Virology xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Cholesterol-rich lipid rafts are required for release of infectious human respiratory syncytial virus particles

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ARTICLE INFO

Article history:
Received 28 July 2011
Accepted 28 October 2011
Available online xxxx

Keywords:
Respiratory syncytial virus
Cholesterol
Lipid rafts
Virus release

ABSTRACT

Cholesterol and sphingolipid enriched lipid raft micro-domains in the plasma membrane play an important role in the life-cycle of numerous enveloped viruses. Although human respiratory syncytial virus (RSV) proteins associate with the raft domains of infected cells and rafts are incorporated in RSV virion particles, the functional role of raft during RSV infection was unknown. In the current study we have identified rafts as an essential component of host cell that is required for RSV infection. Treatment of human lung epithelial cells with raft disrupting agent methyl-beta-cyclodextrin (MBCD) led to drastic loss of RSV infectivity due to diminished release of infectious progeny RSV virion particles from raft disrupted cells. RSV infection of raft deficient Niemann–Pick syndrome type C human fibroblasts and normal human embryonic lung fibroblasts revealed that during productive RSV infection, raft is required for release of infectious RSV particles.

Introduction

Paramyxoviruses are enveloped viruses containing a non-segmented negative strand single-stranded RNA genome. Paramyxoviruses are important human pathogens known to cause many diseases. All paramyxoviruses enter cells via direct fusion of the viral lipid-envelope with the plasma membrane (non-endocytic pathway) and viral assembly/budding occurs in the host plasma membrane. Human respiratory syncytial virus (RSV) is a lung-tropic paramyxovirus that causes high morbidity and mortality among infants, children, and the elderly (Collins et al., 2007; Hall, 2001; Hippenstiel et al., 2006). RSV infects the airway to cause respiratory diseases like pneumonia and bronchiolitis.

Paramyxoviruses like RSV utilize a wide spectrum of cellular proteins during its life cycle. Plasma membrane assembly and release (budding) of paramyxoviruses is an essential component of viral life cycle. Several cellular proteins were shown to assist this stage of viral replication (Gower et al., 2005; Ravid et al., 2010; Utley et al., 2008). Lipid raft micro-domains in the plasma membrane are enriched with cholesterol and sphingolipids (Pike, 2003; Silvius, 2003). Due to the presence of cholesterol, the raft domain membrane possesses an "ordered" rigid structure with limited "fluidity" compared to the surrounding plasma membrane. Lipid rafts serve as platforms for plasma membrane assembly and budding of enveloped viruses like influenza A virus (Leser and Lamb, 2005; Scheiffele et al., 1999; Takeda et al., 2003; Xiangjie and Whittaker, 2003), Sendai virus (Ali and Nayak, 2000), measles virus (Ayota et al., 2004; Manié et al., 2000; Robinzon et al., 2009; Vincent et al., 2000), and Newcastle disease virus (NDV) (Dolganiuc et al., 2003; Laliberte et al., 2006; Laliberte et al., 2007). In addition, several enveloped viruses (e.g. Borna disease virus, Ebola virus, Marburg virus) require intact cell-surface lipid rafts for efficient cellular entry (Bavari et al., 2002; Clemente et al., 2009). Although several studies have reported targeting of paramyxovirus proteins to rafts (Ali and Nayak, 2000; Ayota et al., 2004; Dolganiuc et al., 2003; Laliberte et al., 2006; Laliberte et al., 2007; Manié et al., 2000; Robinzon et al., 2009; Vincent et al., 2000), only a few have demonstrated the functional requirement of cell surface rafts for paramyxovirus infection. Assembly of NDV (an avian paramyxovirus) proteins occurs in raft domains and raft is required for release of infectious NDV particles (Dolganiuc et al., 2003; Laliberte et al., 2006; Laliberte et al., 2007). Parainfluenza virus 5 (a paramyxovirus that was formerly known as simian virus 5 or SV5) also requires caveolin (a protein component of caveolae which similarly to lipid rafts is enriched with cholesterol and sphingolipids) for assembly/budding (Ravid, et al., 2010). Several studies reported that — a) RSV proteins (envelope proteins, matrix protein, polymerase proteins) are localized in raft domains (Brown et al., 2002, 2004; Fleming et al., 2006; Marty et al., 2004; McDonald et al., 2004; Oomens et al., 2006), and b) purified RSV virion particles contain raft associated cellular proteins due to incorporation of rafts in the virion envelope during budding process (Brown et al., 2004; Marty et al., 2004; Yeo et al., 2009). Although

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doi:10.1016/j.virol.2011.10.029

these studies have suggested raft's involvement during RSV infection, the exact role of rafts during infection is not known. Moreover, the role of raft during RSV infection of human lung epithelial cells (the cells productively infected by RSV during infection of the respiratory tract) has not been investigated since previous studies were primarily conducted in HEp-2 (a human laryngeal cell-line contaminated with Hela, a human cervical cell-line) and Vero (African Green monkey kidney cells) cells (Brown et al., 2002, 2004; Fleming et al., 2006; Marty et al., 2004; McDonald et al., 2004; Oomens et al., 2006; Yeo et al., 2009).

In the current study we have examined the role of rafts during RSV infection of human lung epithelial cells and human fibroblasts lacking cell-surface raft domains. Our studies revealed that rafts play a role during RSV life-cycle, since they are critical for release of infectious progeny virion particles from infected cells. Disruption of rafts (by the raft disrupting agent methyl-beta-cyclodextrin or MBCD) in human lung epithelial cells resulted in drastic reduction in RSV infection. Raft disruption during infection culminated in release of progeny virion particles with significantly reduced infectivity. Our studies with lung epithelial cells were further validated by utilizing Niemann–Pick syndrome type C human fibroblasts (NPC) [these cells lack normal rafts as a result of defective cholesterol trafficking to the plasma membrane (Ikonen and Holtta-Vuori, 2004; Koike et al., 1998; Laliberte et al., 2007)] and normal human embryonic lung (HEL) fibroblasts (wild-type counterpart of NPC cells) (Laliberte et al., 2007). Our studies with these cells revealed significant reduction in RSV infectivity in NPC cells compared to HEL cells. Further characterization demonstrated that reduced infectivity is due to failure of NPC cells to efficiently release infectious RSV virion particles. Thus, our studies have demonstrated that — a) intact plasma membrane rafts are required for RSV infection, and b) cell surface rafts are critical for release of infectious progeny RSV particles.

Results

Effect of raft disruption on RSV infectivity

Methyl-beta-cyclodextrin (MBCD), a cholesterol-extracting agent has been extensively utilized to disrupt lipid rafts in the plasma membrane (Allsopp et al., 2010; Laliberte et al., 2006, 2007; Medigeshi et al., 2008; Xiangjie and Whittaker, 2003; Xu et al., 2009). In order to study the role of rafts during RSV infection, we treated human lung epithelial A549 cells with MBCD. A549 cells are airway cells that are routinely used as a model of type II alveolar epithelial cells. After 1.5 h adsorption (at 37 °C) of A549 cells with RSV (0.2 MOI), cells were washed and fresh medium was added in the presence of 5 mM of MBCD. After 1 h incubation with MBCD after virus adsorption, fresh medium containing lovastatin (4 μg/ml) was added. The rationale for adding lovastatin is to inhibit cholesterol bio-synthesis so that cell surface devoid of cholesterol (i.e. loss of cholesterol-rich lipid rafts) is maintained during the course of infection. After 24 h post-infection, medium supernatant was collected to determine viral titer by plaque assay analysis. Treatment of A549 cells with MBCD resulted in significant decrease in RSV infectivity (inhibited by 85%–90%) compared to untreated cells (Figs. 1A and B). The effect of MBCD is specific for RSV, since similar MBCD treatment of A549 cells did not alter Vesicular Stomatitis Virus (VSV) infection (Fig. 1C).

We next evaluated the requirement of raft during RSV infection of primary normal human bronchial epithelial (NHBE) cells. Similar to A549 cells, NHBE cells were incubated with RSV (0.2 MOI) for 1.5 h. After adsorption, fresh medium containing 5 mM MBCD was added to washed cells. Following 1 h MBCD treatment, cells were washed and incubated with lovastatin. At 48 h post-infection, medium supernatant was collected to determine RSV titer by plaque assay analysis. Cell surface rafts are also required for RSV infection of NHBE cells,

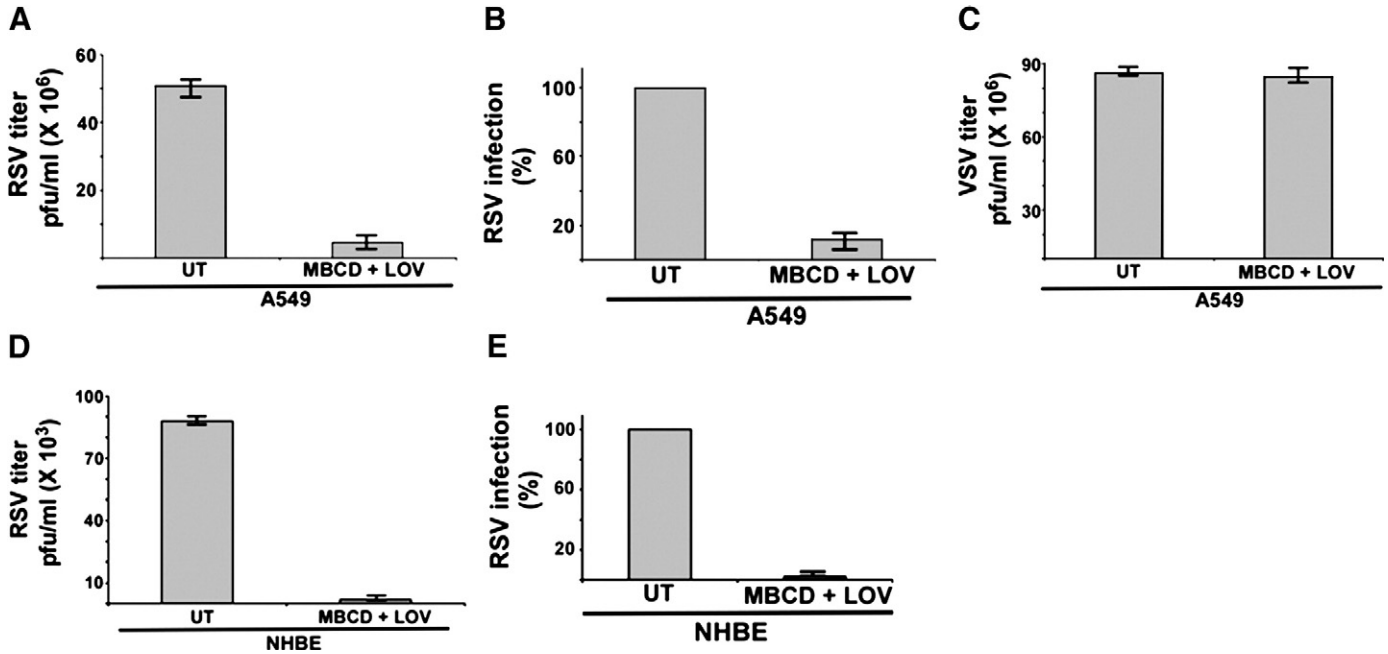

Fig. 1. Plasma membrane rafts are required for RSV infection. (A) RSV infection of untreated (UT) and methyl-beta-cyclodextrin + lovastatin (MBCD + LOV) treated A549 cells. Viral titer was determined at 24 h post-infection by plaque assay. (B) The viral titer values (pfu/ml) shown in A were utilized to calculate percent (%) infection. % infection was calculated based on the ratio of pfu/ml obtained from UT cells vs. MBCD + LOV treated A549 cells. 100% infectivity corresponds to viral titer in UT cells. % infection value represents the mean ± standard deviation for three determinations. (C) VSV infection of UT and MBCD + LOV treated A549 cells. Viral titer was determined at 24 h post-infection by plaque assay. (D) RSV infection of UT and MBCD + LOV treated primary normal human bronchial epithelial (NHBE) cells. Viral titer was determined at 48 h post-infection by plaque assay. (E) The viral titer values (pfu/ml) shown in D were utilized to calculate percent (%) infection. % infection was calculated based on the ratio of pfu/ml obtained from UT cells vs. MBCD + LOV treated NHBE cells. 100% infectivity corresponds to viral titer in UT cells. % infection value represents the mean ± standard deviation for three determinations. Plaque assay values shown in A, C and D are expressed as pfu/ml and it represents mean ± standard deviations for three independent determinations. Standard deviations are shown by the error bars.

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since drastic reduction (by 95%) in viral infectivity was observed in MBCD treated cells, compared to control (MBCD untreated) cells (Figs. 1D and E). The experimental procedure for the above mentioned studies are presented schematically in supplemental Figs. S1A and S1B.

Previously 5 mM–10 mM MBCD was used to disrupt cell surface or virus associated raft domains (Allsopp et al., 2010; Laliberte et al., 2006, 2007; Medigeshi et al., 2008; Xiangjie and Whittaker, 2003; Xu et al., 2009). In order to examine raft status in A549 cells, cells (untreated and MBCD treated) were incubated with FITC conjugated cholera-toxin subunit-B (CHTX), which specifically binds to the cell surface raft resident ganglioside GM1 (Harder et al., 1998). Based on the GM1 binding property of CHTX, it has been widely used to visualize lipid-rafts on the cell surface (Harder et al., 1998). A549 cells were incubated with 5 mM MBCD for 1 h. The cells were then washed and fresh medium containing lovastatin (4 μg/ml) was added. At 16 h post-treatment with lovastatin, cells were incubated with FITC-CHTX. FITC-CHTX incubated cells were fixed and visualized by confocal microscopy. As shown in Fig. 2A, while a cell surface ring representing the plasma membrane rafts were prominent in untreated cells, MBCD treatment led to disappearance of the raft structures. Interestingly, MBCD treatment led to intracellular clustering of GM1. The ability of MBCD to reduce cholesterol levels in A549 cells was also investigated. A549 cells were treated with MBCD and lovastatin as described above for FITC-CHTX labeling studies. The loss of cellular cholesterol levels following MBCD treatment was also evident from presence of significantly less cholesterol in MBCD treated A549 cells compared to untreated cells (Fig. 2B) (Table 1). Similar to A59 cells, we also observed loss of cell surface rafts and significant reduction in cellular cholesterol levels in NHBE cells treated with 5 mM MBCD for 1 h (data not shown). Treatment of A549 and NHBE cells with 5 mM MBCD (for 1 h) and lovastatin (for 24 h–48 h) was not toxic during the time-frame of our experiment as deduced by trypan blue exclusion viability assay (data not shown). Please note that similar toxicity assays were performed for all the experiments described in Fig. 1. Our studies have demonstrated that rafts are critical for RSV infection of both human alveolar epithelial (A549 cells) cells and primary human bronchial epithelial (NHBE cells) cells.

Intact rafts are critical for release of infectious RSV particles

Rafts may play an important role during post-entry stages of RSV life-cycle, since addition of MBCD following adsorption inhibited virus infection (Fig. 1). We speculated that rafts may be required for RSV release/budding because assembly/budding of numerous enveloped viruses occurs in the raft domains of the plasma membrane. Moreover, RSV proteins accumulate in the raft domain of infected cells. In order to examine this possibility, we performed an infectious virus release/budding assay as described previously (Bose et al., 2001). A549 cells were infected with RSV (1 MOI) for 8 h and then treated with MBCD (5 mM for 1 h). After washing, fresh medium was added in the presence of lovastatin (4 μg/ml) and cycloheximide (30 μg/ml cycloheximide was added to accumulate an intracellular viral protein pool in the absence of de novo protein synthesis.). Following 3 h incubation, cells were washed 3× and once again fresh medium (± lovastatin, but with no cycloheximide) was added to the cells to examine the release/budding of the accumulated viral proteins as virion particle in the presence of intact (cells not incubated with MBCD and lovastatin) and disrupted (MBCD and lovastatin treated cells) rafts. After 12 h the medium supernatants were assessed for viral titer by plaque assay. The budding assay revealed that loss of rafts resulted in diminished release of infectious RSV by 80%–85% (Figs. 3A and B). The experimental procedure for the studies described in Fig. 3 is presented schematically in supplemental Fig. S2.

Inhibition of RSV release from raft disrupted cells could be due to several reasons — a) abnormal assembly of viral proteins occurs in raft disrupted cells, resulting in budding blockade; in this case progeny virion particles will not be present in the medium supernatant, b) normal assembly/budding occurs from raft disintegrated cells, but the progeny virion particles are defective and non-infectious probably due to lack of viral proteins (especially envelope proteins that are required for cellular entry), c) progeny virion particles possess viral proteins, but the particle is non-infectious since it is devoid of virion envelope-associated rafts, which is required for initiating subsequent infection.

In order to examine these possibilities, we studied viral protein composition of purified virions obtained from the medium supernatants of infected cells. These studies were performed essentially similar to the budding assay (± MBCD as described above for Fig. 3), but the cells were labeled with ^{35}S-methionine and the experiment was performed in a larger scale (to recover larger amount of purified virus for analysis). The radioactive medium supernatant obtained from untreated and MBCD treated cells was utilized to purify ^{35}S-methionine labeled RSV (^{35}S-RSV) particles. Purified ^{35}S-RSV was then subjected to SDS-PAGE and autoradiography. As shown in Fig. 4A, disruption of rafts did not result in inhibition of assembly/budding of virion particles, since virions derived from both untreated and MBCD treated cells comprised similar levels of viral proteins. Protein assay analysis also did not demonstrate any significant difference in total protein content of purified

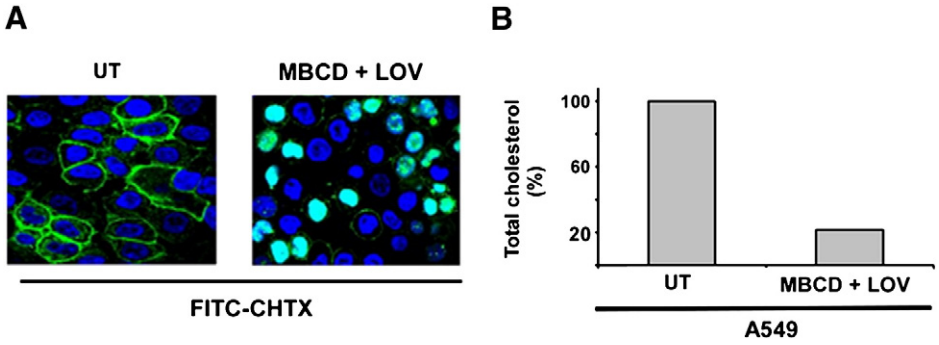

Fig. 2. Effect of methyl-beta-cyclodextrin (MBCD) on raft structure and cholesterol levels. (A) Fluorescence confocal microscopic analysis of A549 cells labeled with FITC-cholera toxin (CHTX) subunit (green) and DAPI (blue, nucleus) following treatment with MBCD + lovastatin (LOV) (cells incubated with 5 mM MBCD for 1 h, followed by addition of lovastatin containing fresh medium for additional 16 h). (B) Untreated (UT) and MBCD + LOV (cells incubated with 5 mM MBCD for 1 h, followed by addition of lovastatin containing fresh medium for additional 16 h) treated A549 cells were used to estimate total cholesterol content by cholesterol quantitation assay kit. Following normalization with protein concentration, the total cholesterol content was expressed as percentage of control. The control cells are UT cells and are represented as 100%. Please see Table 1 for the mean ± standard deviation values.

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Table 1
Cholesterol estimation of A549 cells and purified RSV virion particles.

	[a]Cells ± MBCD + LOV treatment (nmol/mg cellular protein)	[b]RSV purified from medium supernatant ± MBCD + LOV treatment (nmol/mg viral protein)
Untreated	280.6 ± 46.6	262.3 ± 43.3
MBCD + LOV treated	64.7 ± 10.4	88.1 ± 12.8

The results are means ± standard deviations.
[a] Total cholesterol was measured in A549 cells incubated with 5 mM methyl-beta-cyclodextrin (MBCD) for 1 h, followed by addition of fresh medium containing lovastatin (LOV). Cholesterol was measured at 16 h following LOV addition. Untreated cells were not treated with MBCD and LOV.
[b] A549 cells were infected with RSV for 8 h. At 8 h post-infection, cells were incubated with MBCD (5 mM) for 1 h, followed by addition of fresh medium containing LOV. After 16 h, RSV was purified from the medium supernatant. Untreated; cholesterol content of RSV virion particle purified from the medium supernatant of infected cells not treated with MBCD and LOV.

virions obtained from untreated cells vs. MBCD treated cells (data not shown). The experimental procedure for the studies described in Fig. 4A is presented schematically in supplemental Fig. S3.

Although the progeny virions recovered from the medium of untreated and MBCD treated cells had similar protein composition, we examined the cholesterol content of the purified virion particles. As described above, medium supernatant from untreated and MBCD treated cells were utilized to purify RSV particles. The total cholesterol content of these purified virion particles was assayed. The cholesterol content of purified RSV particles obtained from MBCD treated cells was significantly less compared to particles obtained from untreated cells (Fig. 4B) (Table 1). These results suggested that cell surface raft serves as a platform for release of infectious RSV virion particles from infected cells. Moreover, raft is required for enrichment of cholesterol in the RSV virion particles.

RSV infection of cells deficient in plasma membrane cholesterol

In the above studies we have utilized MBCD to disrupt raft microdomains. In order to provide further evidence for the role of rafts during RSV infection, we utilized Niemann–Pick syndrome type C human fibroblasts (NPC cells). NPC cells are human fibroblasts that harbor mutations in either NPC1 and/or NPC2 genes (Ikonen and Holtta-Vuori, 2004; Koike et al., 1998; Laliberte et al., 2007). Due to these mutations, cholesterol trafficking to the plasma membrane in these cells is defective and as a consequence these cells do not possess functional plasma membrane lipid rafts. These cells have been widely used to study the role of rafts in various cellular processes (Henderson et al., 2000), including its role during virus (e.g. NDV, HIV-1) infection (Laliberte et al., 2007; Tang et al., 2009).

RSV infection of NPC and HEL [normal human embryonic lung (HEL) fibroblasts were used as the wild-type counterpart to compare with mutant NPC cells (Laliberte et al., 2007; Tang et al., 2009)] cells revealed significantly diminished cytopathic effect of RSV upon infection of NPC cells compared to HEL cells (Fig. 5A). The observed cytopathic effect in HEL could be due to enhanced syncytia in HEL cells compared to NPC cells. Since syncytia occur due to cell–cell fusion mediated by RSV fusion protein, it reflects the infection efficiency. Thus, enhanced cytopathic effect (due to syncytia) observed in HEL cells suggested enhanced infectivity of these cells compared to NPC cells. In order to determine whether reduced cytopathic effect in cholesterol deficient NPC cells was due to reduced RSV infectivity, we infected HEL and NPC cells with RSV (0.5 MOI). At 36 h post-infection, medium supernatant was collected to determine RSV titer by plaque assay analysis. Indeed, RSV infection was drastically reduced (by 90%) in NPC cells compared to HEL cells (Figs. 5B and C). In contrast, no significant difference in infectivity was observed following infection of HEL and NPC cells with VSV (Fig. 5D). The experimental procedure for the studies described in Fig. 5B is presented schematically in supplemental Fig. S4A.

We speculated that reduced RSV infectivity in NPC cells could be as a consequence of defective release of infectious RSV particles, since rafts play an essential role during this process (Fig. 3). Therefore, we performed virus release/budding assay as described above for A549 cells. HEL and NPL cells were infected with RSV (1 MOI). At 10 h post-infection, cells were washed and the medium was replaced with fresh medium containing cycloheximide (30 μg/ml) to accumulate an intracellular viral protein pool in the absence of de novo protein synthesis. Following 3 h incubation with cycloheximide, cells were washed 3× and once again fresh medium (in the absence of cycloheximide) was added to the cells to promote release/budding of the accumulated viral proteins as virion particles. After 12 h, the medium supernatants were assessed for viral titer by plaque assay. Similar to lung epithelial cells, we also observed defective release of

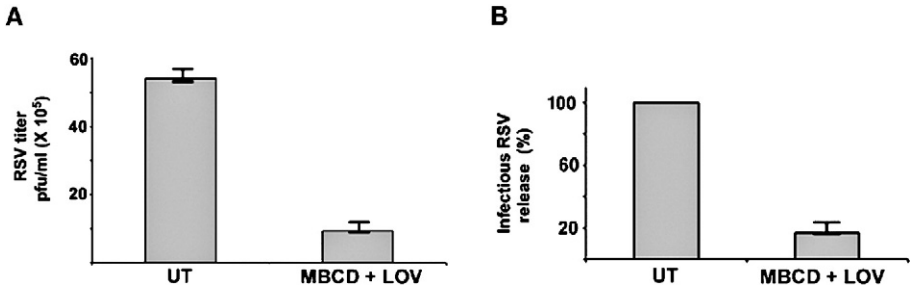

Fig. 3. Rafts are essential for release of infectious RSV particle. (A) Untreated (UT) and methyl-beta-cyclodextrin + lovastatin (MBCD + LOV) treated A549 cells were infected with RSV in the presence of cycloheximide as detailed in the supplemental Fig. S2 and in the Materials and methods section. Infectious RSV release efficiency was determined by utilizing medium supernatant to assess infectious viral titer by plaque assay. Plaque assay values are expressed as pfu/ml and it represents mean ± standard deviations for three independent determinations. Standard deviations are shown by the error bars. (B) The plaque assay values (pfu/ml) shown in A were utilized to calculate percent (%) infectious RSV release. % infectious RSV release was calculated based on the ratio of pfu/ml obtained from UT cells vs. MBCD + LOV treated cells. 100% infectious RSV release corresponds to viral titer in UT cells. % infectious RSV release value represents the mean ± standard deviation for three determinations.

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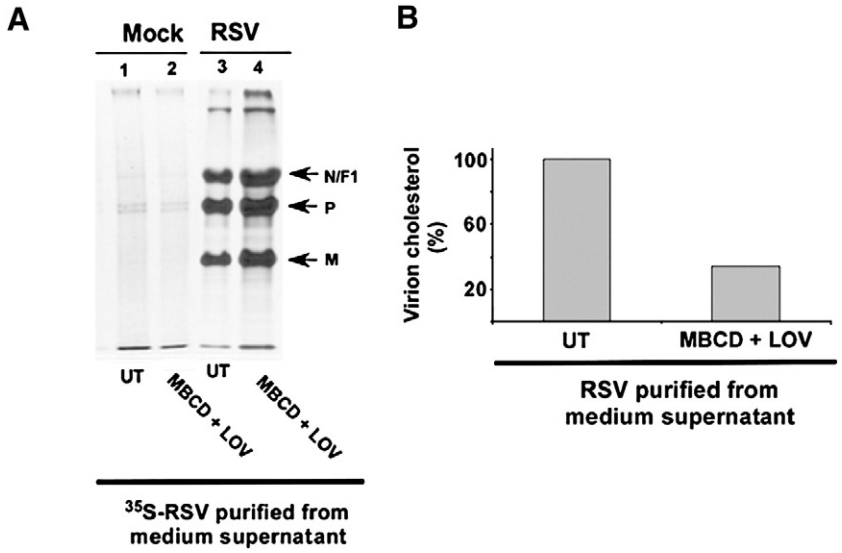

Fig. 4. Effect of raft disruption on RSV budding and cholesterol content of progeny RSV virion particles. (A) Untreated (UT) and methyl-beta-cyclodextrin + lovastatin (MBCD + LOV) treated A549 cells were infected with RSV as detailed in the figure and in the Materials and methods section. After infection, cells were labeled with ^{35}S-methionine to promote budding of radiolabeled RSV particles. Radiolabeled RSV was purified from the medium supernatant of UT and MBCD treated cells. Purified ^{35}S-methionine-RSV was subject to SDS-PAGE and autoradiography to visualize radiolabeled viral proteins. (B) UT and MBCD + LOV treated A549 cells were infected with RSV as detailed in the Materials and methods section. RSV was purified from the medium supernatant of UT and MBCD + LOV treated cells. Purified RSV was utilized to estimate cholesterol content by cholesterol quantitation assay kit. Following normalization with protein concentration, the total cholesterol content was expressed as percentage of control virus. The control virus is virion particles obtained from UT cells and is represented as 100%. Please see Table 1 for the mean ± standard deviation values.

infectious RSV particle from cholesterol lacking NPC cells compared to normal HEL cells (Figs. 6A and B), although viral protein levels in the purified virions derived from NPC and HEL cells were similar (data not shown). Moreover, cholesterol content of NPC cell derived virion particles was significantly less compared to purified virus obtained from HEL cells (data not shown). The experimental procedure for the studies described in Fig. 6 is presented schematically in supplemental Fig. S4B. Thus, our studies have demonstrated that plasma membrane cholesterol rich raft domain is required for RSV infection, by virtue of its role in release of infectious progeny RSV particles from infected cells.

Discussion

Plasma membrane resident cholesterol and sphingolipid enriched lipid rafts play an important role in cellular entry and assembly/morphogenesis of both segmented and non-segmented negative-sense ssRNA viruses (Ali and Nayak, 2000; Ayota et al., 2004; Bavari et al., 2002; Clemente et al., 2009; Dolganiuc et al., 2003; Laliberte et al., 2006, 2007; Leser and Lamb, 2005; Manié et al., 2000; Robinzon et al., 2009; Scheiffele et al., 1999; Takeda et al., 2003; Vincent et al., 2000; Xiangjie and Whittaker, 2003). Proteins of several paramyxoviruses (non-segmented (−)ssRNA viruses) are also targeted to the raft domains of infected cells (e.g. Sendai virus, RSV, measles) (Ali and Nayak, 2000; Brown et al., 2002, 2004; Fleming et al., 2006; Manié et al., 2000; Marty et al., 2004; McDonald et al., 2004; Oomens et al., 2006). Few studies have reported an essential function of rafts during paramyxovirus infection. Rafts play a role during NDV (an avian paramyxovirus) infection (Dolganiuc et al., 2003; Laliberte et al., 2006, 2007) since it is required for release of infectious NDV particles. Budding of parainfluenza 5 virus requires caveolin, a component of raft-like structure known as caveolae (Ravid et al., 2010). In the current study we demonstrated that rafts play an important role during RSV infection. Specifically, raft is required for release of infectious progeny RSV particles. Our studies revealed that raft (and cholesterol) is not required for RSV budding from infected cells. However, the infectiousness of released raft (and cholesterol) deficient RSV particles is severely compromised.

RSV is a lung-tropic virus that causes severe respiratory diseases during infancy, childhood, old age. The high morbidity and mortality associated with RSV infection is due to its ability to cause respiratory diseases like pneumonia and bronchiolitis (Collins et al., 2007; Hall, 2001; Hippenstiel et al., 2006). Several studies have suggested that rafts may play an important role during RSV life-cycle — a) RSV proteins associate with plasma membrane rafts during assembly, b) raft associated proteins were observed in purified RSV virion particles, and c) filamentous virion particle formation requires intact raft structure (Brown et al., 2002, 2004; Fleming et al., 2006; Marty et al., 2004; McDonald et al., 2004; Oomens et al., 2006; Yeo et al., 2009). The cholesterol lowering drug lovastatin also diminished RSV infection of HEp2 cells (the human cervical carcinoma cell-line) (Gower and Graham, 2001). However, the direct role of cholesterol during RSV infection was not evaluated in these cells using a cholesterol-specific drug. Moreover, the role of cholesterol rich raft during RSV life-cycle was unknown. In the current study we have illustrated that rafts are required for release of infectious progeny RSV virion particle.

Lipid raft domains are localized on the exoplasmic side of the plasma membrane and are enriched with cholesterol and sphingolipids (Pike, 2003; Silvius, 2003). The raft domain forms a localized rigid (less fluid) environment due to the clustering of cholesterol molecules and the saturated fatty acyl chains of the sphingolipids. Various proteins (e.g. GPI-anchored CD59 protein, caveolin-1 etc) have high affinity for lipid rafts and therefore, they partition themselves in the raft domains. Such partitioning may occur during intracellular trafficking, whereby these proteins segregate in the lipid rafts of vesicles destined to deliver their cargo to the plasma membrane. Plasma membrane rafts have multiple functions during normal cellular processes. They play an important role during intracellular trafficking/targeting originating from the cell surface and are required for cell-to-cell adhesion. Apart from these functions, rafts act as a cell surface platform for initiating key signal transduction pathways (Ning et al., 2006; Simons and Toomre, 2000). The rigid (less fluid) microenvironment in the plasma membrane provided by the cholesterol

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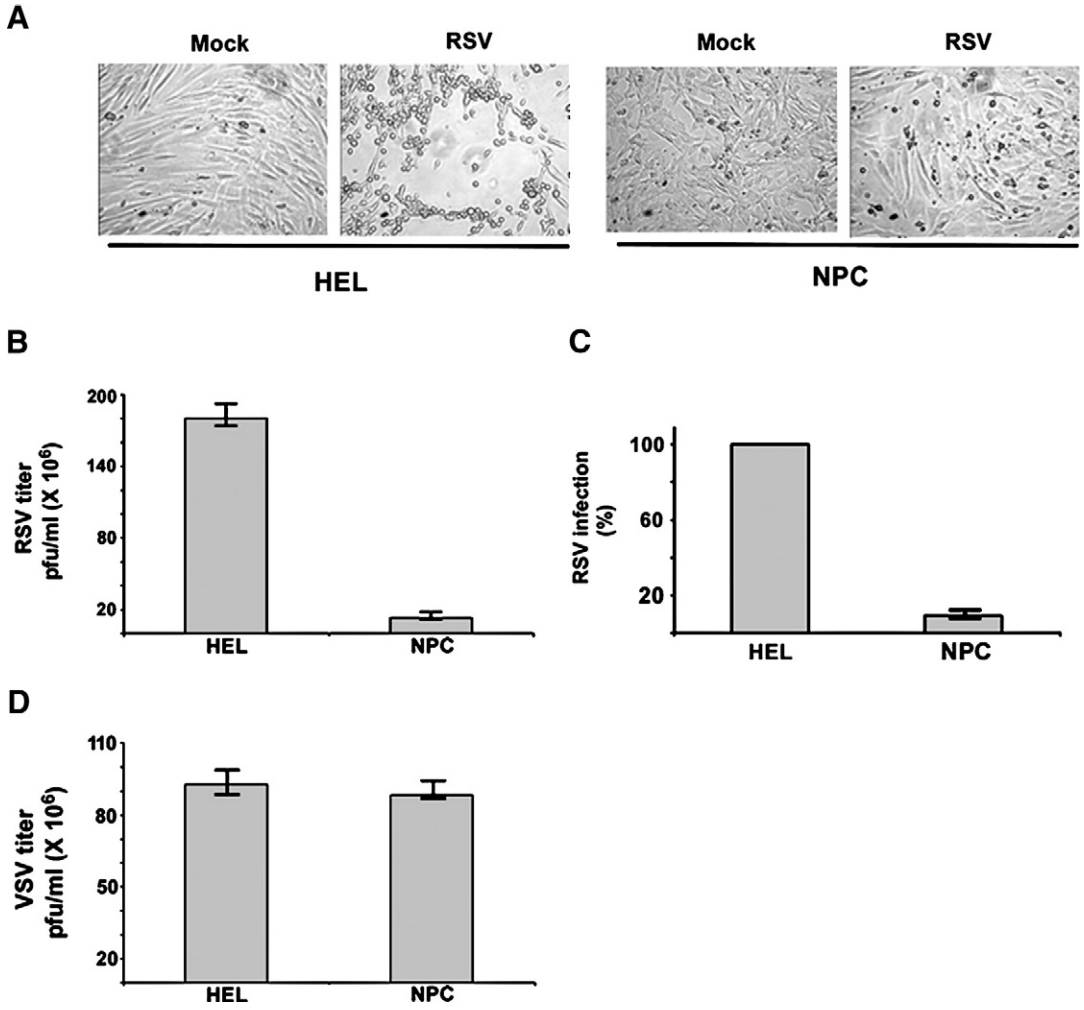

Fig. 5. RSV infection of cholesterol deficient human fibroblasts — (A) morphology of mock-infected or RSV-infected (36 h post-infection) cholesterol-deficient Niemann–Pick syndrome type C human fibroblasts (NPC cells) and normal human embryonic lung (HEL cells) fibroblasts (wild-type counterpart of NPC cells). (B) RSV infection of NPC and HEL cells. Viral titer was determined at 36 h post-infection by plaque assay. (C) The viral titer values (pfu/ml) shown in B was utilized to calculate percent (%) RSV infection. % RSV infection was calculated based on the ratio of pfu/ml obtained from HEL cells vs. NPC cells. 100% infectivity corresponds to viral titer in HEL cells. % infection value represents the mean ± standard deviation for three determinations. (D) VSV infection of NPC and HEL cells. Viral titer was determined at 36 h post-infection by plaque assay. Plaque assay values shown in B and D are expressed as pfu/ml and these represent mean ± standard deviations for three independent determinations. Standard deviations are shown by the error bars.

and sphingolipids (key components of rafts) facilitates homotypic and heterotypic interaction between plasma membrane associated proteins/receptors. These interactions may only occur in the raft "micro-domains" since rigid (less fluid) environment provides limited flexibility to raft resident proteins which promotes their functional interactions.

Our study demonstrated that intact cell surface rafts are essential for release of progeny infectious RSV particles from infected cells. In context to enveloped viruses it is speculated that intracellular-trafficking/seclusion of viral membrane associated proteins (e.g. RSV envelope proteins F and G and matrix protein M associates with

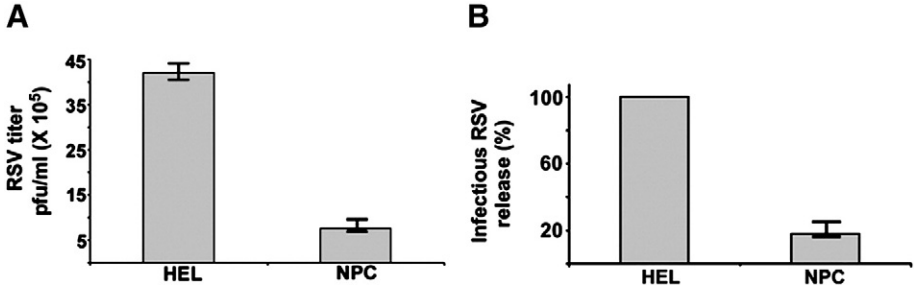

Fig. 6. Release of infectious RSV particle is disrupted in cholesterol deficient human fibroblasts. (A) HEL and NPC cells were infected with RSV in the presence of cycloheximide as detailed in the supplemental Fig. S4B and in the Materials and methods section. Infectious RSV release efficiency was determined by utilizing medium supernatant to assess infectious viral titer by plaque assay. Plaque assay values are expressed as pfu/ml and these represent mean ± standard deviations for three independent determinations. Standard deviations are shown by the error bars. (B) The plaque assay values (pfu/ml) shown in A were utilized to calculate percent (%) infectious RSV release from NPC and HEL cells. % infectious RSV release was calculated based on the ratio of pfu/ml obtained from HEL cells vs. NPC cells. 100% infectious RSV release corresponds to viral titer in HEL cells. % infectious RSV release value represents the mean ± standard deviation for three determinations.

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membrane) in the rafts results in utilization of rafts as the platform for assembly and budding. In addition, cellular proteins involved in viral release (Harty et al., 2001; Irie et al., 2004; Okumura et al., 2008; Usami et al., 2009) may also localize in rafts to facilitate assembly/budding of progeny virion particles. For example, Rho A (Gower et al., 2001, 2005; Pastey et al., 2000) and FIP2 (Rab11 family interacting protein 2) (Utley et al., 2008) have been shown to play an important role during RSV budding. Rho A activation by RSV is required for filamentous virus formation during morphogenesis (Gower et al., 2005). However, active Rho A is dispensable for RSV infection (Gower et al., 2005). Interestingly, both Rho A and FIP2 have been implicated in functioning via raft domain of plasma membrane (Chu et al., 2009; Lacalle et al., 2002). One could envision that raft disruption leads to RSV budding defect due to lack of functional scaffolding of host proteins like FIP2. However, it is a highly unlikely scenario, since virus budding was preserved following plasma membrane raft disruption (Fig. 4A) as deduced from the viral protein content of the purified virion particles. In contrary, our study suggested that release of infectious RSV particle was compromised following raft disruption. In that context, we also observed reduced virion-associated cholesterol levels in RSV particles released from raft disrupted cells (Fig. 4B, Table. 1). In the future, we will investigate the role of virion associated cholesterol (and rafts) in the RSV infection process.

In summary, our study has uncovered a critical role of rafts during RSV infection. Rafts are required for release of infectious RSV virion particles, since intact raft domains are necessary for "loading" cholesterol into the RSV virion particle.

Conclusions

Plasma membrane cholesterol-rich lipid rafts play a critical role during RSV infection, since raft domains are required for release of infectious progeny RSV virion particles.

Materials and methods

Virus and cells

RSV (A2 strain) was propagated in CV-1 cells (Kota et al., 2008; Sabbah et al., 2009). VSV (Indiana serotype, Mudd–Summers strain) was propagated in BHK-21 cells (Basu et al., 2006; Bose et al., 2003). RSV and VSV were purified by centrifugation on discontinuous sucrose gradients as described previously (Ueba, 1978). Human lung epithelial A549 cells and normal primary human bronchial epithelial cells (NHBE cells were purchased from Lonza) were maintained in DMEM (supplemented with 10% fetal calf serum or FCS, penicillin, streptomycin, and glutamine) and Bronchial Epithelial Cell Basal Medium (containing BPE, Hydrocortisone, hEGF, Epinephrine, Transferrin, Insulin, Retinoic Acid, Triiodothyronine, GA-1000) (Clonetics/Lonza), respectively. Normal human embryonic lung (HEL) fibroblasts (ATCC) and Niemann–Pick syndrome type C human (NPC) fibroblasts (NIH-Coriell Cell Repository) were maintained in DMEM (supplemented with 10% FCS, non-essential amino acids, glutamine, vitamins). RSV titer was monitored by plaque assay analysis with CV-1 cells as described earlier (Kota et al., 2008; Sabbah et al., 2009).

Virus infection

To study the role of rafts during RSV infection, A549 and NHBE cells were treated with cholesterol disrupting drug methyl-beta-cyclodextrin (MBCD) (Sigma-Aldrich). Cells were treated with MBCD after virus adsorption stage. RSV (0.2 MOI) or VSV (0.1 MOI) was added to cells (A549 and NHBE) for 1.5 h (adsorption stage). Following adsorption, the cells were washed and fresh medium containing 5 mM MBCD was added. After 1 h MBCD incubation, cells were washed and once again fresh medium was added in the presence (for MBCD treated cells) or absence (control cells) of lovastatin (Sigma-Aldrich) (4 μg/ml). Medium supernatant was collected at either 24 h (for A549) or 48 h (for NHBE) post infection to assess viral titer by plaque assay.

HEL and NPC cells were also infected with RSV (0.5 MOI) or VSV (0.5 MOI). At 36 h post-infection, medium supernatant was utilized to assess viral titer by plaque assay. In addition, light microscope was utilized to visualize cell morphology and cytopathic effect in RSV infected HEL and NPC cells.

Virus release assay

Virus release or budding assay was essentially performed as described previously (Bose et al., 2001). In order to investigate the effect of raft disruption (i.e. MBCD treatment) on RSV release, A549 cells were infected with RSV (1 MOI). At 8 h post-infection, fresh medium containing MBCD (5 mM) was added to washed cells and the cells were incubated with MBCD for 1 h at 37 °C. After 1 h, cells were washed and fresh medium was added in the presence of lovastatin (4 μg/ml) and cycloheximide (30 μg/ml cycloheximide was added to accumulate an intracellular viral protein pool in the absence of de novo protein synthesis). Following 3 h of incubation, cells were washed and fresh medium (± lovastatin, but with no cycloheximide) was added to the cells to examine the assembly and release/budding of the accumulated viral proteins as virion particles in the presence of intact (MBCD and lovastatin untreated cells) and disrupted (MBCD and lovastatin treated cells) rafts. After 12 h the medium supernatants were assessed for viral titer by plaque assay.

Budding assay with NPC and HEL cells was performed similar to A549 cells but devoid of MBCD treatment. NPC and HEL cells were infected with RSV (1 MOI). At 10 h post-infection, fresh medium containing cycloheximide (30 μg/ml cycloheximide was added to accumulate an intracellular viral protein pool in the absence of de novo protein synthesis) was added to washed cells and the cells were incubated with cycloheximide for 3 h. Following 3 h of incubation, cells were washed and fresh medium (with no cycloheximide) was added to the cells to examine the assembly and release/budding of the accumulated viral proteins as virion particles. After 12 h the medium supernatants were assessed for viral titer by plaque assay.

Radioactive labeling

The viral protein composition of released progeny virus was examined by labeling cells with ^{35}S-methionone. A549 cells were infected with RSV (2 MOI). At 8 h post-infection, cells were washed and incubated with fresh medium containing 5 mM MBCD. After 1 h, MBCD medium was replaced with methionine free medium containing ± lovastatin (4 μg/ml). Following 2 h of incubation with methionine free medium, cells were pulsed with ^{35}S-methionone for 4 h. The radioactive medium was replaced with medium containing cold methionine ± lovastatin. After 12 h, ^{35}S-RSV was purified from medium supernatant and the radioactive virus was subjected to SDS-PAGE and autoradiography to visualize viral protein composition.

Immunofluorescence analysis

A549 cells grown on coverslips were either untreated or incubated with 5 mM MBCD for 1 h. Fresh medium containing lovastatin (4 μg/ml) was added to washed cells. After 16 h, cells were incubated with FITC conjugated cholera-toxin subunit-B (CHTX) (Sigma-Aldrich). Cells were fixed with 3.7% formaldehyde in PBS and visualized by confocal microscopy (Leica CISM confocal laser-scanning microscope).

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Cholesterol estimation assay

Untreated and MBCD treated cells were lysed and total cholesterol content was measured by cholesterol quantitation kit (MBL Intl) according to the manufacturer's specification. Virion cholesterol amount was also estimated in purified RSV virion particles (RSV purified from the medium supernatant) by using the cholesterol quantitation kit (MBL Intl).

Acknowledgments

This work was supported by National Institutes of Health grant AI083387 (S.B.). A.S. and V.M. are supported by NIH/NIDCR grant # DE14318 for the COSTAR program. We thank Dr. Mark E. Peeples (The Ohio State University and Nationwide Children's Hospital, Columbus, OH) for helpful suggestions and critically reading the manuscript. We also thank the Optical Imaging Core Facility (University of Texas Health Science Center at San Antonio) for confocal images.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.virol.2011.10.029.

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