

# Immunocytochemical Study of the Partition and Distribution of Sindbis Virus Glycoproteins in Freeze-fractured Membranes of Infected Baby Hamster Kidney Cells

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**ABSTRACT** Sindbis virus-infected baby hamster kidney cells were analyzed by thin section fracture-label. Specific immunolabel with antiviral glycoprotein antibodies or with conventional lectin label (wheat germ agglutinin) were used in conjunction with colloidal gold-conjugated protein A or ovomucoid, respectively. In addition, intact infected cells were analyzed with both labeling procedures. Experiments with Sindbis infected-chick embryo fibroblast cells were carried out as controls. Viral transmembrane glycoproteins appeared present in freeze-fractured inner and outer nuclear membrane, endoplasmic reticulum, Golgi stacks and vesicles, and plasma membranes; a clear preferential partition with the exoplasmic faces of all intracellular membranes was observed. By contrast, at the plasma membrane level, Sindbis glycoproteins were found to partition preferentially with the protoplasmic face. It seems likely that this protoplasmic partition is related to the binding with the nucleocapsid that takes place during the budding of the virus. At the cell surface, viral glycoproteins always appeared clustered and were predominantly associated with budding figures: moreover, large portions of the plasma membrane were devoid of both glycoproteins and budding viruses.

Sindbis virus has only two integral membrane glycoproteins, E1 and E2, which form the spike complex in the membrane envelope and one capsid protein, C, which interacts with the genomic RNA to form the nucleocapsid. The bulk of the glycoprotein mass is exposed on the extracellular face of the membrane. In addition, E2 has a short cytosolic tail which anchors the membrane envelope to the nucleocapsid. The early events in the biosynthesis and membrane assembly of Sindbis (and of the closely related Semliki forest virus) glycoproteins have been intensively studied (1–4). Already in the rough endoplasmic reticulum newly synthesized glycoproteins probably interact to form a dimer (5), which is then transported to the plasma membrane via Golgi stacks and vesicles (6). During transport, extensive posttranslational modifications take place (7–10). The route along which the Sindbis glycoproteins are transported intracellularly and the sites at which these posttranslational modifications take place have not yet been elucidated. Budding occurs at the plasma membrane and is probably due to the interaction of spike complexes with the C proteins assembled in the nucleocapsid (11).

Fracture-label techniques have recently been developed (12,

13). The main advantage of these methods, which permit direct labeling of the fracture faces in freeze-fractured cells, is that they allow identification of membrane components and their sidedness. Several studies concerning the partition after fracture of integral plasma membrane proteins, as well as the distribution and compartmentalization of intracellular membrane glycoproteins, have been reported (14–17). It has been shown, for example, that in human erythrocytes glycoporphin and band 3 have different partitions after fracture. This observation was attributed to their different insertion in the membrane, and to their different association with cytoskeletal elements (12, 18).

We decided to analyze Sindbis-infected baby hamster kidney (BHK)<sup>1</sup> cells by thin section fracture-label for two main reasons: (a) to identify the intracellular membranes bearing viral glycoproteins, and (b) to establish whether transmembrane proteins present in various cellular membranes maintain the same partition after fracture in all their locations. In

<sup>1</sup> Abbreviations used in this paper: BHK, baby hamster kidney; CEF, chick embryo fibroblast; WGA, wheat germ agglutinin.

the present paper, we show the results obtained by using wheat germ agglutinin (WGA) and anti-Sindbis spike antibodies to perform fracture-label and conventional surface label. These data are the first obtained by the combination of immunocytochemistry and fracture-label technique: the use of specific antibodies in this method may represent a very helpful approach in membrane protein studies.

## MATERIALS AND METHODS

### Cell Culture and Virus Infection

Cultures of BHK and chick embryo fibroblasts (CEF) cells were maintained in plastic tissue culture dishes using minimum essential medium supplemented with 5% fetal bovine serum (Flow Laboratories, Inc., Scotland). Sindbis virus HR strain was plaqued, grown, and titered on CEF cells essentially as previously described (19). Subconfluent monolayers were infected at a multiplicity of 50 plaque-forming units/cell for 1 h at 37°C in phosphate-buffered saline (PBS) containing Ca<sup>++</sup> and Mg<sup>++</sup> and 1% fetal calf serum. After incubation, the medium was replaced with an appropriate volume of minimum essential medium containing 5% fetal bovine serum, and the infection was proceeded for 3–6 h.

### Antibody Preparation

An anti-Sindbis antiserum was prepared in rabbit against purified Sindbis virus grown on CEF cells. The immunoglobulin G fraction was obtained by Na sulphate precipitation and DEAE-cellulose column and was preadsorbed against formaldehyde-fixed uninfected BHK cells by two incubations (30 min at 37°C). The immunoglobulin G fraction was then stored at 4°C in aliquots at the concentration of 10 mg/ml. This antibody does not react with any cellular proteins, as shown by immunofluorescence microscopy, and specifically recognizes the Sindbis envelope glycoproteins at all stages of maturation as determined by indirect immunoprecipitation (data not shown).

### Fracture-label and Surface Label

The Sindbis-infected and uninfected BHK and CEF cells were washed three times in PBS, pH 7.4, and fixed with 1% glutaraldehyde in the same buffer (30 min at 25°C).

**FREEZE-FRACTURE:** BHK cells were embedded in 30% bovine serum albumin cross-linked by glutaraldehyde. The resulting gels were cut into small pieces, impregnated in 30% glycerol in PBS, and frozen in Freon 22 cooled by liquid nitrogen. Frozen gels were fractured in liquid nitrogen by repeated crushing with a glass pestle, thawed in 1% glutaraldehyde/30% glycerol in PBS, gradually deglycerinated, and washed twice in PBS before labeling.

**IMMUNOCYTOCHEMICAL AND WGA-CYTOCHEMICAL LABELING:** Fractured gel fragments were incubated in anti-Sindbis spike antibodies (0.5 mg/ml) in PBS for 1 h at 4°C, washed extensively, and finally labeled for 3 h at 4°C with colloidal gold (prepared by the citrate method) conjugated with protein A (20) (Pharmacia Fine Chemicals, Uppsala, Sweden). Isolated BHK and CEF unfractured cells were also directly surface-labeled with the antibodies followed by protein A-colloidal gold as above. Alternatively, fractured gel fragments of Sindbis-infected and uninfected BHK cells were incubated for 1 h at 37°C in a solution of 0.25 mg/ml of WGA (Sigma Chemical Co., St. Louis, MO) in 0.1 M Sorensen's phosphate buffer, 4% polyvinylpyrrolidone, pH 7.4. Controls were preincubated for 15 min at 37°C in 0.4 M *N*-acetyl-D-glucosamine, and then incubated with WGA in the presence of the sugar (1 h at 37°C). The lectin-treated gel fragments and the controls were then incubated for 3 h at 4°C in colloidal gold (prepared by the citrate method) conjugated with ovomucoid (16, 21). Isolated unfractured BHK cells were also directly surface-labeled with WGA (1 mg/ml for 1 h at 37°C) and ovomucoid-colloidal gold as described above.

### Processing for Electron Microscopy

Fracture-labeled gel fragments and surface-labeled isolated cells were post-fixed in 1% osmium tetroxide in Veronal acetate buffer, pH 7.4, for 2 h at 4°C, stained with uranyl acetate (5 mg/ml), dehydrated in acetone, and embedded in Epon 812. Thin sections were examined unstained and poststained with uranyl acetate and lead hydroxide.

## RESULTS

The ultrastructure of protoplasmic and exoplasmic faces of

both plasma and intracellular membranes in fracture-labeled preparations shows the usual aspect, as previously reported and discussed (12, 18), of interrupted unit membrane segments, due to reorganization events after fracture and thawing.

### Fracture-label of Intracellular Membranes

To analyze the distribution of viral glycoproteins on intracellular membranes, we used immunolabeling with anti-Sindbis spike antibodies, followed by protein A-colloidal gold in BHK cells freeze-fractured after different periods of infection (3, 4.5, and 6 h). The amount of labeling over endoplasmic reticulum, nuclear envelope, and Golgi membranes (Fig. 1) was similar in relative intensity and distribution at the different times of infection, whereas an increasing amount of immunolabeling was observed at the plasma membrane level, clearly related with the increase in viral budding with time. In all freeze-fractured intracellular membranes, the immunolabeling was present over the exoplasmic faces. Whenever identification of intracellular protoplasmic faces was possible (as in "cracks" where the two leaflets of the fractured membrane remain in close opposition) (12), no significant labeling was observed (Fig. 1*f*). Over the exoplasmic faces of rough endoplasmic reticulum (Fig. 1*a*), inner (Fig. 1, *b*, *d*, and *f* [arrow]) and outer (Fig. 1*c*) nuclear envelope, and Golgi apparatus membranes (Fig. 1, *a* and *g*), gold particles appeared almost uniformly distributed. When penetration of the label through cross fractures of the Golgi complex occurred, the unfractured inner surfaces of dilated cisternae appeared heavily labeled (Fig. 1, *e* and *f* [arrowhead]). In Fig. 1*g*, unlabeled protoplasmic faces (arrows) and labeled exoplasmic faces (arrowheads) of fractured membranes of a Golgi region are evident. Cross-fractured cytoplasm as well as mitochondria membranes were not labeled. Low labeling in Golgi areas of the cytoplasm was associated with small vesicles probably responsible for the transport of viral glycoproteins. Free nucleocapsids scattered in the cytoplasm were not labeled (data not shown). In the control uninfected cells the density of the labeling was very low (~10% with respect to the infected cells, not shown in the figures).

### Surface Label

Surface labeling with anti-Sindbis spike antibodies and WGA of unfractured Sindbis virus-infected BHK cells (at 3, 4.5, 6 h after infection) was performed to study the distribution of the viral glycoproteins and their relationship with the budding virions. The immunolabeling was always exclusively restricted to the budding virion, showing the high specificity of the antibodies used (Fig. 2, *b* and *c*). Budding viruses and immunolabeling were not distributed at random on the entire cell surface, but concentrated in discrete areas (Fig. 2, *a* and *b*). This nonrandom distribution was evident even at the early times of infection (3 h), as well as at a later time (6 h), despite the great differences in the amount of immunolabeling and budding observed. The same results were obtained by immunolabeling of Sindbis-infected CEF cells at 4.5 h after infection (data not shown). Controls, using uninfected BHK cells, showed only unlabeled surfaces (not shown).

WGA-labeling of cell surfaces showed a uniform distribution over the entire plasma membrane, without preferential concentration in budding areas (Fig. 2*e*), even if budding

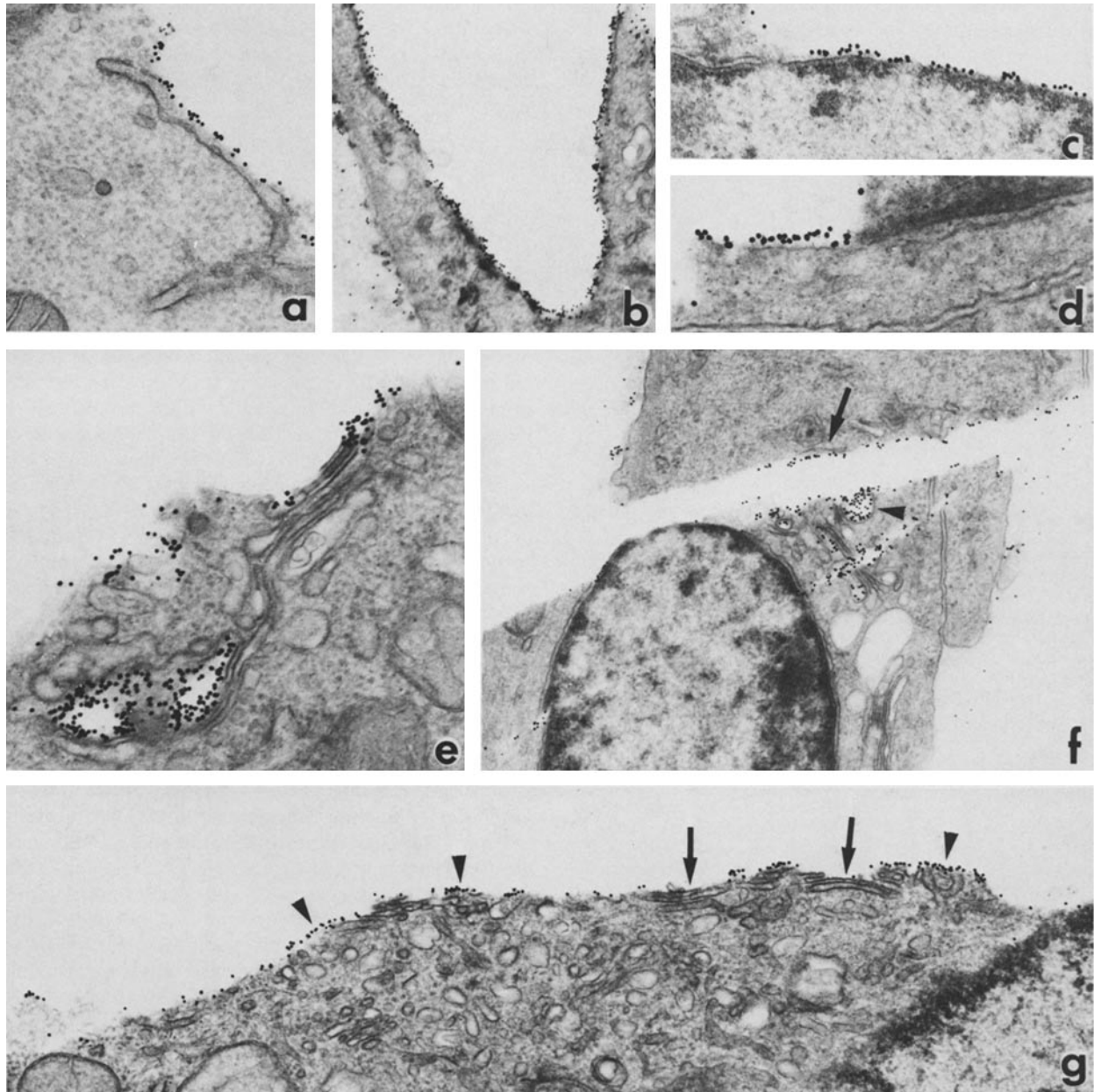


FIGURE 1 Fracture immunolabel of intracellular membranes of infected BHK cells: preferential partition with the E faces of viral glycoproteins. (a) E face of endoplasmic reticulum membrane (4.5 h after infection); (b) E face of inner nuclear envelope (6 h after infection); (c) E face of outer nuclear envelope (6 h after infection); (d) E face of inner nuclear envelope (6 h after infection); (e) P face of fractured Golgi cisternae and inner surfaces of dilated rims and cisternae (6 h after infection); (f) "crack" showing complementary P face and E face (arrow) of an inner nuclear envelope and inner surfaces of dilated Golgi cisternae (arrowhead) (6 h after infection); (g) E faces (arrowheads) and P faces (arrows) of Golgi fractured cisternae (3 h after infection). (a)  $\times 38,000$ ; (b)  $\times 23,000$ ; (c)  $\times 38,700$ ; (d)  $\times 55,000$ ; (e)  $\times 41,000$ ; (f)  $\times 25,000$ ; (g)  $\times 31,600$ .

viruses were very intensely labeled by the lectin (Fig. 2d). No differences in amount of WGA label could be observed between uninfected and infected BHK cells (at 3, 4.5, and 6 h after infection). Controls, in which the cells were treated with the lectin in the presence of *N*-acetyl-D-glucosamine, showed drastic (>90%) reduction of the label (not shown).

#### Fracture-label of Plasma Membranes

The partition after fracture of viral glycoproteins with the protoplasmic and exoplasmic faces of plasma membrane of

Sindbis virus-infected BHK cells was studied by immunolabeling with anti-Sindbis spike antibodies followed by protein A-colloidal gold, 3, 4.5, and 6 h after infection. At variance with the results obtained with endomembranes, significant immunolabel was observed over the protoplasmic faces, frequently at sites of budding, revealed by the co-localization with the underlying nucleocapsids (Fig. 3, a-c [arrows]). The gold particles were usually clustered in small groups, and only rarely isolated. These images were well observed at 4.5 h after infection, when a good balance occurred between the amount of labeling and the preservation of the plasma membrane.

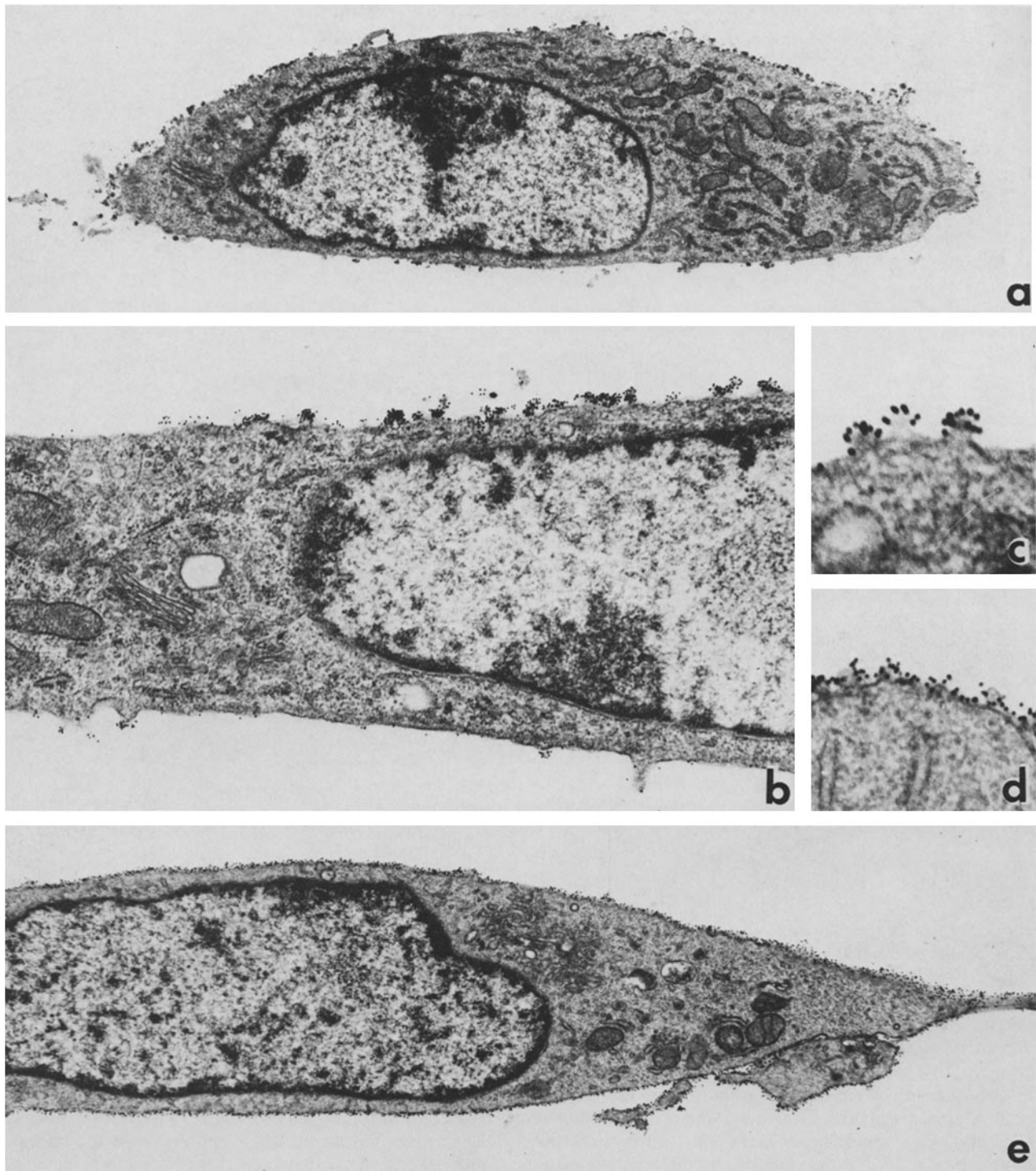


FIGURE 2 Surface immunolabel and surface WGA label of infected BHK cells: nonrandom distribution of viral glycoproteins and budding figures, in contrast with the uniform distribution of the WGA-label. (a-c) Immunolabeling (4.5 h after infection); (d and e) WGA labeling (4.5 h after infection). (a-e)  $\times 12,000$ ,  $22,000$ ,  $66,000$ ,  $35,000$ , and  $14,000$ , respectively.

The WGA labeling of the protoplasmic faces was as significant as the immunolabeling, and appeared with the same distribution in small clusters in opposition to the underlying nucleocapsids (Fig. 3*d*). Unlabeled nucleocapsids, as well as clusters of gold particles unrelated with nucleocapsids, were only rarely observed (Fig. 3*c*). Because of the heterogeneous distribution of budding and immunolabeling at the cell surface (see above and Fig. 2, *a* and *b*), the observation of

exoplasmic faces was confined to those which showed mature virions budded from and still in contact with the surface, embedded in the bovine serum albumin matrix. These exoplasmic faces were very weakly immunolabeled (Fig. 4, *a-c*), even if gold particles penetrated in the gel were seen decorating the surface of adjacent unfractured budding virions (Fig. 4*c* [arrows]). In contrast, the WGA labeling over the exoplasmic faces was intense and uniformly distributed (Fig. 4*d*) (16).

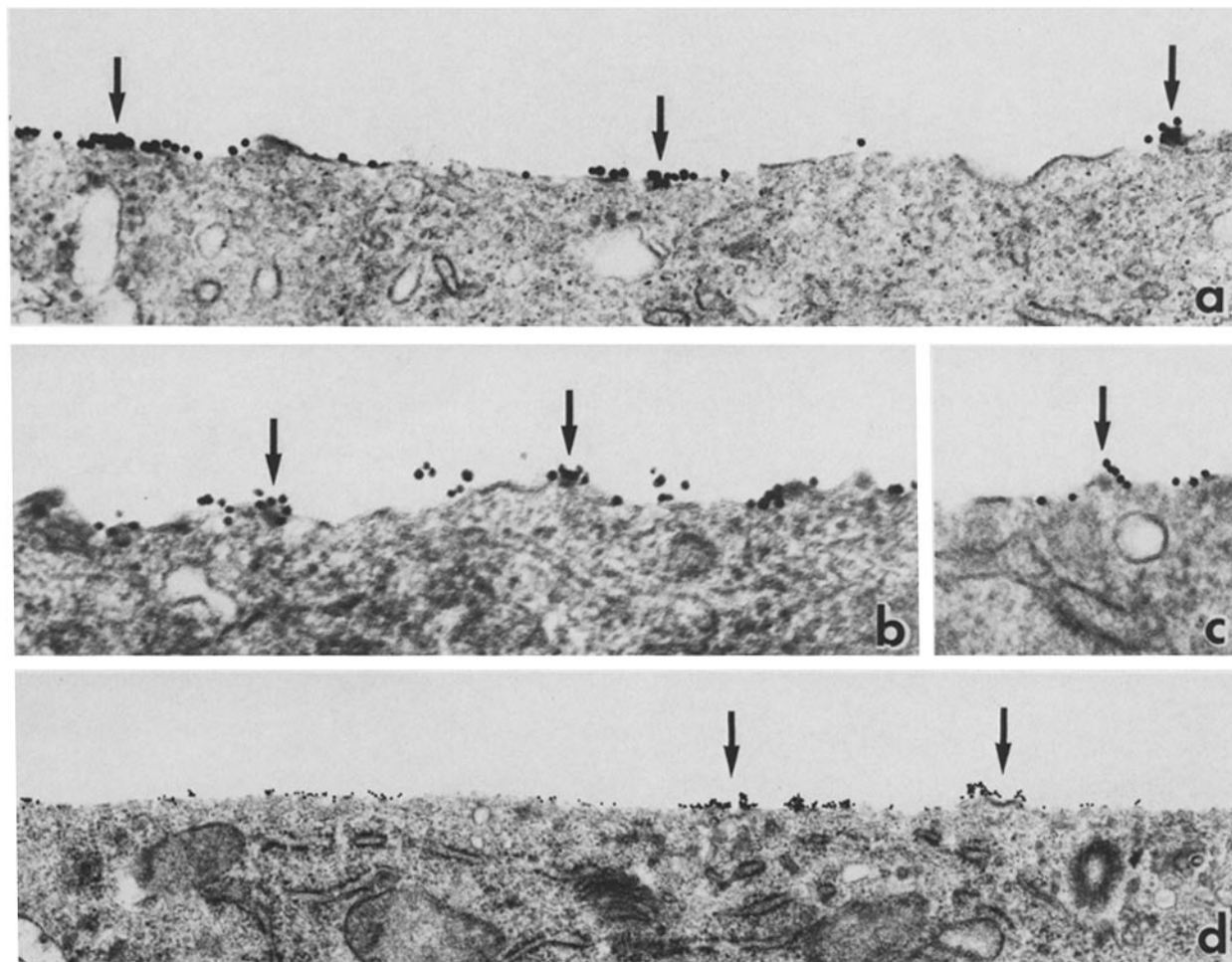


FIGURE 3 Fracture immunolabel and fracture WGA label over P faces of infected BHK cells: the immunolabeling as well as the WGA labeling is clustered and frequently related to underlying nucleocapsids (arrows). (a-c) Immunolabeling (6, 6, and 3 h after infection, respectively). (d) WGA-labeling (4.5 after infection). (a-d)  $\times 65,000$ ,  $73,000$ ,  $58,400$ , and  $24,000$ , respectively.

## DISCUSSION

Examples of different partition after fracture of transmembrane glycoproteins had been previously reported (12, 18). Human glycoporphin, which in the plasma membrane of erythrocytes is oriented in a fashion very similar to Sindbis glycoproteins, was preferentially found over the exoplasmic face of the plasma membrane. This partition can be explained by the knowledge that the bulk of glycoporphin mass, comprising the carbohydrate side chains, is exposed at the extracellular side of the plasma membrane. However, band 3, a glycoprotein which also has a conspicuous mass and carbohydrate side chains exposed at the extracellular face of the plasma membrane, is preferentially found over the protoplasmic face of the membrane after fracture. This peculiar partition has been interpreted as probably due to the association of band 3 to components of the erythrocyte cytoskeleton. The main finding we report in this paper is the apparent difference of partition after fracture of Sindbis glycoproteins present in different cellular membranes. In all fractured intracellular membranes, viral glycoproteins remained exposed at the exoplasmic face, whereas at the plasma membrane a clear preferential partition with the protoplasmic face was constantly found. The peculiar WGA pattern of labeling of the plasma membrane, diffuse labeling of the exoplasmic face

and discrete labeling of the protoplasmic faces at the site of viral budding, demonstrates that the change of partition polarity of endomembranes and plasma membranes is not a general phenomenon of all membrane glycoproteins but, rather, is specific for viral glycoproteins. These results strongly suggest that the partition after fracture of a transmembrane glycoprotein depends more on the interaction with other components adjacent to the membrane than on the orientation of the protein itself in the membrane. Along this line it seems likely that Sindbis glycoproteins acquired a protoplasmic partition at the plasma membrane level because of their interaction across the membrane with the underlying nucleocapsid. This interaction takes place during the budding process. In most cases, the dense nucleocapsid was observed in close contact to the regions of the protoplasmic face of the plasma membrane where viral glycoproteins were detected; moreover, when nucleocapsid was not evident, we cannot exclude that this was due to the plane of sectioning.

Sindbis spike complexes and budding viruses showed a clear nonrandom distribution at the surface of both BHK and CEF cells. Large regions of the plasma membrane were devoid of both glycoproteins and budding viruses. These results confirm an earlier report (22) on surface replica images of BHK infected cells. The amount of surface labeling that cannot be ascribed to budding figures was always low. We



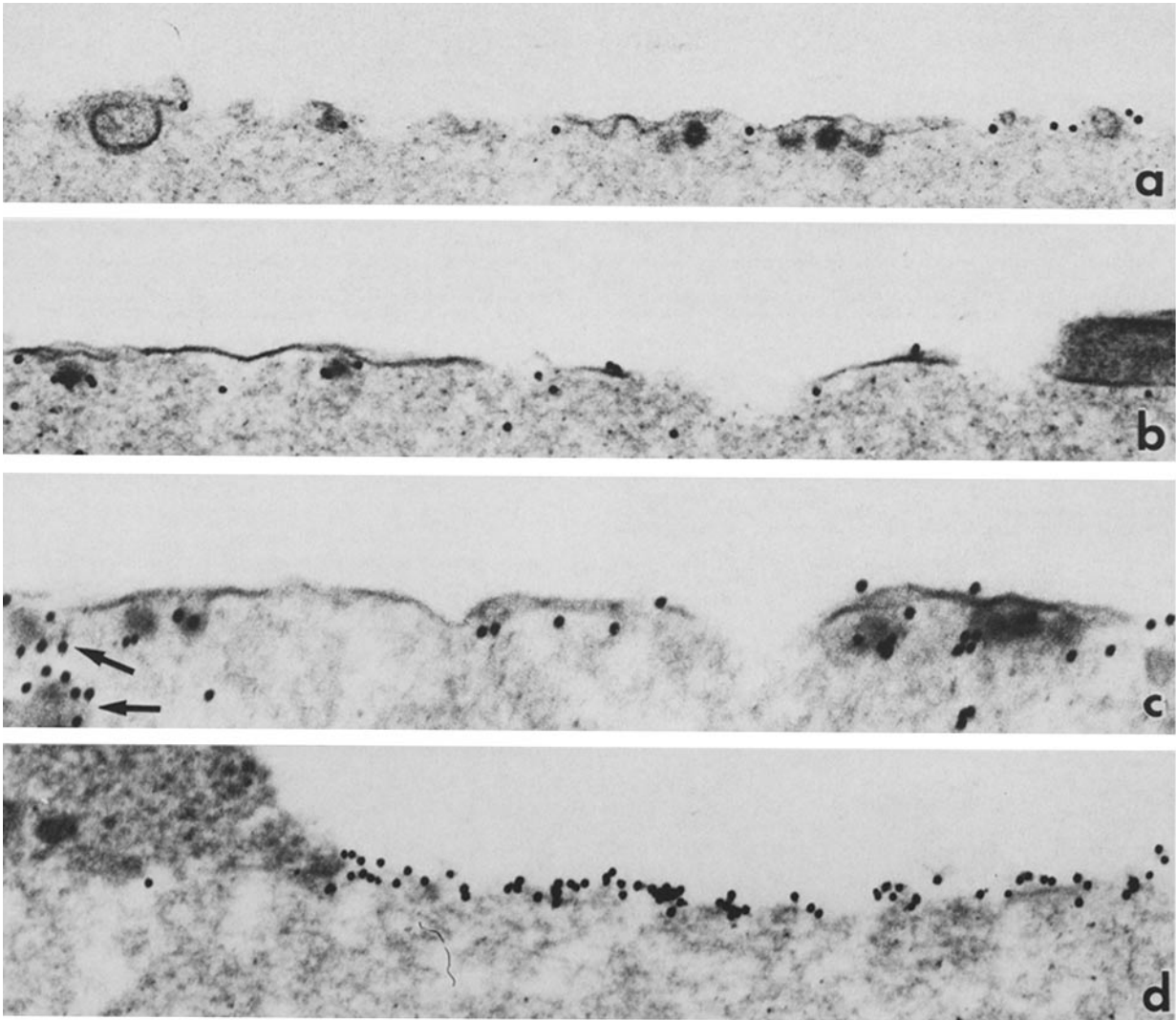


FIGURE 4 Fracture immunolabel and fracture WGA label over the E faces of infected BHK cells: the immunolabeling is very weak or absent, in contrast with the heavily labeled budded virions in the BSA-gel matrix (arrows). The WGA labeling appears dense and uniform. (a–c) Immunolabeling (6 h after infection). (d) WGA-labeling (4.5 h after infection). (a–d)  $\times 67,000$ ,  $65,000$ ,  $91,000$ , and  $90,000$ , respectively.

favor, therefore, the view that the pool of free viral spikes in the plasma membrane is small (23). In addition, surface labeling experiments made at an earlier or later time of infection (3 or 6 h) showed great differences in the amount of budding and of glycoprotein labeling, but always demonstrated a nonrandom distribution of both.

The thin section fracture-label technique gives a good preservation of the subcellular structures and thus permits us to recognize the cellular organelles being labeled. By applying this methodology to Sindbis-infected BHK cells, we show in this paper that viral glycoproteins are present in the inner and outer nuclear membrane, endoplasmic reticulum, Golgi stacks and vesicles, and plasma membranes. These results confirm and extend the previous data on Semliki forest virus-infected BHK cells obtained by immunolabeling of ultrathin frozen sections (6). An unexpected and interesting finding was the high concentration of spike proteins in the inner nuclear membrane, which is devoid of attached ribosomes. Because the labeling we observed was comparable to that found on the outer nuclear membrane, it could be argued that viral glycoproteins synthesized by the polyribosomes

attached to the outer membrane freely diffused from the outer to the inner membrane despite the pore complex structure. However, we have no evidence that the glycoproteins present in the inner nuclear membrane are destined to enter into the intracellular transport pathway, as their counterparts present on the outer membrane presumably do. To answer this point, analysis of infected cells, fractured at different times after cycloheximide addition (6), is currently in progress. The presence of vesicular stomatitis virus G glycoprotein on the inner nuclear membrane of infected Chinese hamster ovary cells had been previously reported (24); in that study, however, the viral glycoproteins were detected only in localized blebbing regions of the nuclear envelope, presumably because of the limitations of the ultrathin frozen sections technique used.

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