Distribution of the attachment (G) glycoprotein and GM1 within the envelope of mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy

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Abstract

Field emission scanning electron microscopy (FE SEM) was used to visualize the distribution of virus-associated components, the virus-attachment (G) protein, and the host-cell-derived lipid, GM1, in respiratory syncytial virus (RSV) filaments. RSV-infected cells were labeled in situ with a G protein antibody (MAb30) whose presence was detected using a second antibody conjugated to colloidal gold. No bound MAb30 was detected in mock-infected cells, whereas significant quantities bound to viral filaments revealing G protein clusters throughout the filaments. GM1 was detected using cholera toxin B subunit conjugated to colloidal gold. Mock-infected cells revealed numerous GM1 clusters on the cell surface. In RSV-infected cells, these gold clusters were detected on the filaments in low, but significant, amounts, indicating the incorporation of GM1 within the viral envelope. This report describes the first use of FE SEM to map the distribution of specific structural components within the envelope of a Paramyxovirus.

Keywords: Respiratory syncytial virus; FE SEM; Virus assembly; GM1; G protein; Caveolae

Introduction

The assembly of respiratory syncytial virus (RSV) occurs in association with host cell membranes to produce mature infectious viral particles (Parry et al., 1979; Roberts et al., 1995). These RSV particles comprise a ribonucleoprotein (RNP) core that is surrounded by a viral envelope within which the three different viral glycoproteins, the attachment (G), fusion (F), and small hydrophobic (SH) proteins, are located. The RSV assembly process is still poorly understood and the factors involved in the formation of progeny virus are ill-defined, although several host-cell factors have been implicated in the process (Ulloa et al., 1998; Burke et al., 1998; Pastey et al., 1999; Gower et al., 2001; Brown et al., 2002a, b).

The G protein is a type II glycoprotein that is anchored to the viral membrane via the N-terminal hydrophobic sequence. In the mature protein, the ectodomain contains two mucin-like domains, which are separated by a stretch of amino acids in which conserved cysteine residues form a structure termed a cysteine noose (Langedijk et al., 1998). The G protein is initially synthesized as a precursor (approx. 32 kDa) that subsequently undergoes extensive N- and O-linked glycosylation in a manner dependent on both virus strain and cell type (Garcia-Beato et al., 1996; Garcia-Beato and Melero, 2000). This protein was identified as the virus attachment protein in studies that showed that antibodies against G protein inhibited virus attachment (Levine et al., 1987). However, while the G protein plays a role in virus
attachment, in tissue culture this process can also be mediated through the F protein (Karron et al., 1997; Karger et al., 2001; Techarapotkul et al., 2001). It is therefore likely that RSV requires both proteins for its efficient cell attachment and cell entry.

Several reports have described electron microscopic analysis of RSV and in particular the locations of the various virus structural components (Norrby et al., 1970; Bach and Howe, 1973; Roberts et al., 1995; Arslanagic et al., 1996; Brown et al., 2002a, 2002b) TEM studies reveal the virus spike proteins as projections that extend up to 10–12 nm on the viral envelope. However, these studies are limited since they have mainly employed immunogold labeling of thin sections, which does not allow an appreciation of the surface distribution of virus spike proteins. This is primarily because such immunogold labeling techniques allow only the detection of antigen that is exposed on the surface of the section (Hermann et al., 1996). In this study, we have overcome this major obstacle by employing field emission scanning electron microscopy (FE SEM) to analyze the surface of RSV-infected cells. Although FE SEM has been applied mostly to the analysis of materials in the physical sciences, it is now emerging as a suitable technology with which to analyze complex biologic systems.

FE SEM allows biologic samples to be viewed at much higher magnification than conventional SEM, with resolution approaching that obtainable in TEM of thin sections. In addition, the instrument contains two different detectors, which are referred to as the secondary electron (SE) and the back scatter electron (BSE) detectors. The SE detector allows visualization of surface morphology, while the BSE detector allows detection of electrons that are scattered by dense, nonbiologic, atoms such as colloidal gold (reviewed in Mueller et al., 1989; Hermann et al., 1996). The increased resolution, coupled with the ability to easily detect small immunologic markers such as 5- to 10-nm colloidal gold enables us to visualize directly the distribution of specific antigens in situ using standard immunogold labeling techniques that are, in essence, similar to those used for light microscopy. FE SEM therefore combines the advantages of fluorescence microscopy and SEM by allowing us to label and visualize the distribution of specific virus antigens on the surface of RSV-infected cells. In this report, we describe the use of FE SEM to map the distribution of both the virus attachment protein and the host-cell-derived lipid, GM1, within the envelope of mature RSV filaments.

Results and discussion

Characterization of MAb30 in RSV-infected Vero C1008 cells

The data presented in this report were obtained using the polarized Vero C1008 cell line, and the G protein was detected in RSV-infected cells with the monoclonal antibody MAb30, a reagent previously characterized (Taylor et al., 1992). The specificity of MAb30 in our experiments was confirmed by Western blotting analysis (Fig. 1A). Probing with MAb30 showed the presence of two specific protein bands migrating as 55- and 80-kDa protein species. These represent different O-linked glycosylated forms of the G protein that have been reported previously (Feldman et al., 2000; Teng et al., 2001). We noted that the 55-kDa G protein species appeared to be the predominant form in infected Vero cells, whereas in several other established cell lines (e.g., HEp2 and BHK) the 80-kDa G protein species represents the major form of the protein. Previous studies have shown that the apparent size of the G protein detected by SDS–PAGE is host-cell-dependent (Garcia-Beato et al., 1996; Garcia-Beato and Melero, 2000). However, both G protein species (55 and 80 kDa) are able to interact efficiently with the putative virus receptor, heparan sulfate (Feldman et al., 2000). As expected, these protein bands were not detected in mock-infected cells, confirming the specificity of MAb30.

The presence of both G protein species on the surface of RSV-infected cells was confirmed by cell-surface biotinylation using sulfo-NHS-LC-LC-biotin. Lysates prepared from mock- and RSV-infected cells were immunoprecipitated with MAb30, and the G protein was transferred by Western blotting onto a PVDF membrane, which was then probed using streptavidin–HRP (Fig. 1B). We observed the presence of two protein bands, of sizes similar to those detected by Western blotting using MAb30, that were not detected in mock-infected cells. This clearly shows that both G protein species are expressed on the surface of RSV-infected cells.

The distribution of the G protein on the surface of RSV-infected cells was analyzed by immunofluorescence (IF) microscopy using MAb30. We noted that the fluorescence signal was largely confined to the long filamentous structures that correspond to budding virus (Fig. 1C), hence indicating the sites of virus assembly. These filaments formed both singly and in clusters on the cell surface and were similar in appearance to the filamentous structures previously observed on RSV-infected cells (Parry et al., 1979; Roberts et al., 1995; Brown et al., 2002a). In addition, we noted the appearance at relatively lower levels of a second type of staining pattern, more speckled in appearance, that was distinct from the readily visible virus filaments (Fig. 1C). This was not present in mock-infected cell monolayers and suggests that staining is due to the presence of the G protein, rather than nonspecific background staining. These may represent sites of virus assembly at which smaller immature virus filaments cannot be clearly visualized, because of the limitations in resolution of light microscopy.
Distribution of the G protein in mature RSV filaments

A major limitation in the use of confocal microscopy concerns the maximum theoretical resolution that can be obtained using light microscopy (250 nm; Lacey, 1989). Although we were able to detect the presence of the G protein within RSV filaments using confocal microscopy, this limitation in resolution prevents a detailed description of the surface distribution of the G protein. Furthermore, in several experiments we have used immuno-TEM to analyze thin sections (80 nm thickness), prepared from embedded RSV-infected cells that were labeled with MAb30. Exam-
nation of these thin sections revealed that the filaments exhibited a level of labeling that was lower than expected (data not shown), given that the images obtained by confocal microscopy showed continuous staining along the length of the filaments (Fig. 1C). We reasoned that this anomaly may arise because of a combination of antigen accessibility on the surface of the thin sections and possibly the distribution of the G protein within the virus envelope. We therefore investigated the surface distribution of the G protein using an alternative technology, FE SEM. This EM technique allows the analysis of the surface of RSV-infected cells at a much higher resolution (approx. 1–2 nm) than can be obtained by light microscopy.

Mock- and RSV-infected cells were labeled with MAb30 and subsequently analyzed by FE SEM at an intermediate magnification (×30,000). A comparison of the images obtained from mock- and RSV-infected cells using the SE detector revealed a significant difference in cell surface morphology (Fig. 2A and B). In mock-infected cells, we observed the presence of numerous microvilli that were approximately 100 nm thick and which extended 500–1000 nm from the cell surface. These structures appeared to form both singly and as clusters. In contrast, on the surface of RSV-infected cells, we noted the presence of numerous filamentous structures that correspond to mature virus. These structures extended up to 6–8 μm in length, were approximately 150–200 nm thick, and were clearly distinguishable from the smaller microvilli observed in mock-infected cells. These observations are consistent with previous reports that have used SEM to visualize mature virus filaments on RSV-infected cells (Parry et al., 1979; Roberts et al., 1996; Brown et al., 2002a).

Analysis of the above samples using the BSE detector revealed significant differences in the levels of detectable colloidal gold particles. In mock-infected cells (Fig. 2C), no signal was detected, suggesting the absence of any gold particles and hence any surface-bound MAb30. In contrast, in RSV-infected cells (Fig. 2D), we observed an abundance of white points that were present on the viral filaments and which correspond to the presence of colloidal gold particles.
indicating the presence of surface bound MAb30. Also visible was a low-level, nonspecific, background signal that was present in both mock- and virus-infected cells (Fig. 2C and D, indicated by Hz). It is unclear where this background was present in both mock- and virus-infected cells (Fig. 2C). The background signal that arises from the heavy metal and D, indicated by Hz). It is unclear where this background was present in both mock- and virus-infected cells (Fig. 2C). The background signal can clearly be distinguished from the relatively strong backscatter signal that arises from the presence of surface-bound colloidal gold particles. Although filamentous structures were heavily labeled with gold particles, we also noted a much lower level of label in the filament-free regions. This presumably represents non-filament-associated G protein that is present on the cell surface, since no background signal was detected in mock-infected cells. The overall staining pattern observed by FE SEM was consistent with our observations obtained using confocal microscopy.

A comparison of the mock- and virus-infected cells by FE SEM showed that whereas microvilli were abundant in mock-infected cells, there appeared to be a significant reduction in these structures in RSV-infected cells. Microvilli are characterized by the presence of F-actin bundles and by the presence of other specific protein markers such as ezrin (Bretscher, 1983; Berrymman et al., 1995). We have employed confocal microscopy to locate the presence of F-actin (using phalloidin–FITC) in virus-infected cells with respect to the virus filaments. In this analysis a distinct labeling pattern was observed for F-actin that corresponds to the cellular actin network (Fig. 3A). The labeling pattern for MAb30 was largely confined to the virus filaments; however, despite repeated attempts, we failed to detect the presence of F-actin within the stained RSV filaments (Fig. 3B). We also noted that ezrin and F-actin showed a strong colocalization, appearing as small spot-like structures that represent microvilli (Fig. 3C and D). We noted that these structures were less apparent in virus-infected cells and that the staining pattern for ezrin was clearly distinct from that of the virus filaments. Taken together, these biochemical data show that the RSV filaments are clearly distinct from the microvilli, although we have not yet established whether the virus can assemble at those regions of the cell membrane containing microvilli.

Inspection of the labeled filamentous structures at higher magnification (×100,000), using the BSE and SE detectors, allowed us to visualize the pattern of labeling in detail (Fig. 4). At this magnification, the surface of the RSV filaments appeared to be covered with dome-shaped structures that were interspersed with relatively smooth areas (Fig. 4A), and the gold particles were visualized as intensely bright circular features (Fig. 4B). In particular, we noted that the locations of the gold particles and dome-shaped projections appeared to coincide. These dome-shaped structures presumably represent the specific locations of G protein spikes together with the bound primary and secondary antibodies. This is most clearly visualized when the images obtained from identical parts of the infected cell using the SE and BSE detectors are merged (Fig. 4C). These projections were not previously visualized in RSV-infected cells examined by TEM (Roberts et al., 1995; Brown et al., 2002a), and the reason for their appearance is unclear but it may reflect differences in the sample preparation. The samples prepared for SEM are processed by critical point drying (CPD), a treatment that can cause shrinkage of lipid membranes. It is possible that CPD produces a change in the integrity of the lipid membrane in the vicinity of the G protein–antibody complex (e.g., caused by shrinkage), which may therefore exaggerate the appearance of the G protein within the viral envelope after coating of the sample. It should also be appreciated that the other virus glycoproteins and specific host factors may occupy the regions of the envelope that remain unlabeled.

Examination of several images of RSV-infected cells showed two types of gold labeling patterns on the viral envelope, single and double, corresponding to one and two bound gold particles, respectively. However, we noted that the doublet pattern was more predominant. The distance between the individual gold particles within each doublet was approximately 20–25 nm (Fig. 4C, inset). Furthermore, we noted that these small clusters of gold label appeared to be separated by distances of up to approximately 80 nm on the virus filaments. It is at present unclear whether each gold particle represents an individual G protein spike or a small cluster of G protein spikes within the viral envelope, since a 10-nm gold particle can theoretically bind up to 10 molecules of the primary antibody. However, immunonegative staining of RSV particles, which were labeled with MAb30 and 10-nm gold probe, suggested that in reality each gold probe can label only one to two G protein spikes (data not shown). In addition, we have noted that the overall gold-labeling pattern observed in FE SEM is very similar when a 5-nm gold probe, which can theoretically bind 1–2 molecules of primary antibody, was used to locate the surface-bound MAb30 (data not shown). Our data suggest that only a small number of G protein spikes are likely to be present within a G protein cluster; however, at present the precise number is not known.

Although the studies presented in this report have used Vero cells as the host cell to support RSV replication, the G protein labeling pattern in Vero cells was broadly similar to that which we have observed in other established cell lines such as HEp2 and BHK (data not shown). This suggests that G protein distribution is not dependent on variations in the degree of O-linked glycosylation. Taken together, these data indicate that G protein molecules appear to cluster at intervals within the viral envelope. It is likely that clustering of G protein spikes within the envelope may facilitate binding of the virus to the cell surface by increasing the number of contacts per unit area during cell attachment. It is interesting to note that several viruses have evolved to be able to interact with their respective host-cell receptor with a higher affinity than with the natural ligand of their respective
concentrate the virus attachment protein at specific locations within the virus envelope may form part of a general mechanism whereby virus attachment to the cell surface is enhanced.

The sites of RSV assembly viewed by FE SEM

We have used FE SEM to visualize the sites at which virus assembly occurs, and in particular we examined the area of the cell surface from where the mature RSV filaments form (Fig. 5A–E). In virus-infected cells labeled with MAb30, we noted that while the virus filaments are heavily labeled with colloidal gold, the immediate area of the cell surface that surrounds the filaments lacks similar levels of label. We further noted that each filament, even within a cluster (as seen by light microscopy; Fig. 1C), forms from a distinct location on the cell surface. This finding is supported by examining the G protein labeling pattern on smaller, less developed, RSV filaments (Fig. 5D and E) that are at a relatively early stage in the assembly process. In these cases, we observed that the virus formed from specific regions of the cell surface and that the gold label was sharply restricted to the growing filament. In addition, sporadic gold labeling of the cell surface was observed at a significantly lower level than labeling of the virion. This may represent sites of virus assembly at which filaments have not yet formed. The fact that no label was detected on mock-infected cells suggests that this non-filament-associated label is actually a specific labeling product and it may be identical to the non-filamentous labeling pattern that we observed using light microscopy (see Fig. 1C).

Since the methodology that we have employed only locates cell surface protein, this allows us to speculate about the incorporation of G protein into the viral envelope. Our observations suggest that the G protein may be recruited preferentially to the sites of virus assembly (e.g., lipid rafts) during the formation of the viral envelope. We obtained no evidence to suggest a large accumulation of G protein at locations other than where the RSV filaments form during virus egress.

The ability to clearly visualize RSV filaments and the regions of the cell surface from which they form allows us to estimate the size of the sites of virus assembly as being between 150 and 200 nm in diameter. The available evidence has indicated that RSV assembly occurs at specialized regions of the cell surface that contain both GM1 and caveolin-1 (Brown et al., 2002a,b). This suggests that specialized regions of the host-cell membrane, which possess caveolae-like properties, are involved in the RSV assembly process. In this regard, it is interesting to note the similarity in sizes between the sites of RSV assembly and the physical dimensions of caveolae (Westermann et al., 1999).

Early studies have shown the role of host-cell-derived glycolipids in the formation of the paramyxovirus envelope (Klenk and Choppin, 1970). In addition, several viruses have been reported to assemble from specialized regions of the host cell that are referred to as lipid rafts. In these cases, the raft lipid GM1 has been used as a lipid raft marker (Manie et al., 2000; Nguyen and Hildreth, 2000). In our previous studies, we examined the sites of RSV assembly and provided data showing that this process occurred within GM1-rich microdomains on the cell surface (Brown et al., 2002b). We had employed cholera toxin B subunit conjugated to FITC (CTX-B–FITC) to localize GM1, because the CTX-B subunit binds to GM1 with a stronger affinity than that for other gangliosides and is thus able to stabilize GM1-rich domains on the cell surface (Hagmann and Fishman, 1982; Parton, 1994; Harder et al., 1998). In our previous study, we postulated that GM1, which is enriched in caveolae, may, like caveolin-1, be subsequently incorporated into the mature virus filament. However, CTX-B–FITC-labelled virus filaments could not be clearly distinguished from the CTX-B–FITC bound on the cell surface, primarily because of the resolution limitations of light microscopy. To overcome this problem, we have employed FE SEM to analyze the surface of RSV-infected cells that have been labeled with cholera toxin B subunit conjugated to colloidal gold (BGOLD), a reagent that was previously employed in electron microscopic studies on neuronal transport (Llewellyn-Smith et al., 1990). In our study, RSV-infected cell monolayers were treated in a similar manner with either CTX-B–FITC or BGOLD and subsequently processed for examination by either confocal microscopy or FE SEM, respectively (Fig. 6).

Mock- and RSV-infected cells were labeled with MAb30 (red) and CTX-B–FITC (green) and examined by confocal microscopy (Fig. 6a). Mock-infected cells revealed a punctate staining pattern similar to that which we have previously observed (Brown et al., 2002b). This pattern is consistent with the presence of distinct regions on the cell surface that are enriched with GM1. In virus-infected cells, the images obtained using confocal microscopy clearly show colocalization (yellow) of the RSV antigen (G protein) and CTX-B–FITC, but we were unable to determine if the latter was able to bind to the RSV filaments. We noted that upon CTX-B treatment, the viral filaments became less clearly defined, and it is possible that this treatment results in some morphologic changes on the cell surface that may obscure the direct visualization of the filaments by light microscopy. It is known that a single CTX-B molecule is able to bind up to five GM1 molecules and that this binding ratio may result in the cross-linking of individual filaments within a cluster such that they may not be clearly distinguishable by light microscopy.

A closer examination of mock-infected cells by FE SEM showed the same distribution of distinct BGOLD clusters as that observed by light microscopy (compare Fig. 6a and b). Although the size of the BGOLD clusters, and hence the GM1 domains, were variable, they did not exceed 70 nm. Our estimates for the sizes of the GM1 domains in mock-infected cells are in agreement with estimates previously...
published using indirect methods such as fluorescence resonance energy transfer (Varma and Mayor, 1998; Kenworthy et al., 2000).

A detailed analysis of BGOLD-labeled RSV-infected cells clearly showed the presence of colloidal gold on the surface of RSV filaments, although at a lower level than that which we observed for the G protein (Fig. 6c, A–C). Although significant levels of GM1 are present in the viral envelope, the gold clusters appeared to be randomly distributed and did not appear to cover the viral envelope in its entirety. In addition, we noted that the BGOLD clusters within the viral filaments appeared to be generally smaller than those observed on the surface of mock-infected cells. Two possibilities exist to explain our observations. It is

Fig. 3. RSV filaments do not colocalize with markers for microvilli. (A and B) RSV-infected Vero C1008 cells were labeled with phalloidin–FITC (green) and MAb30 (red). Selected images from the same cells that represent the cell subsurface (A) or cell surface (B) were obtained from a Z-stack. Mock-infected (C) and RSV-infected (D) cells were labeled with phalloidin–FITC (green) and anti-ezrin (red). Colocalization of different antigens is shown by the yellow staining pattern. The virus filaments (VF) and microvilli (mv) are indicated. Enlarged views of the labelled cells are shown (inset). Magnification ×64.

Fig. 4. Distribution of the G protein on mature RSV filaments. RSV-infected cells were labeled with MAb30 and 10-nm colloidal gold as described. The RSV filaments were visualized using (A) the secondary electron (SE) and (B) back-scatter electron (BSE) detectors at ×100,000 magnification. The images obtained from identical regions of the cell surface were visualized as a merged image (C). In the merged image, the gold particles and filaments, recorded using the BSE and SE detectors, respectively, are superimposed. The predominant doublet pattern of labeling is highlighted (white circles) in an enlarged view of the superimposed image (inset).
possible that GM1 is a minor component and other host-cell-derived lipids comprise the bulk of the viral envelope. At present, the precise nature of the host-cell-derived lipids that form the virus envelope is unknown. Alternatively, it is possible that higher levels of BGOLD may be prevented from binding to the viral envelope because of the presence of the envelope-associated proteins. However, the presence of RSV-bound BGOLD provides the first direct evidence for the incorporation of GM1 into mature RSV filaments.

GM1 is a major component of specialized lipid raft systems referred to as caveolae. These raft-associated lipids are able to form highly ordered, membranous structures (Brown and London, 1998). It is possible that the presence of GM1 within the RSV envelope may have important consequences for its structural stability (Scheiffele et al., 1999). In addition, the presence of lipid raft platforms in the mature virus offers the possibility that host-cell-derived signaling complexes, which are associated with caveolae, may, in addition to the viral glycoproteins, be present within the viral envelope. However, the precise functional significance of the RSV-associated GM1 requires further investigation.

The sizes of the GM1 clusters observed in mock-infected cells (up to 70 nm) and our estimate of the sizes of virus egress sites (approx. 150–200 nm) differed. It is currently unclear whether RSV assembly occurs within a single or multiple GM1-enriched domains. However, we can speculate that the RSV assembly process may induce local clustering of several individual GM1-rich domains at the sites of egress. In this respect, reorganization of the actin cytoskeleton during RSV infection has been implicated (Gower et al., 2001; Brown et al., 2002b), and the role of actin reorganization in the clustering of lipid rafts has recently been demonstrated (Villalba et al., 2001; Valensin et al., 2002).

It is clear that RSV assembles predominantly in the filamentous form and that virus infectivity remains largely cell-associated throughout the replication cycle (Roberts et al., 1996). In addition, the filamentous form of the virus has been identified in biopsies, suggesting that this form of RSV is of clinical relevance and does not arise as a result of laboratory adaptation (Joncas et al., 1969). Thus, it is important to develop high-resolution techniques to study RSV assembly in the context of the infected cell, in particular its association with the cell surface platforms on which virus assembly occurs. In this report, we have used FE SEM to visualize the distribution of the virus attachment protein on the RSV filaments and to further characterize the assembly process. Although FE SEM has been used to map the surface distribution of several host-cell receptors (Soeda et al., 1998; Erlandsen et al., 2001), this is the first report in which this technology has been applied to mapping the surface distribution of a virus glycoprotein within a mature virus particle. The future use of FE SEM will provide us with a powerful technique for further characterization of the RSV assembly process at both the molecular and the cellular level.

Materials and methods

Cells and viruses

The RSV A2 strain was used throughout this study. Vero C1008 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies

The G protein monoclonal antibody MAb30 was provided by G. Taylor. The ezrin antibody was purchased from BD Transduction Laboratories.

Fig. 5 (continued)

Fig. 5. Interaction of RSV filaments with the cell surface. RSV-infected cells were labeled with MAb30 and analyzed by SEM. The base of mature RSV filaments was visualized using the SE (A) and BSE (B) detectors at ×50,000 magnification. The images obtained from identical regions of the cell surface were merged (C) to show the location of bound gold and RSV filaments. A smaller, immature filament viewed using the SE (D) and BSE (E) detectors at ×100,000 magnification. The area of contact between the RSV filament and the cell surface is highlighted (white arrow).
Western blotting was performed as described previously (Brown et al., 2002a). Briefly, the cell monolayers were extracted using 1% SDS, 15% glycerol, 1% β-mercapto-ethanol, 60 mM sodium phosphate, pH 6.8, and the proteins were separated by SDS–PAGE. The proteins were transferred by Western blotting onto a PVDF membrane, which was washed and blocked with 1% Marvel. The membrane was probed with MAb30 (1/1000 dilution) for 1 h after

Fig. 6. The raft lipid GM1 is incorporated into mature RSV filaments. (a) Mock- and RSV-infected cells were double-labeled with MAb30 (red) and CTX-B–FITC (green). In both cases, only the merged image is shown. Colocalization of MAb30 and CTX-B–FITC is indicated by the yellow staining pattern. (b and c) Mock- and RSV-infected cells were labeled with BGOLD and visualized by FE SEM (×110,000 magnification). (b) Mock-infected cells, labeled with BGOLD were visualized using the BSE detector. Gold clusters on the cell surface are highlighted (white arrows). (c). RSV-infected cells, labeled with BGOLD were visualized using the SE (A) and BSE (B) detectors. Clusters of bound BGOLD are highlighted (white arrowheads) in the BSE image. The location of the BGOLD on the RSV filaments is more clearly shown in the merged image (C) in which the SE and BSE images are superimposed. The RSV filaments (VF) and scale bar are indicated.
which it was subsequently washed and probed with goat anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma). The protein bands were visualized using the ECL protein detection system (Amersham). Apparent molecular masses were estimated using Rainbow protein markers (Amersham) in the molecular weight range 14.3–220 kDa.

Cell surface biotinylation

The cell surface biotinylation procedure has been described previously (Altin and Pagler, 1995). Briefly, cell monolayers were treated with 500 μg/ml sulfo-NHS-LC-biotin (Pierce) in PBS, pH 8.0, at 4°C, and the G protein was isolated by immunoprecipitation using MAb30 and separated by SDS–PAGE. The G protein was transferred by Western blotting onto a PVDF membrane and probed with streptavidin–HRP. The protein bands were detected using the ECL protein detection system (Amersham).

Immunofluorescence

Cells were seeded on 13-mm glass coverslips, either mock- or RSV-infected, and incubated overnight at 33°C, after which the cells were fixed with 3% paraformaldehyde in PBS for 30 min at 4°C. The fixative was removed and the cells washed five times with PBS + 1 mM glycine and once with PBS, after which they were permeabilized using 0.1% saponin in PBS. Following incubation at 25°C for 1 h with the primary antibody, the cells were washed and incubated for an additional 1 h with the secondary antibody, goat anti-mouse IgG (whole molecule) conjugated to Cy5 (1/100 dilution). The stained cells were mounted on slides using Citifluor and visualized using a Zeiss Axioplan 2 confocal microscope. The F-actin network was detected using phalloidin–FITC (Sigma) as described previously (Brown et al., 2002a). The GM1 microdomains were visualized using CTX-B–FITC. The CTX-B–FITC (Sigma) was prepared as a stock solution (1 mg/ml) in distilled, sterilized water. RSV-infected cells were incubated with 4 μg/ml CTX-B–FITC for 20 min at 4°C, washed with PBSA, and fixed with paraformaldehyde. The images obtained by confocal microscopy were processed using LSM 510 v2.01 software.

Field emission scanning electron microscopy

Cells were seeded on 10-mm glass coverslips and incubated overnight at 33°C. Following infection with RSV for 24 h, the samples were fixed with 0.25% glutaraldehyde in PBS at 4°C for 30 min. The fixed monolayers were incubated in PBS supplemented with 2 mM glycine for 20 min, after which they were washed extensively with PBS. The cells were incubated at 25°C for 4 h with MAb30, washed, and then incubated for an additional 4 h in goat anti-mouse IgG (whole molecule) conjugated to 10-nm colloidal gold (1/40 dilution). The monolayers were washed with PBS and fixed with 2.5% glutaraldehyde in PBS. GM1 microdomains were detected using CTX-B–BGOLD (List Biological Laboratories, Inc.). Cell monolayers were incubated with 4 μg/ml BGOLD in PBS as described above. The cells were washed and then fixed with 2.5% glutaraldehyde in PBS. In all cases, the monolayers were subsequently processed for SEM as described previously (Sugrue et al., 2001) and visualized in a Hitachi S4700II field emission scanning.
electron microscope using appropriate settings. Coverslips were rotary coated with 10 nm of carbon before examination at accelerating voltage 10 kV, working distance 8 nm, and beam current 15 μA. Image pairs were acquired simultaneously from the upper SE detector and the YAG BSE detector. Digital images were recorded using Hitachi FE PCSEM (ver 3.2) software and further processed using AnalySIS software.

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