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From assembly to virus particle budding: Pertinence of the detergent resistant membranes

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Abstract

Detergent resistant membranes (DRMs) are the site of assembly for a variety of viruses. Here, we make use of Sendai virus mutant proteins that are not packaged into virus particles to determine the involvement of this assembly for the virus particle production. We found that, in the context of an infection, (1) all the Sendai virus proteins associated in part with DRMs, (2) mutant HN and M proteins not packaged into virus particles were similarly part of this association, (3) after M protein suppression resulting in a significant reduction of virus production, the floatation profile of the other viral proteins was not altered and finally (4) cellular cholesterol depletion did not decrease the virus particle production, although it somehow reduced their virus infectivity. These results led us to conclude that the assembly complex found in DRM fractions does not constitute a direct precursor of virus particle budding.

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Keywords: Sendai virus; Assembly; Budding; Detergent resistant membranes; HN protein; M protein

Introduction

Cellular lipid bilayers are composed of a variety of phospho- and sphingolipids. Also, these membranes are not homogeneous, but rather constituted of microdomains of different size and lipid composition (Eddin, 1997; Simons and Ikonen, 1997). In the past decade, membrane domains enriched in cholesterol and glycosphingolipids received a particular attention, in part, because they are resistant to detergent solubilization at 4 °C, a property that facilitates their isolation. These detergent resistant membranes (DRMs) can cluster to form microdomains, called “rafts”. Rafts have been localized at the plasma membrane, in the Golgi apparatus as well as in the endocytic pathway (van Meer and Sprong, 2004). They have been associated with intracellular sorting and signal transduction events, cell adhesion, cell polarity, lipid and protein excretion and host–pathogen interactions (Golub and Pico, 2005; Ikonen, 2001; Simons and Ikonen, 1997; Simons and Toorme, 2000; Lafont and van der Goot, 2005; Manes et al., 2003).

Rafts have also been implicated in virus entry, intracellular trafficking, assembly and budding, this for a variety of viruses including non-enveloped and enveloped DNA or RNA viruses (Pelkmans, 2005; Nayak and Barman, 2002; Chazal and Gerlier, 2003). For the enveloped negative stranded RNA viruses, the involvement of rafts in virus entry and budding has been reported for Influenza virus (Orthomyxovirii, Scheiffele et al., 1997, 1999; Keller and Simons, 1998; Ali et al., 2000), Ebola and Marburg viruses (Filovirus, Panchal et al., 2003; Bavari et al., 2002), Respiratory syncytial, Newcastle disease, measles and Sendai viruses (Paramyxovirus, Brown et al., 2002; Dolgainue et al., 2003; Manie et al., 2000; Vincent et al., 2000; Ali and Nayak, 2000; Sanderson et al., 1995); for a recent review on Paramyxovirus budding see Takimoto and Portner (2004).

For measles virus, a member of the Paramyxoviridae family, it was shown that the localization of the viral components into rafts resulted from a cooperative assembly process. The F1 glycoprotein, inherently capable of raft association, was responsible for dragging the other viral glycoprotein, H, into the rafts. Similarly, the assembly of the internal viral proteins N and M into rafts was dependent on the presence of the viral genome (Manie et al., 2000; Vincent et al., 2000). An equivalent study has not been performed for Sendai virus. However, cellular membranes were purified from infected cells and treated with detergents to investigate the degree of
association of the viral proteins, F, HN and M, with these membranes (Ali and Nayak, 2000; Sanderson et al., 1995). While M acquired resistance to both Triton X-100 and octylglucoside extraction, HN and F were resistant only to Triton X-100 solubilization. When individually expressed from vaccinia virus recombinants, each viral protein (F, HN or M) was independently capable of acquiring Triton X-100 insolubility (Sanderson et al., 1995). However, this association with DRMs was disrupted at Triton X-100 concentrations (0.03–0.05%) that do not correspond to the solubilization conditions generally accepted as characteristic of rafts.

The association of viral constituents with rafts has naturally led to the conclusion that the raft membranes were likely involved in virus particles budding as well (Manie et al., 2000). This conclusion may not follow since often, and this is the case for Sendai virus, a minor fraction of the viral components assembles into virus particles (Tuffereau and Roux, 1988), making its tracking difficult. We decided to test the pertinence of the assembly complex for virus particle production by using mutant virus proteins, HN and M, known not to be packaged into virus particles. The rationale of this approach rests on the prediction that the assembly complex pertinent for virus particle production should not contain the mutant proteins.

We observed that, in Sendai virus infected cells, association with DRMs did not necessarily correlate with incorporation into virus particles. Moreover, in conditions where the M protein was suppressed, resulting in a significant decrease in virus particle production, the protein profile of the DRM fractions was not altered. Finally, cholesterol depletion did not lead to a decrease of virus particle release. These results led to the conclusion that the assembly complex found in DRM fractions does not constitute a direct precursor of virus particle budding. Another possible route of assembly is discussed.

Results

Association of the Sendai virus proteins with detergent resistant membranes

Sendai virus infected LLC-MK2 cells were solubilized with 1% Triton X-100 at 4 °C and the cellular extracts were loaded in OptiPrep gradients (see Materials and methods) to assess the degree of association of the viral proteins with the detergent resistant membranes (DRMs). In such gradients, DRMs are found at the top of the gradient (Fig. 1, DRM, fractions 2–3), identified, in our case, by the presence of caveolin-1 (Cav), a constituent of the caveolae, known to harbor light lipid membranes enriched in cholesterol and sphingolipids (Kurzchalia and Parton, 1999; Parton and Richards, 2003; Simons and Ikonen, 1997). The proteins float in the DRM fractions by their full association with the low density membranes. In contrast, the proteins found at the bottom of the gradient (fractions 7–8, Sol), are fully dissociated from lipid membranes and sediment according to their intrinsic density. The fractions of intermediate density (fractions 4–6, Int) contain proteins partially associated with lipid complexes or associated with membranes of higher density.

All the Sendai virus proteins were partly found in the light DRM fractions (Fig. 1, DRM). Their sedimentation profile, however, was not homogenous. The P and N proteins, part of the nucleocapsid complex, appeared evenly distributed throughout the gradients, with, however, a preponderance for the intermediate (Int) and DRM fractions (particularly, fraction 3). The two glycoproteins, HN and F, exhibited a biphase distribution with an under representation in the intermediate fractions. In contrast, the M protein appeared enriched in these intermediate fractions. Identical results were obtained when floatation gradients were prepared with sucrose solutions in place of OptiPrep (not shown, see Materials and methods). It is noteworthy that the M protein, accepted to be the central organizer of the virus particle formation, was poorly represented in the DRM fractions.

Are DRM fractions relevant for assembly leading to virus particle production?

Virus assembly fractions identified as relevant for virus particle production should be the site of discrimination between proteins that will become incorporated into virus particles and proteins that are excluded. We made use of previously produced mutant HN and M proteins to verify this assertion. A mutant HN protein, HNctf35Slep, has been generated which carries a five amino acid substitution in its cytoplasmic tail (SYWST → AFYKD; Fouillot-Coriou and Roux, 2000; see Materials and methods). Upon infection of LLC-MK2 cells with a recombinant SeV (rSeV-HNctf35Slep) expressing HNctf35Slep, the mutant HN protein could accumulate at the cell plasma membrane, but was not incorporated into virus particles in a detectable manner. Interestingly, virus particle production was normal (Fouillot-Coriou and Roux,
Fig. 2A reproduces this result showing a Western blot with, to the left, the extracts (CE) of cells infected with wild-type (lane 1) or rSeV-HNct35SIcp (lane 2) viruses and, to the right, the protein contents of the virus particles (VP). Note that 13-folds less cellular extracts (1/13) than virus particles (1/1) were analyzed, showing that less that 10% of the viral proteins were found in virus particles. Next, the DRM membrane association profile of HNct35SIcp obtained from these infected cells was compared with that of the HN wild type (Figs. 2B and C). No difference in the DRM composition was observed between the two proteins. The floatation profile of the other viral proteins was also similar for the wild-type and the mutant virus infected cell extracts (not shown).

A similar analysis of an M protein mutant, HA-M30, was then performed. HA-M30 carries two mutations T112M and V113E which prevent incorporation of the protein into virus particles (Mottet et al., 1999; see Materials and methods). In this case, the mutant M, tagged with an HA epitope (HA-M), was expressed from a minigenome supported by a helper virus (mixed virus stock) expressing a wild-type M. In this context, HA-M30 was shown (1) not to interact with the wild-type M by coimmunoprecipitation, (2) to mainly accumulate around the cell nucleus and (3) to exhibit a partial negative dominant phenotype on virus particle budding. These features could not be attributed to the HA tag, since a wild-type HA-M (HA-Mwt) expressed under the same conditions behaved normally (Mottet et al., 1999). Fig. 3A (CE, part α-M) shows the relative level of the HA-Ms and Ms expressed in LLC-MK2 cells infected with the mixed virus stocks, coexpressing HA-Mwt (lane 1) or HA-M30 (lane 2) along with the normal M. Part [α-HA] scores the tagged M proteins only. Fig. 3A (VP) shows the same analysis performed on the virus particles produced and demonstrates the total absence of HA-M30 (VP part lane 2) scored with the α-HA in regard to the positive uptake of HA-M wild type (lane 1). Despite the total absence of HA-M30 in virus particles, its floatation profile in the DRM fractions is unchanged compared to HA-Mwt (compare Figs. 3B and C, fractions 2–3). However, an increase of HA-M30 in the soluble portion of the gradient was reproducibly observed (Fig. 3C, fractions 7–8), which may relate to the different subcellular localization of the two proteins (Mottet et al., 1999). As for the sedimentation profile of the other viral proteins, it did not differ in the two situations (not shown).

Finally, the floatation profile of the viral proteins was tested in the absence of detectable M protein, with the idea that the assembly complex would be severely compromised under these conditions. To achieve this goal, a recombinant SeV harboring a siRNA target sequence in its M gene 5′ UTR was grown in cells constitutively expressing the cognate siRNAs (see Materials and methods). Under these conditions, the suppression of M, although not complete, became more and more prominent with time in regard to the amounts of the
other viral proteins which steadily increased (Fig. 4A, CE, compare 18 and 40 h pi). After 40 h of infection, the suppression was sufficient to provoke a significant reduction of virus particle production compared to that of the control infection (Fig. 4A, VP compare lanes 1 and 2). At this later time, the floatation profiles of the HN, F and N proteins were compared. The absence of detectable M resulting in the significant reduction in virus particle production was not paralleled by a change in the floatation profile of the other viral proteins (compare Figs. 4B and C).

In summary, the viral composition of the DRM fractions could not be perturbed either when the viral proteins were mutated not to be packaged into virus particles, or when the M protein was suppressed so that virus particle production could be significantly reduced.

**Effect of cholesterol depletion on virus particle production**

DRM insolubility properties depend mainly on their high cholesterol composition. Membrane embedded cholesterol can be removed by treatment with methyl-β-cyclodextrin (MβCD, Harder and Simons, 1997). Fig. 5A shows that a 90 min treatment of cells with 20 mM MβCD causes a depletion of about 60% of their total cholesterol content. Upon cholesterol depletion, DRMs undergo some alteration as evidenced by the caveolin floatation profile which shows a displacement from the top fractions to the more intermediate positions (Fig. 5C, Cav). As for the viral proteins, if the HN and Fo protein profiles exhibited a significant shift towards the soluble fractions (Figs. 5B and C, HN, Fo), little effect of cholesterol depletion was seen on the distribution of the N and M proteins (Figs. 5B and C).

The effect of cholesterol depletion on the virus particle production was then measured. We rationalized that virus particle production could be affected by the disruption of the DRMs if these latters represent the site at which assembly complex relevant for budding takes place. Infected cells were then treated with MβCD and the virus particles produced under these conditions were collected. Fig. 6A shows (duplicate samples) that MβCD treatment had little effect on the HN and N content in the cellular extracts (CE). In contrast, a ~4-fold decrease in the M protein amount was observed. Interestingly, the protein profile of the virus particles produced shows a corresponding increase in the M protein composition following MβCD treatment (VP). A slight increase in HN was also observed. Finally, caveolin, not usually found in virus particles (see also Fig. 2B), was now detected (see Discussion).
inf ectivity of the produced virions was assessed by plaque formation assay (Fig. 6B), and a loss (20–40%) was reproducibly noticed. Despite these alterations, possibly due to side effects of the treatment, it remains that DRM disruption by MβCD treatment resulted in no way in a decrease of physical virus particle production.

Discussion

Enveloped virus constituents have been shown to assemble on DRMs, usually described as rafts after their coalescence into microdomains, and its has been assumed that this assembly is relevant for virus particle production (Pelkmans and Helenius, 2003; Manes et al., 2003). However, an intrinsic complication of this logic comes from the minor fraction of the intracellular virus constituents which, eventually, end up in virus particles. We therefore designed experiments to assess the relevance of DRM driven assembly for virus particle production, using mutant virus proteins excluded from virus particles. The rationale of this approach rests on the conclusion that their exclusion should parallel their absence in the assembly complex, if this latter is relevant for virus particle formation. The results were clear cut. The representation in the DRM fractions of the HN and M mutants excluded from virus particles did not change from that of their wild-type counterpart. Moreover, the M protein suppression did not provoke an alteration of the DRM composition, although, under these conditions, assembly leading to budding was significantly perturbed, as seen by the significant decrease in virus particle production. Taken together, these observations strongly suggest that the DRM fractions do not constitute sites of viral component assembly directly related to virus particle production. This conclusion was reinforced by the fact that DRM disruption following cholesterol depletion had no detrimental effect on the amount of virus particle production. The better uptake of M (and HN) observed under these conditions is interesting because it may refer to an alteration of the regulation of assembly, but remains unexplained at present.

There are different ways to interpret these data. On the one hand, they may reflect the poor resolution of the DRM fractions obtained from floatation gradients. These fractions are certainly heterogeneous, reflecting the heterogeneity and the constant remodeling of the populations of cholesterol enriched membranes. On the other hand, the DRM association may represent an assembly step in the pathway to budding at which discrimination between the proper virus particle constituents has not yet segregated away from the contaminants (be it improper viral or cellular proteins). In that respect, it is noteworthy that HNc13Slcp is efficiently expressed at the cell surface (Fouillot-Coriou and Roux, 2000). Yet, its packaging into virus particles does not take place, despite the fact that its association with DRM is similar to that of wild-type HN. This suggests that the cell plasma membrane is not the site at which HN is incorporated in the assembly complex relevant for budding. This situation is reminiscent of the observation made in the study of retrovirus pseudotyping (Sandrin et al., 2004). The feline endogenous retrovirus RD114 glycoprotein was found not to pseudotype efficiently with SIV cores, whereas it does so readily with MLV core. The explanation for this discrepancy relies on the necessity of the glycoprotein to find its core on intracellular membranes, after reinternalization from the cellular plasma membrane. It is then possible that the SeV HN has to be reinternalized to attach to the nucleocapsid/M assembly complex. The effect that its SYWST motif, found not to migrate to the cell periphery. Yet, its ability to partially reduce the virus particle production was recognized (Mottet et al., 1996). In the end, both sets of data point to a site of assembly relevant for budding located on membranes other than those identified as cholesterol enriched membranes in the present study, and possibly not localized at the plasma membrane.

It is noteworthy that this discrepancy between DRM association and uptake in virus particle has been reported before for a Paramyxovirus. Following infection of cells with a chimeric measles virus (MGV) in which H and F were replaced by the vesicular stomatitis virus (VSV) G glycoprotein, it was found that the VSV G protein, in contrast to the measles virus proteins, did not associate with DRMs. Yet, MGV particles (containing G) production was found efficient (Vincent et al., 2000). So, for measles virus, as well as for Sendai viruses, it appears that the lipid rafts may not represent sites where partitioning between proteins incorporated or not into virus particles takes place. Discrimination at the site of rafts has yet been described for HIV produced by infected T-cell lines.
(Nguyen and Hildreth, 2000). In this case, not only the viral proteins (Gag and Env, raft associated) are incorporated into virus particles, but also the GPI anchored cellular proteins Thy-1 and CD59, as well as the ganglioside GM1, similarly known to partition into lipid rafts. In contrast, the CD45 protein is poorly incorporated despite its high cell surface expression, and this exclusion correlates with an exclusion from the lipid rafts. In the end, the direct involvement of rafts for virus particle production may vary from one virus family to another, an assertion that should not be a surprise considering the extreme variability with which the viruses have evolved in their adaptation to their host.

Materials and methods

Cells

LLC-MK2 and A549 cells were grown at 37 °C in Dulbecco modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS) under 5% CO2 atmosphere. The preparation of the A549-LV-siGFP cells will be described in details elsewhere (Mottet Geneviève, Laurent Roux and coworkers, Department of Microbiology and Molecular Medicine, University of Geneva Medical School, in preparation). In brief, A549 cells were transduced with a lentiviral vector expressing siRNAs targeted to a defined sequence (gftp) of the green fluorescent protein (GFP) gene and the nerve growth factor receptor (NGFR) as described in Wisnerowieciz and Trono (2003). The transduced cells were selected on the basis of efficient NGFR surface expression. Efficient expression and suppression capacity of the siRNAs were probed by GFP suppression upon infection of the cells with a recombinant SeV expressing GFP (rSeV-GFP, see below).

Viruses and virus infection

Infections with Sendai virus (SeV), with its various recombinants (rSeV) or with the mixed Sendai virus stocks were performed at 33 °C. Virus stocks were adequately diluted (multiplicity of infection of 3) in MEM without FCS and laid over the cells for 1 h. At the end of the infection period, the infectious mix was removed and replaced with fresh MEM supplemented with 2% FCS. SeV Harris strain was prepared as described by Abrami et al. (2003). Virus stocks were adequately diluted (multiplicity of infection of 3) in MEM without FCS and laid over the cells for 1 h. At the end of the infection period, the infectious mix was removed and replaced with fresh MEM supplemented with 2% FCS. SeV Harris strain was prepared as described by Abrami et al. (2003). This virus expresses a mutant HN glycoprotein carrying an amino acid sequence substitution in its cytoplasmic domain (change of 10SYWST14 into 10AFYKD14). This HN protein also harbors an influenza virus hemagglutinin (HA) epitope tag at its C-terminus (aa 57aMAYPYDVP-DYASLG-GPGA593, Fouillot-Coriou and Roux, 2000). This virus expresses a wild-type HN glycoprotein tagged with the same HA epitope (rSeV-HA-HNwt) was also generated previously (Fouillot-Coriou and Roux, 2000). A rSeV expressing a wild-type HN glycoprotein tagged with the same HA epitope (rSeV-HA-HNwt) was also generated previously (Fouillot-Coriou and Roux, 2000). A rSeV expressing a wild-type HN glycoprotein tagged with the same HA epitope (rSeV-HA-HNwt) was also generated previously (Fouillot-Coriou and Roux, 2000). A rSeV expressing a wild-type HN glycoprotein tagged with the same HA epitope (rSeV-HA-HNwt) was also generated previously (Fouillot-Coriou and Roux, 2000).

Reagents

Antibodies used in this study include anti-caveolin-1 (N20; Santa Cruz), anti-SeV P (a rabbit serum raised against SDS-denatured P protein, α-PSDS), anti-SeV HN (a rabbit serum raised against SDS-denatured HN protein, α-HNPSDS), anti-SeV Fo (a rabbit serum raised against Fo cytoplasmic tail peptide, α-Fo), anti-SeV N (a rabbit serum raised against SDS-denatured N protein, α-NPSDS), anti-SeV M (a rabbit serum raised against SDS-denatured M protein, α-MPSDS (Mottet et al., 1986; Tuffereau and Roux, 1988) and MAb 383 obtained from Claes Orvelli (Laboratory of Clinical Virology, Huddinge Hospital, Huddinge, Sweden), anti-Influenza virus HA epitope MAb (16b12, Berkeley Antibody Co.). Peroxidase-coupled secondary antibodies were from BioRad. Methyl-β-cyclodextrin was from Sigma.

Methyl-β-cyclodextrin treatment and cholesterol measurement

Methyl-β-cyclodextrin (MβCD) treatment (0, 10, 20 mM) was performed in serum-free medium during a 90 min period at 37 °C. This treatment led to at least 60% extraction of total cellular cholesterol quantitatively measured by one-dimensional thin layer chromatography (TLC) after CuAc/H3PO4 staining as described by Abrami et al. (2003).

Detergent resistant membrane preparation and floatation gradients

Infected cells (107) were lysed in 300 μl of ice-cold TNE buffer (25 mM Tris–HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA) containing 1% Triton X-100 (lysis buffer) plus a cocktail of protease inhibitors (Complete; Roche). The lysis buffer volume to cell number ratio was adjusted empirically according to the sedimentation profile of caveolin-1 and was kept constant throughout the experiments. Detergent resistant membranes (DRMs) were separated using OptiPrep (Nycomed). Cellular extracts (200 μl) treated for 30 min at 4 °C with the lysis buffer were mixed with 400 μl of 60% OptiPrep. This 40% OptiPrep mixture was overlaid with 30% (1200 μl) and 0% (500 μl) OptiPrep layers, and centrifuged for 2 h at 55,000 rpm (4 °C) using a TLS55 Beckman rotor (Lafont et al., 2002). Alternatively, sucrose gradients were used. In this case, cellular extracts were made 40% in sucrose (800 μl), overlaid with 35%
(2.5 ml) and 15% (1 ml) sucrose solutions and centrifuged at 40,000 rpm (16 h, 4 °C) using a SW60 rotor. Fractions were collected from the top, the protein content of each fraction was precipitated with 8% TCA in the presence of 375 μg sodium deoxycholate, dissolved in 25 μl of SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

**SDS-PAGE analyses and Western blotting**

Protein samples were analyzed by SDS-PAGE. After electrophoresis, the proteins were transferred using a semidry system onto polyvinylidene difluoride membranes (Millipore). Blots were then incubated with specific antibodies, followed by the appropriate horseradish peroxidase (HRP)-coupled secondary antibodies. Protein detection was performed by using the enhanced chemiluminescence system (Amersham Biosciences).

**Virus particle characterization**

To estimate virus production and to characterize the virus particle composition, the virus particles in the clarified cell supernatants were collected by centrifugation through a 25% glycerol cushion (Beckman SW55 rotor, 2 h, 50,000 × g) and directly resuspended in SDS sample buffer.

**Virus titration**

SeV and rSeV plaque assays were done on LLC-MK2 under a 0.3% agarose overlay in the presence of 2 μg/ml of acetylated trypsin according to Sugita et al. (1974).

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**References**


