathrin-coated pits, do not label for G protein. The occasional budding profiles which do label with the anti-cytoplasmic tail antibody are tentative candidates for transport vesicles rather than the clathrin-coated vesicles, as suggested by Rothman and Fine (33). Care must be taken with this interpretation, however, for two technical reasons. First, antigens on the luminal side of a budding vesicle may not be accessible to antibodies because the bulk of the vesicle may be contained within the frozen section (i.e., the section is thicker than the vesicles; see reference 15). Second, antigens on the cytoplasmic side may not be accessible to antibodies due to the presence of the coat structure itself. Further, we cannot rule out that at low temperatures the G protein is unable to enter the coated pits, but immediately upon warming, it enters the coated pits which then bud.

Within 5–30 min after switching the temperature from 20 to 32°C, significant changes are evident in the structure of the *trans* reticulum. At 20°C, this large reticulum is relatively compact and situated next to the Golgi stacks (although, because of its size it may be many micrometers away from the stack). Labeling with both G protein antibodies, especially the anti-spike one, is often relatively low for a structure that is packed with G protein. This, we believe, is due to steric hindrance; within minutes of warming to 32°C, accessibility to the anti-tail antibodies increases significantly. With increasing time at 32°C, the reticulum appears to fragment, and the fragments tend to be localized away from the stack. Preliminary quantitation indicates that it becomes significantly smaller in terms of membrane surface. These changes are accompanied by a significant increase in labeling of the plasma membrane. Experiments are now in progress to quantitate these membrane changes that accompany the tempera-

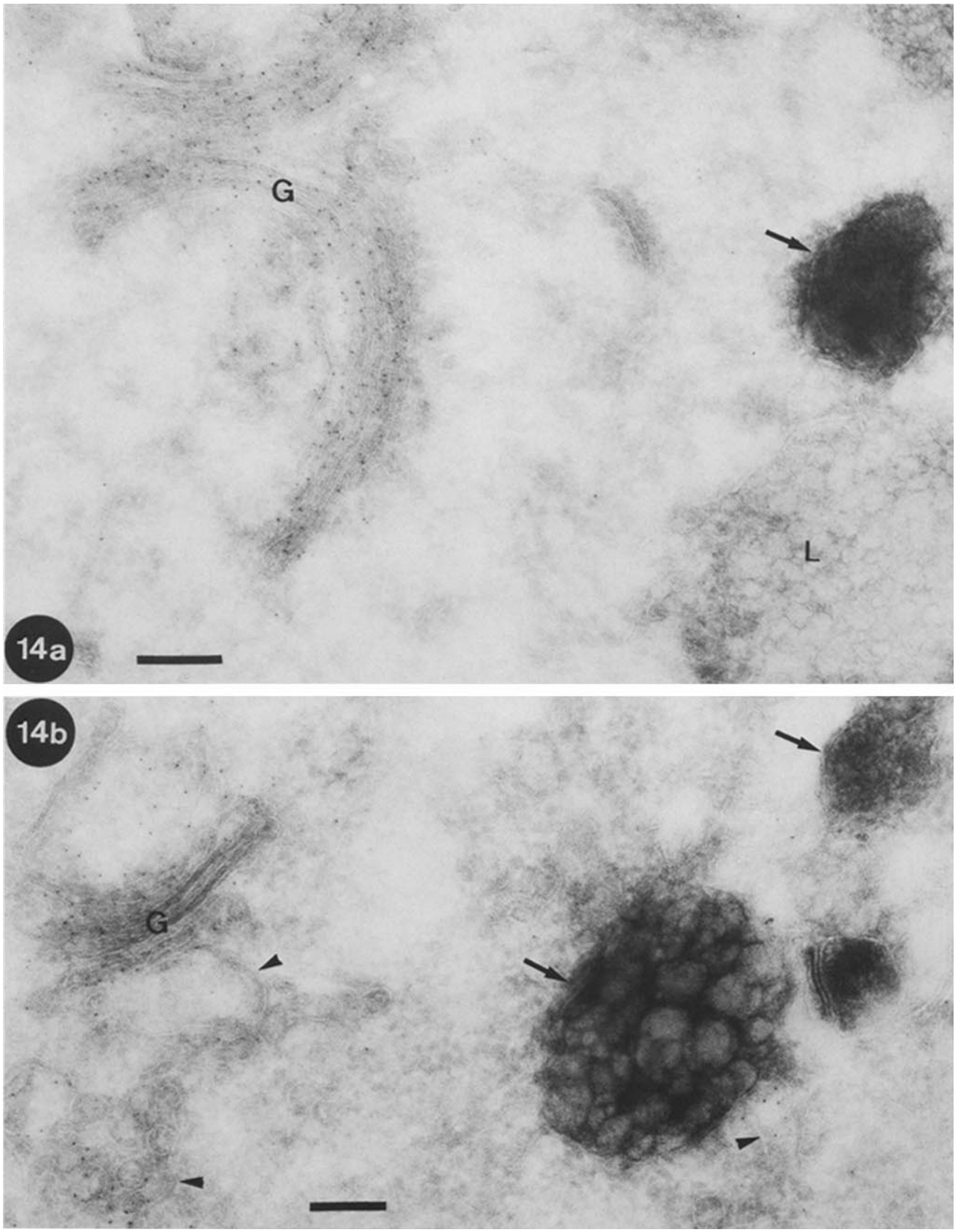


FIGURE 14 *a* and *b* show two high magnification images of the Golgi region of cells infected with VSV ts 045-6 (2 h at 20°C) which have been given HRP in the culture medium. Sections labeled with anti-tail antibody. The HRP-reactive endosome structures are easily identified due to their electron density (arrows). In *a*, note the profile of a secondary lysosome (*L*) which can be identified by its absence of reaction product. There is no significant labeling of any of these endosome profiles for G protein. (Arrowheads) *Trans* reticulum. *G*, Golgi stack. Bars, 200 nm. *a*, × 77,000; *b* × 70,000.

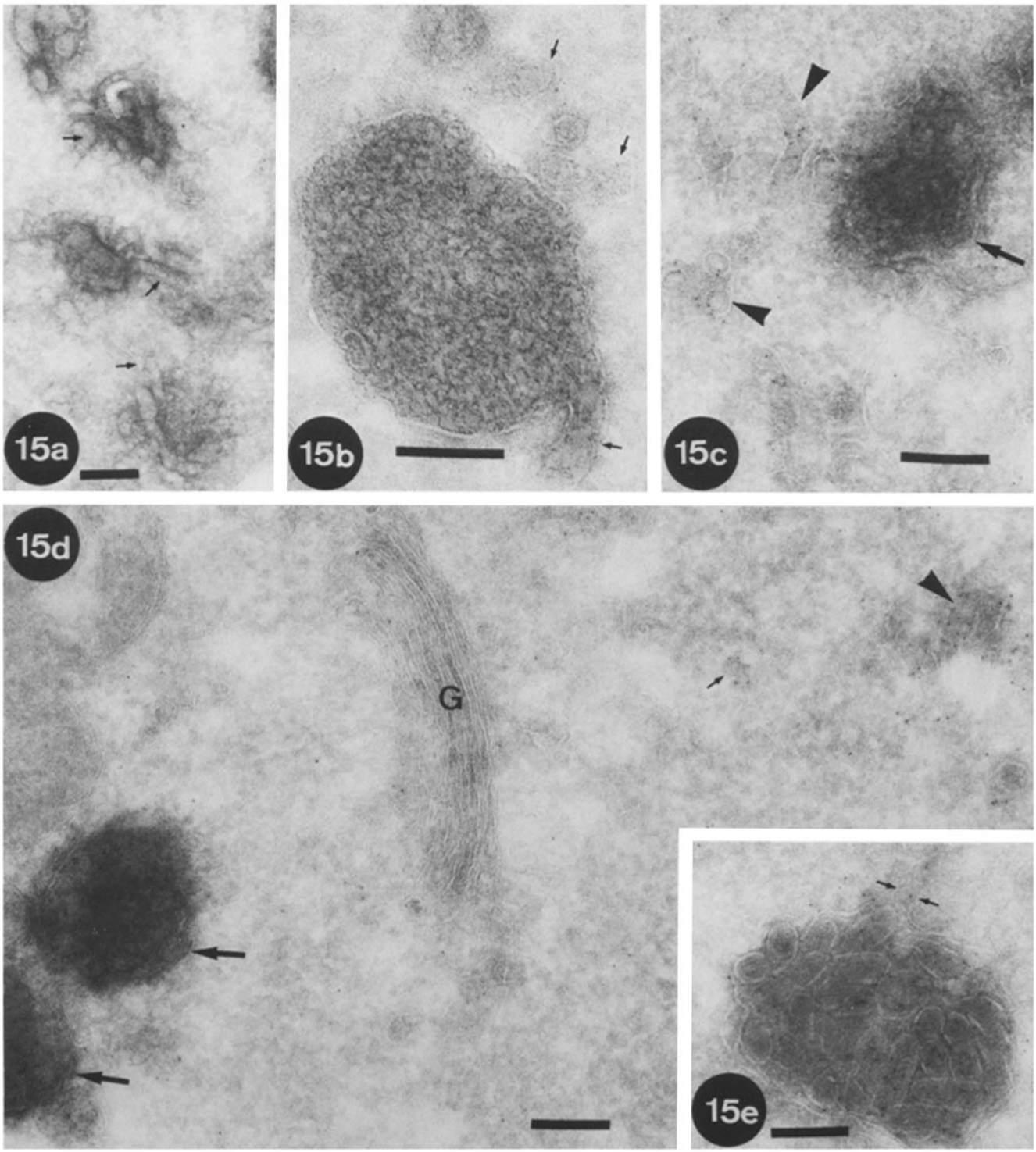


FIGURE 15 Images of BHK cells infected with VSV ts 045-6 (2 h at 20°C) which have endocytosed HRP and the sections labeled with anti-tail antibody. *a* is a low magnification image to show the tubules (arrows) of the peripheral endosomes as they appear in these preparations. *b* is an HRP-filled vacuolar profile, with attached tubules. This is from a preparation which has been shifted to 32°C for 5 min. No significant labeling for G protein is evident. *c* is from a cell that was warmed for 15 min to 32°C. The characteristic *trans* Golgi structures are evident (arrowheads) close to an HRP structure (arrow). With lighter printing, a few gold particles are evident over this structure which may be either an endosome or lysosome under these conditions. *d* shows the Golgi region of a cell that has been warmed up to 32°C for 15 min. Neither, the stack (G) nor the HRP-positive structures have significant amounts of label. A "fragment" of the *trans* reticulum (arrowhead) is labeled. The small arrow indicates a possible transport vesicle between *trans* Golgi cisterna and plasma membrane. e shows an endosome structure from a cell that had endocytosed VSV and HRP: 50 μg VSV per 2 × 10^6 cells was bound for 1 h at 4°C. Then 5 mg/ml HRP was added, and the temperature raised to 37°C for 15 min before fixation. The HRP-reactive endosome is significantly labeled with the anti-tail antibody. The arrows indicate a tubular profile attached to the vacuole. Bars, 200 nm. (a) × 51,000; (*b*) × 91,000; (c) × 75,000; (*d* and *e*) × 67,000.

ture switch using a stereological approach we have used previously (14).

The periodic structures seen in the *trans* cisterna at 20°C have also been occasionally found in infected cells maintained at the permissive temperature throughout infection. An analagous structure is observed frequently in BHK cells during infection with Semliki Forest virus (see Fig. 17 of reference 11). Under all conditions we have tested, a cisternal structure positive for acid phosphatase is present (12). Hence, the *trans* reticulum we observe at 20°C is a modification of the *trans* cisterna present in normal cells. It is also, we believe, the same structure which has been designated GERL (23) Although the original GERL concept, which postulated direct continuities between rough endoplasmic reticulum and the *trans*-most cisterna (thereby bypassing the rest of the Golgi stack) has not been verified by subsequent data, the GERL structure per se is well defined in a number of cell types (for review see references 17, 23). It was, in fact, the unique structural features of the *trans* cisterna which originally led Novikoff and colleagues to postulate that this structure was functionally distinct from the rest of the Golgi stack. Two documented features of GERL are of particular relevance to this paper. The first is the presence of coated pits, probably budding from this cisterna, which have been shown to contain clathrin (2, 6, 10, 25). The second is the presence of structural continuities between GERL and immature secretory granules (16, 24). In this context, Orci et al. (25) have shown that in pancreatic B cells proinsulin-to-insulin conversion is blocked after monensin treatment and the proinsulin accumulated in a clathrin-coated, acid phosphatase-reactive cisterna. Proinsulin also accumulated in immature secretory granules which were continuous with the latter structure.

In an interesting parallel to our work, where the *trans*-most cisterna enlarges in response to the accumulation of a membrane protein, Hand and Oliver (17) have reported that GERL enlarges considerably in secretory cells in response to secretagogue stimulation. It appears, therefore, that the *trans* cisterna of the Golgi stack is a very dynamic structure which is capable of rapidly changing its size in response to different conditions.

The experiments combining endocytosis of HRP with immunolabeling of G protein indicates that, at 20°C, the *trans* Golgi cisterna and all HRP-positive structures are distinct from each other although often in close proximity. Upon raising the temperature, the G protein is transported to the surface without being detected in any HRP-positive structures. Our tentative conclusion would be that the endosome compartment is not an obligatory station in exocytosis of newly synthesized plasma membrane proteins. As for any negative result, we realize, of course, that this interpretation must be treated with caution. Although likely, we have not formally shown that the HRP fills all compartments involved in endocytosis. Our confidence in these results is supported by the fact that, at 20°C, immunoreactive G protein is not seen in any structure besides Golgi cisternae, plus very low levels on endoplasmic reticulum and plasma membrane. Similarly, after 5 min of warming to 32°C, a condition where the labeling of plasma membrane becomes significant, the small vesicular structures which label with the anti-tail antibody are the only additional structures which become labeled.

It is now generally believed that all plasma membrane and secretory proteins move through identical compartments from the rough endoplasmic reticulum to the distal compartments of the Golgi stack where terminal glycosylation occurs. The *trans* cisterna, which is the last Golgi station, is emerging as an interesting candidate responsible for sorting of membranes and secretory proteins to different post-Golgi locations.

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