Dynamics of transmembrane proteins during Sindbis virus budding

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Summary

Label-fracture and immunogold fracture-flip techniques are used to address at the ultrastructural level the dynamics of viral and cellular transmembrane proteins during the budding of Sindbis virus on the plasma membrane of infected cells. Immunolabeling with anti-Sindbis spike antibodies shows that the viral proteins are mostly in clusters, all associated with budding viruses. Ultrastructural observation of the unlabeled freeze-fractured plasma membranes shows that membrane particles aggregate over the budding viruses. These results indicate that the concentration of viral transmembrane proteins gives rise to a parallel concentration of membrane particles. Immunolabeling with anti-CD8 antibodies of cells expressing by transfection the CD8 transmembrane protein and infected with Sindbis virus shows absence of labeling on the particle aggregates over the forming virions. These findings indicate the exclusion of CD8 proteins from the portions of the membrane where budding occurs.

Key words: fracture-flip, label fracture, Sindbis virus.

Introduction

In virus-infected cells the process of viral budding from the plasma membrane of host cell surface represents a dynamic event that involves not only viral envelope glycoproteins but also cellular membrane proteins (Fuller, 1987; Simons and Garoff, 1980). For instance, during viral budding, the formation of clusters of viral envelope glycoproteins inserted at the plasma membrane appears to involve the exclusion of cellular proteins from budding domains (Fuller, 1987; Simons and Garoff, 1980).

Sindbis virus is a simple and well-studied viral model. It is a 60 nm RNA virus with an envelope that contains two transmembrane proteins, E1 and E2, forming the virus spike. In infected cells, the viral envelope glycoproteins are processed and transported in large amounts to the cell surface. Nucleocapsids are assembled in the cytosol and reach the plasma membrane where they interact with the cytosolic portion of viral glycoproteins driving the budding process (Strauss and Strauss, 1977; Simons and Garoff, 1980; Fuller, 1987).

In previous papers we have used immunogold surface replicas to address the distribution over the cell surface of the Sindbis envelope glycoproteins, before and during viral maturation. We showed that, at a late stage of infection, these proteins are almost exclusively associated with viral budding (Pavan et al., 1987; Torrisi et al., 1990).

To analyze the behaviour of viral and cellular proteins in the membrane regions where budding occurs we use here label-fracture (Pinto da Silva and Kan, 1984) and fracture-flip (Fujimoto and Pinto da Silva, 1988) methods that combine freeze-fracture with immunogold labeling to provide high-resolution views of freeze-fractured membrane faces and of actual membrane surfaces. These new methods allow us to relate the distribution and dynamics of membrane components, as established by immunogold labeling, to intramembrane particles (IMPs) revealed by freeze-fracture and to surface particles (SPs) visible in fracture-flip replicas of cell surfaces. Recently, we showed that surface particles revealed by fracture-flip include the surface protrusions of integral membrane proteins seen as intramembrane particles in freeze-fracture (Pavan et al., 1990). We reasoned, therefore, that the parallel application of the two methods might be an ideal approach by which to pursue our objectives.

Materials and methods

Cell culture and virus infection

Cultures of BHK cells were maintained in plastic tissue
Antibody preparation

Supplemented with 5% fetal bovine serum (FBS) (Flow Laboratories, Scotland). Sindbis virus HR strain was plaque-defined (Pfefferkon and Hunter, 1963). 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 Ca²⁺ and Mg²⁺ and 1% fetal calf serum (FCS). After incubation, the medium was replaced with an appropriate volume of Minimum Essential Medium (MEM) containing 5% FBS, and the infection was allowed to proceed for 3-6 h.

Parental FRT cells (Nitsch et al., 1985) were cultured in Coon's modified Ham's F12 medium containing 10% fetal calf serum and maintained in a 95% air, 5% CO₂ incubator. Cotransfection with plasmids carrying the human CD8 cDNA and the bacterial neo gene was performed as described previously (Migliaccio et al., 1990). Stable transformants were selected in the presence of G418 and positive clones screened by immunofluorescence. The FRT-U10 clone (Pastase et al., 1992) was used throughout this work.

All cells were grown on 4 mm glass coverslips.

To induce the clustering of surface particles, FRT-U10 cells were treated with 30% glycerol in PBS for 1 h at 37°C (McIntyre et al., 1974; Pinto da Silva and Martinez-Palomo, 1974; Pavan et al., 1989a, 1990).

Immunogold labeling

For immunogold labeling of viral glycoproteins Sindbis-infected and control uninfected BHK and FRT-U10 cells were washed three times in PBS, pH 7.4, and fixed with 1% glutaraldehyde in the same buffer (25°C, 30 min). The cells were incubated with anti-Sindbis spike antibodies (0.5 mg/ml) in PBS for 1 h at 4°C, then washed extensively and labeled for 3 h at 4°C with colloidal gold (prepared by citrate method) conjugated with Protein A (Slot and Geuze, 1981) (Pharmacia Fine Chemicals, Uppsala, Sweden).

For immunogold labeling of CD8 antigens, Sindbis-infected and uninfected FRT-U10 cells fixed as above, were incubated with OKT8 monoclonal antibody (1:20), for 1 h at 4°C and labeled with Protein A/collodial gold conjugates as above.

Control experiments were performed omitting the monoclonal antibodies from the immunolabeling procedure.

Label-fracture

Immunogold-labeled cells, fixed with glutaraldehyde, were cryoprotected with 30% glycerol in phosphate buffer, the coverslips were covered with a naked complementary gold specimen carrier and frozen by plunging them into partially solidified liquid nitrogen. The cells were then freeze-fractured in a freeze-fracture device (~105°C, 10⁻⁶ mm Hg), and replicated by evaporation from a platinum/carbon gun. The replicas were floated into distilled water, picked up by adhesion to Formvar-coated grids and observed with a transmission electron microscope. Stereo pairs were made with ±6° tilt (see Andersson Forsman and Pinto da Silva, 1988, for details). We used stereo-reversed images, since these are 'mentally' equivalent to familiar images of surface labeling given by scanning electron microscopy or in replicas of dried specimens.

Fracture-flip

For fracture-flip, unlabelled and labeled cells were freeze-fractured as above and the fractured surfaces were stabilized by unidirectional deposition of carbon (about 20 nm in thickness) evaporated from an electron-beam gun mounted at 90° to the specimen plane. The specimens were immersed in distilled water to release the carbon-stabilized replicas from the specimen carriers. The replicas were floated sequentially through distilled water, picked up from the top (i.e. carbon side) onto Formvar-coated grids and inverted (flipped). Excess water was carefully removed by blotting with the edge of a piece of filter paper, and the flipped replicas were immediately shadowed unidirectionally with platinum at an angle of 45° to a depth of approximately 2 nm. Stereo pairs were made with ±6° tilt.

Results

Distribution of Sindbis virus glycoproteins

In Sindbis-infected BHK cells labeled with anti-Sindbis antibodies, viral envelope glycoproteins were mostly distributed over the cell surfaces in clusters corresponding to viral budding areas, as seen either in labeling-fracture (Fig. 1A) or in immunolabeling fracture-flip (Fig. 1B). Sparse labeling was present on the exoplasmic leaflet of the freeze-fractured plasma membranes (Fig. 1A,B). Stereo views (Fig. 1A) showed that the clusters of immunogold label corresponded to different levels of budding. Control samples, in which we omitted the anti-Sindbis antibodies from the immunolabeling procedure, showed more than 90% reduction of labeling. Ultrastructural examination of the freeze-fractured and fracture-flipped plasma membranes showed a uniform distribution of both intramembrane particles (IMPs) and of surface particles (SPs) (Fig. 1A,B). However, in these preparations we could not correlate the pattern of distribution of immunogold with that of the membrane particles over the viral budding sites, since the immunolabeling obscured the morphology of the cell surface areas to which it was bound.

Distribution of intramembrane particles and surface particles in infected cells

To display IMPs and SPs over the budding viruses, we used conventional freeze-fracture and fracture-flip methods on unlabeled cells. In these preparations, portions of the membranes corresponding to viral budding sites showed a concentration of IMPs (Fig. 2A) as well as of SPs (Fig. 2B, arrows), whereas over the non-budding areas both IMPs and SPs appeared to be uniformly distributed. At later stages of the budding process, an organized structure of the virions was clearly seen (Fig. 2B, insert).
Fig. 1. Immunogold labeling of Sindbis virus envelope glycoproteins in freeze-fractured Sindbis virus-infected BHK cells as seen in label-fracture (A, stereo view) and in immunogold fracture-flip (B). Colloidal gold particles are mostly in clusters corresponding to viral budding figures. There is no apparent alteration of the density or of the pattern of distribution of IMPs (A) and SPs (B). A, $\times 44,000$; bar, 0.5 $\mu$m. B, $\times 44,000$; bar, 0.5 $\mu$m.

Distribution of CD8 proteins in infected cells
To analyze the behaviour and distribution of cellular membrane proteins during viral budding, we decided to use cells expressing, by transfection, a high level of a human lymphocyte antigen, the CD8 transmembrane protein, and successfully infectable with Sindbis virus. In Sindbis-infected FRT-U10 cells, immunolabeled with the anti-Sindbis protein antibodies, the pattern of distribution of the immunolabeling was similar to that observed above for BHK-infected cells, showing labeling mostly associated with budding virions (Fig. 3A,B).

In parallel experiments, we immunolabeled Sindbis-infected FRT-U10 cells with a monoclonal antibody directed against the CD8 proteins. Here the immunolabeling was distributed all over the surface of the fracture-flipped membrane and absent over the budding
Fig. 2. Sindbis virus-infected BHK cells as seen in conventional freeze-fracture (A) and fracture-flip (B). Stereo view of an exoplasmic face shows concentration of IMPs only over the viral budding figures (A). SPs are aggregated only over the viral budding portions of the membrane (arrows in B). Inset shows an organized structure of the virions at a late stage of the budding process. A, ×70,000; bar, 0.1 μm. B, ×85,000; bar, 0.1 μm. Inset, ×120,000; bar, 0.1 μm.

virions (Fig. 3C). Moreover, in some instances (Fig. 3C, arrows) it appeared that the gold particles corresponding to CD8 proteins were located all around and in close proximity to budding viruses, as if in a process of exclusion from them. In these images SPs were aggregated in regions corresponding to sites of viral budding (Fig. 3C). Control samples, in which we omitted the anti-Sindbis and anti-CD8 antibodies from the immunolabeling procedure, showed more than 90% reduction of labeling.

To demonstrate that CD8 transmembrane proteins correspond to surface particles, we incubated uninfected FRT-U10 cells in glycerol solution, a non-physiological treatment to induce experimentally the parallel aggregation of intramembrane and surface particles in fracture-flipped lymphocytes (Pavan et al.,
Cell surfaces during viral budding

Fig. 3. Immunogold fracture-flip of CD8 transfected FRT-U10 cells. (A,B) Immunolabeling of Sindbis virus envelope glycoproteins in virus-infected FRT-U10 cells. Clusters of colloidal gold particles are associated with viral budding figures. (C) Immunolabeling of CD8 glycoproteins in virus-infected FRT-U10 cells. Gold particles are distributed all over the cell surface and absent over the budding virions. In some instances, gold particles are located around and in close proximity to budding figures (arrows). (D) Immunolabeling of CD8 glycoproteins in glycerol-treated FRT-U10 cells. Immunogold labeling shows that CD8 glycoproteins are confined to the areas of aggregation of the surface particles. A-B, ×59,000; bar, 0.5 μm. C, ×54,000; bar, 0.5 μm. D, ×54,000; bar, 0.5 μm.

1989a, 1990). Observation of fracture-flip replicas showed also that in FRT-U10 cells treatment with glycerol caused aggregation of the surface particles, leaving particle-free areas in between (Fig. 3D). This confirms also that in these cells surface particles correspond to real components of the membrane. After glycerol treatment and fixation with glutaraldehyde, cells were labeled with anti-CD8 monoclonal antibody. Fracture-flip replicas of the surfaces of these cells showed that colloidal gold, and therefore CD8 transmembrane proteins, is confined to the particle aggregates on the cell surfaces (Fig. 3D, arrows).

Discussion

In infected cells the immunogold clusters observed corresponding to areas of viral budding illustrate the presence of a concentration of viral envelope glycoproteins. These findings confirm our previous observations using immunogold surface replicas and conven-
tional thin sections (Pavan et al., 1987; Torrisi et al., 1990). Here, however, our aim was to correlate the distribution and dynamics of viral and cellular transmembrane proteins with ultrastructural features of the plasma membrane. After fracture, transmembrane proteins may give rise to IMPs (revealed by label-fracture) within the plane of the fracture and to corresponding SPs (revealed by fracture-flip) over the surface of the fractured membranes. In unlabeled cells we observed that both IMPs and SPs were concentrated over the budding figures, suggesting that clusters of both particles corresponded to the clustering of viral glycoproteins. Over non-budding areas of the freeze-fractured membranes, the membrane particles appeared uniformly distributed and were taken to reflect the distribution of cellular transmembrane proteins.

In previous papers we focused on the distribution and dynamics of lymphocyte antigens during capping (Pavan et al., 1989a,b, 1990). We observed that the membrane particles (either IMPs and SPs) remained uniformly distributed over the entire plasma membrane, despite the high concentration of transmembrane proteins on the capping area. Therefore, we put forward the hypothesis that, during patching/capping, equilibration of the concentration of membrane components involving influx of capping transmembrane proteins, concomitantly with efflux of non-capping proteins, prevents the formation of noticeable aggregates of membrane particles. A similar equilibration of membrane components might occur during viral budding, where viral envelope glycoproteins interacting with the nucleocapsids form close-packed patches of proteins with the exclusion of cellular plasma membrane proteins. However, in this case, concentration of viral transmembrane proteins appears to give rise to a parallel concentration of membrane particles.

Other viral systems also show this phenomenon. During Sendai virus maturation patches of particles have been observed in the freeze-fractured plasma membrane of infected cells. This pattern of distribution of particles, similar to that observed in our experiments, presumably results from the restricted mobility of viral envelope proteins interacting with the nucleocapsids (Bachi, 1980). In cells infected with paramyxovirus, the ridges and bulges of particles observed over the freeze-fractured plasma membrane are related to attachment of the nucleocapsid to the plasma membrane, with immobilization of the viral envelope glycoproteins during assembly of the mature virus particles (Dubois-Dalcq and Reese, 1975).

To verify the efflux/influx theory during viral budding we used a cell line infectable with the Sindbis virus and expressing by transfection a high level of a transmembrane protein (the CD8 protein). This appeared to be a useful system in which to study simultaneously the dynamics of both a cellular transmembrane protein and the two viral proteins. In FRT-U10 cells infected with the Sindbis virus, the viral envelope glycoproteins and the membrane particles were, again, patched and concentrated in areas corresponding to the budding regions. Our observation of glycerol-treated FRT-U10 cells showed that CD8 transmembrane proteins co-localized with glycerol-induced surface particle aggregates. Therefore, the absence of labeling of CD8 proteins on the particle aggregates over the forming virus might be interpreted as a result of the exclusion of these proteins (and probably of other cellular proteins) from the budding area caused by the formation of clusters of the viral envelope glycoproteins.

The different pattern of distribution of membrane particles observed during capping (aggregation of antigens not accompanied by a coincident aggregation of membrane particles), and during budding (concentration of viral envelope glycoproteins accompanied by aggregation of membrane particles), may be due to the different mechanisms of these dynamic events. Capping is regulated by migration of proteins into the cap areas with positional control by cytoskeletal components (Bourguignon and Bourguignon, 1984; Holilfield et al., 1990). In viral budding, nucleocapsids underneath the plasma membrane act as a nucleation site, inducing an accumulation and immobilization of viral envelope glycoproteins inserted in the plasma membrane as proposed for Sindbis virus (Johnson et al., 1981), Semliki forest virus (Garoff and Simons, 1974) and for VSV (Knipe et al., 1977). We can, therefore, assume that immobilization, instead of concentration, of the viral glycoproteins is responsible for the accumulation of both membrane particles in areas of viral budding.

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References


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