

Free Diffusion to and from the Inner Nuclear Membrane of Newly Synthesized Plasma Membrane Glycoproteins

Maria Rosaria Torrisi,* Lavinia Vittoria Lotti,* Antonio Pavan,*
Giovanni Migliaccio,† and Stefano Bonatti‡

* Istituto di Patologia Generale, Università di Roma La Sapienza, Viale Regina Elana 324, Roma, Italy; and

† Istituto di Biochimica Cellulare e Molecolare, Università di Napoli, Via S. Pansini 5, Napoli, Italy

Abstract. Sindbis virus-infected baby hamster kidney (BHK) cells were analyzed by thin section fracture-label. Specific immunolabel with antiviral glycoprotein antibodies was used in conjunction with colloidal gold-conjugated protein A. As we previously reported (Torrisi, M. R., and S. Bonatti, 1985, *J. Cell Biol.*, 101:1300-1306), Sindbis transmembrane glycoproteins are present in the inner nuclear membrane as well as in the outer nuclear membrane, endoplasmic reticulum, Golgi stacks and vesicles, and plasma membranes. Viral glycoproteins located on the inner nuclear membrane resemble those present on the outer

membrane in terms of amount, distribution, and preferential partition after fracture.

We show in this paper that Sindbis glycoproteins after treatment with cycloheximide are removed from the inner nuclear membrane with the same kinetics as their counterparts present on the outer membrane. This finding strongly suggests that newly synthesized transmembrane glycoproteins may freely diffuse to and from the inner nuclear membrane before entering into the intracellular transport pathway to the plasma membrane.

THE nuclear envelope is a complex structure surrounding the nuclear compartment in eukaryotic cells (2). It is composed by two membranes (outer and inner membrane), pore complexes, and nuclear lamina (3). According to a recent report (12), the outer membrane is not only morphologically but also functionally equivalent to the rough endoplasmic reticulum; on the contrary, due to technical difficulties, the composition of inner nuclear membrane and pore complexes as well as the role of the pore complex in delimiting the communication between inner and outer membranes are not known yet (2, 4). Recently, it has been reported that the inner membrane has a lower protein diffusion coefficient than the outer membrane (13); based on this observation it has been suggested that the inner membrane is a static mosaic surface for the attachment of several nuclear components (13).

In a recent study, we have applied a combination of immunocytochemistry and fracture-label techniques to study the intracellular distribution and partition of Sindbis virus transmembrane glycoproteins in various freeze-fractured cellular membranes (15). We reported that Sindbis glycoproteins are preferentially present on the exoplasmic face of outer nuclear membranes, rough endoplasmic reticulum, Golgi stacks and vesicles, whereas on the plasma membrane they partition preferentially with the protoplasmic face. Surprisingly, we observed in the same study a high concentration of Sindbis glycoproteins in the inner nuclear membrane: this observation was unequivocal since the plane of the fracture removed the outer membrane and the perinuclear space, exposing

large areas of the inner membrane to the immunolabeling. The glycoproteins appeared to be uniformly distributed along the inner membrane, had a preferential partition with the exoplasmic face, and were present in a number comparable to that revealed on the outer membrane. Presence of vesicular stomatitis virus G glycoprotein in the nuclear membrane has been previously reported (1); in that study, however, because of the limitations of the technique used it was possible to detect G protein only in localized regions of the inner membrane. It was suggested in both reports that glycoproteins synthesized by polyribosomes bound to the outer membrane are free to diffuse from the outer to the inner membrane despite the pore complex structure. However, no evidence was available that the glycoproteins present in the inner membrane were able to diffuse back to the outer membrane and to the rough endoplasmic reticulum; i.e., that they were destined to enter the intracellular transport pathway. To address this point, we analyzed with the fracture-immunolabel approach Sindbis virus-infected baby hamster kidney (BHK) cells after addition of cycloheximide, a drug which inhibits protein synthesis without interfering with the intracellular transport (5, 6).

Materials and Methods

Cell Culture and Virus Infection

Cultures of BHK cells were maintained in plastic tissue culture dishes using MEM supplemented with 5% FBS (Flow Laboratories, Irvine, Ayrshire,

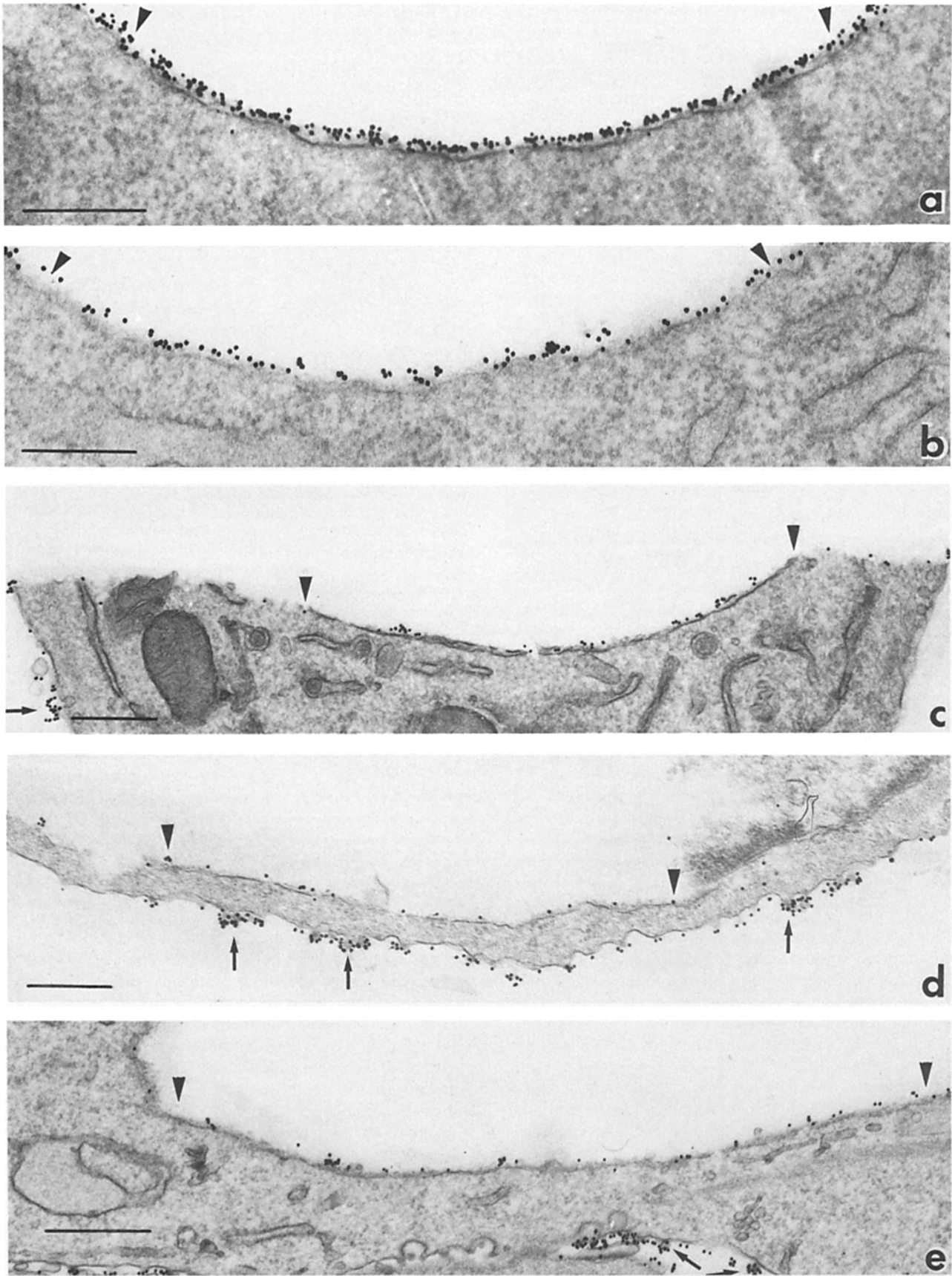


Figure 1. Fracture-immunolabel of inner nuclear membranes of infected BHK cells after cycloheximide addition: decrease of labeling in response to the treatment. (a-e) Exoplasmic faces (*arrowheads*) after 0, 10, 20, 30, and 60 min, respectively. In c-e, surface-labeled plasma membranes are evident showing immunolabel associated with budding figures (*arrows*). Bars, 0.5 μm .

Scotland). Sindbis virus HR strain was plaqued, grown, and titrated on cells essentially as previously described (15). Subconfluent monolayers were infected at a multiplicity of 50 plaque-forming units/cell for 1 h at 37°C in PBS containing Ca⁺⁺ and Mg⁺⁺ and 1% FCS. After incubation, the medium was replaced with an appropriate volume of MEM containing 5% FBS, and the infection was proceeded for 4.5 h. Cycloheximide (BDH Chemicals Ltd., Poole, England) was added to a final concentration of 10 µg/ml directly to the culture medium. In this condition, total protein synthesis in infected cells was inhibited >90% (5, 6).

Fracture-Immunolabel

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

Freeze-fracture. BHK cells were impregnated in 30% glycerol in PBS

and frozen in Freon 22 cooled by liquid nitrogen. Frozen cells 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 Labeling. Fractured cells were incubated with anti-Sindbis glycoprotein antibodies (15) (0.5 mg/ml) in PBS for 1 h at 4°C, then washed extensively, and finally labeled for 3 h at 4°C with colloidal gold (prepared by the citrate method) conjugated with protein A (14) (Pharmacia Fine Chemicals, Uppsala, Sweden).

Processing for Electron Microscopy

Fracture-labeled cells were postfixed 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.

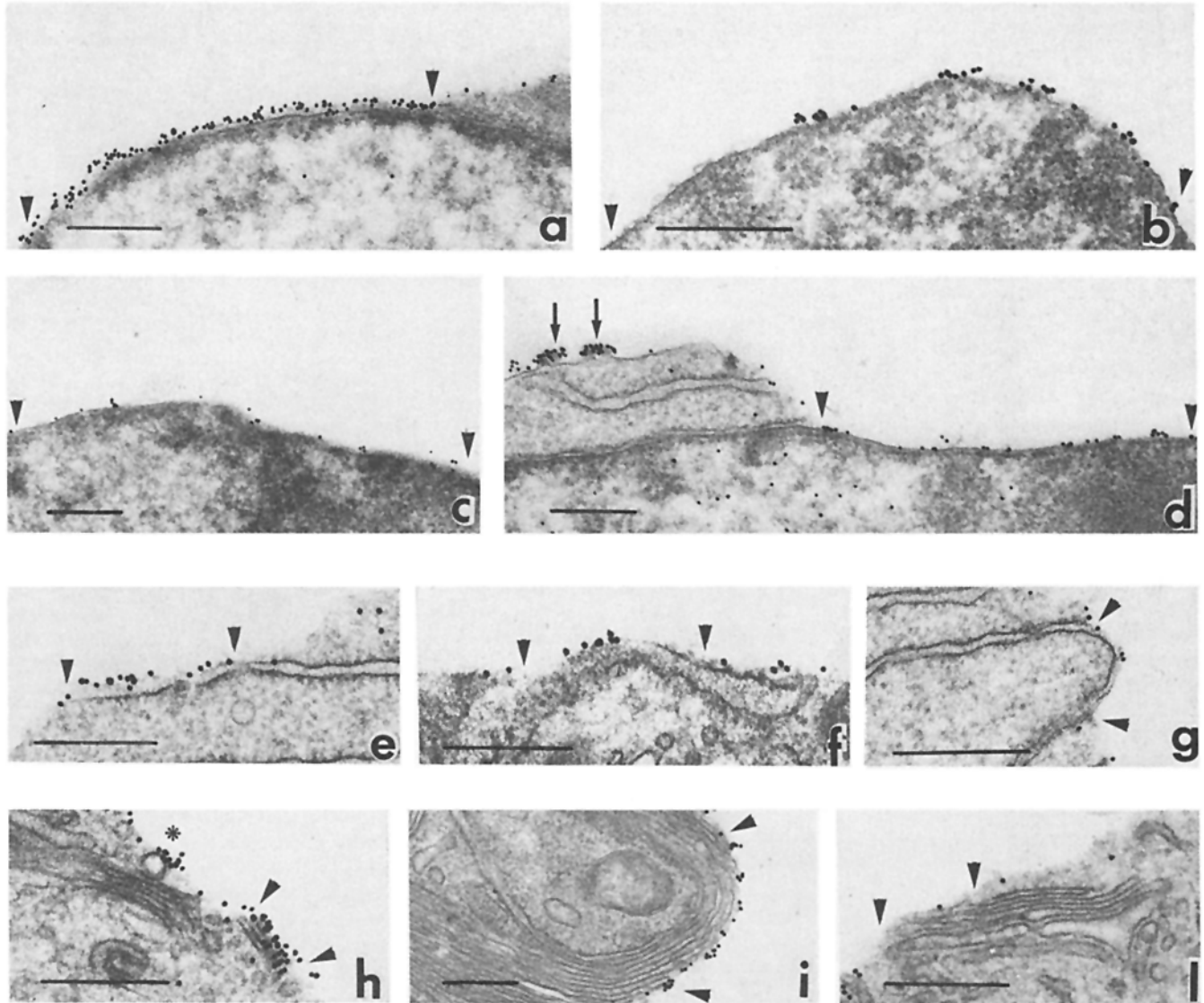


Figure 2. Fracture-immunolabel of outer nuclear membrane, endoplasmic reticulum membranes, and Golgi apparatus cisternae and vesicles of infected BHK cells after cycloheximide addition: decrease of labeling in response to the treatment. (a-d) Exoplasmic faces (arrowheads) of outer nuclear membranes after 0, 20, 30, and 120 min, respectively; (e-g) exoplasmic faces (arrowheads) of endoplasmic reticulum membranes after 0, 10, and 120 min, respectively; (h-l) exoplasmic faces (arrowheads) of fractured Golgi cisternae and vesicles (asterisk in h) after 0, 10, and 60 min, respectively. In d, the surface-labeled plasma membranes show heavily immunolabeled budding figures (arrows). Bars, 0.5 µm.

Results and Discussion

Sindbis virus-infected BHK cells, freeze-fractured at different times after cycloheximide addition (10, 20, 30, 60, and 120 min), were immunolabeled using anti-Sindbis spike antibodies, followed by protein A-colloidal gold. Cycloheximide was added 4.5 h post-infection.

The ultrastructure of the freeze-fractured labeled plasma as well as intracellular membranes was identical to that observed and discussed in previous reports (8-11, 16), showing interrupted unit membrane segments due to reorganization events during thawing. Inner nuclear membranes in fracture-label preparation were easily identified when the corresponding unfractured outer membranes were visible beneath the fracture plane (arrowheads in Fig. 1) (10, 11). Moreover, this identification was further confirmed by the presence of cross-fractured chromatin following the fractured inner nuclear membrane (Fig. 1 *d*). Conversely, the outer nuclear membranes were identified by the presence of corresponding unfractured inner nuclear membranes beneath the fracture plane (arrowheads in Fig. 2, *a-d*). The good preservation of the intracellular structures allowed the morphological identification of exoplasmic faces of endoplasmic reticulum (arrowheads in Fig. 2, *e-g*) and Golgi-fractured membranes (arrowheads in Fig. 2, *h-l*).

Fracture immunolabeling was performed on isolated cells to allow simultaneously the comparison of the pattern of labeling on the surface as well as on intracellular fracture faces (Fig. 1, *c-e*; Fig. 2 *d*). As previously reported (15), the immunolabeling of all freeze-fractured intracellular membranes was confined to the exoplasmic faces. In fact protoplasmic faces, when identified as example in cracks (where the two membrane halves remain in close opposition), were virtually unlabeled (not shown). Surface labeling was mostly associated with budding viruses, revealing the high specificity of the antibodies used (arrows in Fig. 1, *c-e*, and in Fig. 2 *d*). Cross-fractured cytoplasm and mitochondria were virtually unlabeled as well as the fractured membranes of uninfected control cells. Rarely, some unspecific labeling was found associated with the chromatin.

In the absence of cycloheximide, the labeling over the exoplasmic faces of inner nuclear membranes (Fig. 1 *a*) was intense and uniformly distributed as was the labeling of outer nuclear membranes (Fig. 2 *a*), endoplasmic reticulum (Fig. 2 *e*), and Golgi membranes (Fig. 2 *h*). The intensity and distribution of the labeling were totally comparable to those observed in our previous report (15). At different times after cycloheximide addition to the infected cells, the labeling density over the freeze-fractured intracellular membranes clearly decreased (Fig. 1, *b-e*; and Fig. 2, *b-d, f, g, i, and l*). On the plasma membrane, highly labeled regions with numerous budding figures were detected at all time points (arrows in Fig. 1, *c-e*; Fig. 2 *d*; data not shown). Virus budding and immunolabeling appeared to be clearly concentrated in areas of the cell surface (7, 15).

The quantitation of labeling density in response to cycloheximide addition is shown in Fig. 3. We focused on the inner and outer nuclear membranes since they were morphologically better defined and comparable to each other applying our fracture-label technique. Golgi apparatus membrane were also considered as an internal control. We found a very similar timing of exit from the inner and the outer nuclear

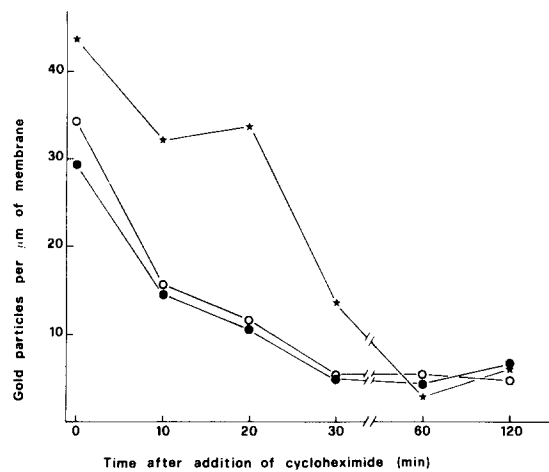


Figure 3. Quantitation of Sindbis virus glycoproteins contained in freeze-fractured membranes at different times of the cycloheximide addition. Each time point value represents the average number of gold particles per μm of membrane obtained from six different micrographs per membrane type. (Open circles) Inner nuclear membrane; (solid circles) outer nuclear membrane; (stars) Golgi apparatus membrane.

membrane: ~ 10 min after cycloheximide addition, 50% of Sindbis glycoproteins have left both nuclear membranes; at 30 min after the addition, the loss of labeling was virtually concluded and a constant level of $\sim 15\%$ of the original labeling was maintained up to the 120-min time point. As expected, the labeling in Golgi apparatus membranes (as seen in both exoplasmic faces [Fig. 2, *h-l*] and inner surfaces of dilated cisternae) decreased in response to cycloheximide with a slower rate (50% of loss between 20- and 30-min time points and constant level of $\sim 10\%$ of the original labeling reached after 30-min time points). These results are in good agreement with those reported previously for the endoplasmic reticulum (including the nuclear envelope) and Golgi apparatus by quantitation of immunolabeled cryosection of BHK cells infected with the closely related Semliki Forest virus (5).

The results reported here show that the pool of Sindbis glycoproteins present on the inner nuclear membrane is removed after cycloheximide treatment as the corresponding pools present on the outer nuclear membrane, endoplasmic reticulum, and Golgi apparatus. On kinetic grounds, inner and outer nuclear membrane behavior appears undistinguished. This finding suggests free diffusion of transmembrane glycoproteins to and from the inner nuclear membrane. The only other unlikely alternative explanation would be a specific and localized degradation of Sindbis glycoproteins on the inner nuclear membrane. The implication of the suggested free diffusion hypothesis made above is that newly synthesized transmembrane glycoproteins destined to the plasma membrane or to intermediate locations along the intracellular transport pathway are recruited from a unique functional pool, distributed on rough endoplasmic reticulum and outer and inner nuclear membranes. Although it is widely accepted that viral and cellular membrane protein biogenesis follows exactly the same routes, it remains to be demonstrated that outer-inner membrane diffusion is a general phenomenon and that it is not only restricted to viral

systems (where large amounts of only one or two membrane proteins are synthesized at the same time by the cell). Whatever is the case, the data presented in this paper suggest that the inner nuclear membrane, aside from its important role in the organization of interphase chromatin (3), has some dynamic characteristics that were thought up to now to be exclusive properties of the outer nuclear membrane and endoplasmic reticulum.

We thank Drs. J. Meldolesi and J. Lazdins for helpful discussion and Mr. S. Ferraro and Mr. S. Valia for excellent photographic work.

This work was partially supported by grant 850239744 from Progetto Finalizzato "Oncologia" and by grant 840172004 from Consiglio Nazionale delle Ricerche.

Received for publication 11 August 1986, and in revised form 10 October 1986.

References

1. Bergmann, J. E., and S. J. Singer. 1983. Immunoelectron microscopic studies of the intracellular transport of the membrane glycoprotein (G) of vesicular stomatitis virus in infected Chinese hamster ovary cells. *J. Cell Biol.* 97:1777-1787.
2. Franke, W. W., U. Scheer, G. Krohne, and E. D. Jarasch. 1981. The nuclear envelope and the architecture of the nuclear periphery. *J. Cell Biol.* 91(3, Pt. 2):39s-50s.
3. Gerace, L., and G. Blobel. 1982. Nuclear lamina and the structural organization of the nuclear envelope. *Cold Spring Harbor Symp. Quant. Biol.* 46:967-978.

4. Gerace, L., Y. Ottaviano, and C. Kondor-Koch. 1982. Identification of a major polypeptide of the nuclear pore complex. *J. Cell Biol.* 95:826-837.
5. Green, J., G. Griffiths, D. Louvard, P. Quinn, and G. Warren. 1981. Passage of viral membrane proteins through the Golgi complex. *J. Mol. Biol.* 152:663-698.
6. Jamieson, J. D., and G. E. Palade. 1968. Intracellular transport of proteins in the pancreatic exocrine cells. *J. Cell Biol.* 39:580-588.
7. Pavan, A., L. V. Lotti, M. R. Torrisi, G. Migliaccio, and S. Bonatti. 1987. Regional distribution of Sindbis virus glycoproteins on the plasma membrane of infected baby hamster kidney cells. *Exp. Cell Res.* 164:3-16.
8. Pinto da Silva, P., and M. R. Torrisi. 1982. Freeze-fracture cytochemistry: partition of glycoporphin in freeze-fractured human erythrocyte membranes. *J. Cell Biol.* 93:463-469.
9. Pinto da Silva, P., C. Parkison, and N. Dwyer. 1981. Fracture-label: cytochemistry of freeze-fractured faces in the erythrocyte membrane. *Proc. Natl. Acad. Sci. USA.* 78:343-347.
10. Pinto da Silva, P., C. Parkison, and N. Dwyer. 1981. Freeze-fracture cytochemistry: thin sections of cells and tissues after labeling of fracture-faces. *J. Histochem. Cytochem.* 29:917-928.
11. Pinto da Silva, P., M. R. Torrisi, and B. Kachar. 1981. Freeze-fracture cytochemistry: localization of wheat germ agglutinin and concanavalin A binding sites on freeze-fractured pancreatic cells. *J. Cell Biol.* 91:361-372.
12. Puddington, L., M. O. Lively, and D. S. Lyles. 1985. Role of the nuclear envelope in synthesis, processing and transport of membrane glycoproteins. *J. Biol. Chem.* 260:5641-5647.
13. Schindler, M., J. F. Holland, and M. Hogan. 1985. Lateral diffusion in nuclear membranes. *J. Cell Biol.* 100:1408-1414.
14. Slot, J. W., and H. J. Geuze. 1981. Sizing of protein A-colloidal gold probes for immunoelectron microscopy. *J. Cell Biol.* 90:533-536.
15. Torrisi, M. R., and S. Bonatti. 1985. Immunocytochemical study of the partition and distribution of Sindbis virus glycoproteins in freeze-fractured membranes of infected baby hamster kidney cells. *J. Cell Biol.* 100:1300-1306.
16. Torrisi, M. R., and P. Pinto da Silva. 1984. Compartmentalization of intracellular membrane glycoproteins is revealed by fracture-label. *J. Cell Biol.* 98:29-34.