Respiratory Syncytial Virus Matures at the Apical Surfaces of Polarized Epithelial Cells

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Respiratory syncytial (RS) virus infects the epithelium of the respiratory tract. We examined the replication and maturation of RS virus in two polarized epithelial cell lines, Vero C1008 and MDCK. Electron microscopy of RS virus-infected Vero C1008 cells revealed the presence of pleomorphic viral particles budding exclusively from the apical surface, often in clusters. The predominant type of particle was filamentous, 80 to 100 nm in diameter, and 4 to 8 μ m in length, and evidence from filtration studies indicated that the filamentous particles were infectious. Cytopathology produced by RS virus infection of polarized Vero C1008 cells was minimal, and syncytia were not observed, consistent with the maintenance of tight junctions and the exclusively apical maturation of the virus. Infectivity assays with MDCK cells confirmed that in this cell line, RS virus was released into the apical medium but not into the basolateral medium. In addition, the majority of the RS virus transmembrane fusion glycoprotein on the cell surface was localized to the apical surface of the Vero C1008 cells. Taken together, these results demonstrate that RS virus matures at the apical surface of polarized epithelial cell lines.

Respiratory syncytial (RS) virus is the most important viral cause of severe respiratory infection in infants (17, 20), infecting the epithelium of both the upper and lower respiratory tract.

Epithelial cells line the major cavities of the body, functioning in selective secretion and adsorption and providing a barrier to the external environment (11, 27, 36). As such, they are often the initial target for pathogens. The plasma membrane of these cells is divided into two discrete domains with distinct protein and lipid compositions. Specialized complexes termed tight junctions serve to preserve these domains and prevent mixing of components between them. The tight junctions are also involved in the formation of a monolayer of tightly adherent cells. A number of cell lines retain the properties of differentiated epithelial tissues in culture (16, 36).

Many viruses bud asymmetrically from polarized epithelial cells in culture, with potential implications for viral pathogenesis (32, 33, 35, 36). Vesicular stomatitis virus and retroviruses are released from the basolateral domain of polarized cells, while influenza virus and the paramyxoviruses simian virus 5 and Sendai virus have been shown to bud from the apical domain (14, 23, 29, 30, 33, 35, 36). RS virus is classified in the separate *Pneumovirus* genus of the family *Paramyxoviridae* because of the distinctive molecular and biological features which set it apart from other members of this family. Since RS virus is a pathogen of major clinical importance which naturally infects the respiratory epithelium, we have examined RS virus infection in two polarized epithelial cell lines to determine whether maturation of this virus is asymmetric.

The maturation of RS virus in polarized epithelial cells was investigated with the polarized epithelial cell line Vero C1008. This line has been used to demonstrate the asymmetric matu-

ration of a number of viruses (8, 9, 31). The Vero C1008 cell line is a clone of the nonpolarized epithelial cell line Vero 76, which we used in the studies presented here as a control. Vero 76 is a derivative of the epithelial cell line Vero, which has been used extensively in studies of RS virus maturation (2, 3, 13, 21).

Vero C1008 cells were seeded onto plastic culture dishes and allowed to grow until confluent monolayers were formed. The monolayers were infected with RS virus, and sections from both uninfected and RS virus-infected monolayers were examined by transmission electron microscopy. RS virus was detected budding exclusively from the apical surface of infected cells and was often observed budding in clusters (Fig. 1A). The viral particles were pleomorphic, and both round and filamentous forms of 80 to 100 nm in diameter were observed, with the filamentous forms predominating. Many of these viral particles possessed an electron-dense fuzzy coat, and in cross-section, a regular pattern of electron-dense dots was often observed (Fig. 1B). These electron-dense dots were also observed just under the cell membrane in areas where virus budding was occurring (Fig. 1B). No released or budding viral particles were detected on the basolateral surface or in the intercellular spaces of the RS virus-infected Vero C1008 cells, indicating that the virus was released from the cells asymmetrically. No syncytial cells were observed at this time by electron or light microscopy (data not shown).

For comparison, nonpolarized Vero 76 cells were also infected with RS virus and observed by transmission electron microscopy. Following infection of these cells with RS virus, viral particles were detected all around the cells and in the intercellular spaces (Fig. 1C), in agreement with earlier work in other nonpolarized cell lines (2, 3, 5, 19, 21). At lower magnifications, syncytial cells containing multiple nuclei were also observed by electron and light microscopy in the nonpolarized cells (data not shown). In addition, cytoplasmic inclusion bodies were observed in both polarized and nonpolarized cell lines following RS virus infection (Fig. 1A and 1C). These

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FIG. 1. Release of RS virus from the apical surfaces of polarized Vero C1008 cells. Polarized Vero C1008 cells and nonpolarized Vero 76 cells were infected at a multiplicity of infection (MOI) of 1.0 and prepared for electron microscopy at 48 h p.i. (30). (A) Two adjacent RS virus-infected Vero C1008 cells are shown. Pleomorphic RS virus particles are shown budding from the apical surface (arrowheads). No virions are observed at the basolateral surface or in the intercellular space. (B) Higher magnification of RS virions (arrowheads) budding from the apical surface of Vero C1008 cells. (C) Portions of three Vero 76 cells with RS virus particles (arrowheads) present in the intercellular spaces and along the upper surface of one cell are shown. Abbreviations: N, nucleus; I, cytoplasmic inclusion body. Magnifications: (A) $\times 15,750$; (B and C) $\times 57,000$. Bars represent 1,000 nm (A) and 200 nm (B and C). *Continued.*

cytoplasmic inclusions are thought to consist of aggregated viral nucleocapsids and have been observed in RS virus-infected cells as well as in cells infected with the related virus pneumonia virus of mice (5, 10, 15, 21).

Examination of the structures budding from the surface of the RS virus-infected Vero C1008 cells by scanning electron microscopy revealed large, thick clumps of long filamentous particles in addition to some individual filaments (Fig. 2B). These clusters could be resolved into individual filaments at higher magnification (Fig. 2D). The diameter of these fila-

ments was similar to that of the microvilli on the uninfected cells; however, the RS virus filaments were much longer than the microvilli, which averaged $0.7\ \mu\text{m}$ in length. The viral filaments ranged between 4 and $8\ \mu\text{m}$, although it was difficult to determine the beginning and termination of individual filaments. The filamentous morphology of the cell-associated RS virus particles observed on the surface of the Vero C1008 cells was not a function of the polarized phenotype of the cell, as we observed this morphology in both the polarized Vero C1008 and nonpolarized Vero 76 cell lines and this morphology has

FIG. 1—Continued.

Uninfected

RS virus-infected

FIG. 2. RS virus virions budding from the surfaces of infected Vero C1008 cells were filamentous. Vero C1008 cells were infected at an MOI of 1.0 and prepared for scanning electron microscopy at 48 h p.i. (37). For comparison, uninfected monolayers of Vero C1008 cells were identically prepared. (A and C) Uninfected cells with numerous microvilli; (B and D) RS virus-infected cells. Note the thick clusters of virions on the surface of the RS virus-infected cells (B), which resolve into individual filaments at higher magnification (D). Magnifications: (A and B) $\times 1,600$; (C and D) $\times 8,400$. Bars represent 5 μm (A and B) and 1 μm (C and D).

FIG. 3. RS virus attachment glycoprotein is present in filaments on the surfaces of infected Vero C1008 and Vero 76 cells. Polarized Vero C1008 cells and confluent Vero 76 cells were grown on coverslips and infected at an MOI of 1.0. At 24 h p.i., the monolayers were fixed and processed for indirect immunofluorescence microscopy as described previously with the RS virus G glycoprotein-specific monoclonal antibody L9 (26), provided by Ed Walsh. (A) Uninfected Vero C1008 cells; (B) RS virus-infected Vero C1008 cells; (C) uninfected Vero 76 cells; (D) RS virus-infected Vero 76 cells. Note the presence of filamentous projections on the surface of the RS virus-infected cells in both cell lines.

been reported previously in other nonpolarized cells (2, 12, 13, 25). In addition, the filamentous morphology of RS virus does not appear to be a result of tissue culture adaptation. While the strain used in our study has been maintained in the laboratory for many passages, electron microscopic analysis of RS virus isolated directly from nasopharyngeal secretions or observed in autopsy material has demonstrated that such virus is also predominantly filamentous (6, 7, 18).

Immunofluorescence microscopy of the RS virus-infected cell surface demonstrated that the RS virus attachment glycoprotein G was not uniformly distributed on the infected cell surface and was not present on the uninfected control cells (Fig. 3A and C). Rather, the G protein was concentrated in the long filaments on the surfaces of both polarized and nonpolarized Vero cell lines (Fig. 3B and D), in agreement with earlier reports on the distribution of this glycoprotein in viral filaments (3, 12, 25).

There was little evidence of cytopathology at 27 h postinfection (p.i.) in the RS virus-infected Vero C1008 cells when observed by light microscopy (data not shown), in agreement with the absence of syncytia in the RS virus-infected Vero C1008 cells, as shown by transmission electron microscopy. No syncytia were observed and domes were still present, consistent with the infected cells, remaining polarized with the tight junctions intact, preventing virus spread via the basolateral surfaces. In contrast, syncytia were evident at this time in RS virus-infected non-polarized Vero 76 cells (data not shown). This lack of syncytium formation was not due to inefficient infection of the Vero C1008 cells. Comparable amounts of

infectious virus were produced with similar kinetics in the two cell lines (2×10^7 to 3×10^7 PFU/ 10^6 cells at 48 h p.i.), and in both cell lines, the majority of the virus infectivity was cell associated.

To assay the release of infectious RS virus particles into the cell culture supernatants, it was necessary to grow the cell monolayers on permeable supports. However, it has been reported previously that RS virus infectivity was retained by 0.45- μ m filters (24), such as those commonly used as permeable supports. Therefore, we tested the ability of RS virus to pass through both Millipore 0.45- μ m HA membranes and Costar Transwell 3- μ m polycarbonate membranes. RS virus was added to the upper chamber of the support and allowed to flow through by gravity filtration. The flow through was then titrated on Vero 76 cells. Less than 0.5% of the original RS virus infectivity passed through the support with the 0.45- μ m membrane, while more than 85% of the infectivity of the virus stock was recovered after passing through the 3- μ m membrane support. These results suggest that the long filamentous viral particles observed by electron microscopy may be the predominant infectious form of RS virus. On the basis of these results, 3- μ m membrane supports were used for subsequent growth experiments. Because Vero C1008 cells readily grow through these larger pore membranes and establish monolayers on both sides of the membrane (37), we used the polarized epithelial cell line MDCK for these studies, although RS virus replicates less well in MDCK cells than in Vero C1008 cells.

Polarized MDCK cells grown on the Transwell 3- μ m permeable supports were infected with RS virus, and at 24 h p.i.,

FIG. 4. RS virus F glycoprotein accumulates at the apical surfaces of infected Vero C1008 cells. Vero C1008 cells were seeded onto filters, allowed to polarize, and infected with RS virus at an MOI of 1.0. At 21 h p.i. the cells were labeled with Tran^{35}S -label, and at 28 h p.i. the cells were biotinylated on either the apical or basolateral surface. Cytoplasmic extracts were prepared and immunoprecipitated with the F glycoprotein-specific monoclonal antibody 19 the gift of G. Taylor and J. Furze. Biotinylated protein was detected by incubating an aliquot of the immunoprecipitate with streptavidin-agarose. The proteins in both the total immunoprecipitate and the streptavidin-agarose precipitate were resolved by electrophoresis on 10% polyacrylamide gels, and the amount of biotinylated F protein expressed at the apical and basolateral cell surface was quantitated with a Molecular Dynamics PhosphorImager and ImageQuant software. Lanes 1 and 2, proteins from monolayers biotinylated on the apical surface; lanes 3 and 4, proteins from monolayers biotinylated on the basolateral surface. Lanes 1 and 3, streptavidin (SA)-precipitated protein; lanes 2 and 4, total protein (TOT) immunoprecipitated with monoclonal antibody 19. Numbers on the left indicate molecular mass (in kilodaltons).

the cell culture supernatants were harvested from the apical and basolateral sides of the support. Both supernatants and the monolayer were assayed for infectious virus titers. As observed with the Vero C1008 cells, the majority of infectivity was associated with the cell monolayer (3.64×10^4 PFU/ 10^6 cells). Quantitation of the virus released into the cell culture supernatant removed from either the apical or basolateral side of the monolayer demonstrated that RS virus was released from the apical surface only (1.85×10^4 PFU/ 10^6 cells). No virus infectivity was detected in the culture fluid removed from the basolateral side. Thus, infectious RS virus release from MDCK cells was consistent with our electron microscopy and immunofluorescence results from RS virus infection of the Vero C1008 cells.

Since many viral glycoproteins accumulate in the same membrane domain from which the virus buds (14, 22, 28, 36), we also analyzed the distribution of the RS virus fusion glycoprotein F on the infected cell surface. RS virus-infected Vero C1008 cell surfaces were biotinylated at 28 h p.i. (4), and the amount of biotinylated F protein expressed at the apical and basolateral cell surface was quantitated (Fig. 4). In five assays, an average of 88% of the biotinylated F protein was expressed

on the apical surface. Thus, the surface expression of RS virus F protein is polar, and this protein is expressed on the same surface from which the virus is released from polarized Vero C1008 cells. These results suggest that the lack of syncytia in the RS virus-infected polarized Vero C1008 cells is due in part to the apical restriction of surface expression of the fusion glycoprotein F. Studies with Sendai virus have shown that accumulation of the viral envelope glycoproteins in areas of cell-cell contact is essential for cell fusion (1), and analysis of a Sendai virus mutant that buds in a nonasymmetrical fashion from polarized cells has shown that basolateral expression of the viral fusion glycoprotein F is essential for cell fusion (34).

Our results demonstrate that RS virus matures at the apical membrane domain in the two polarized cell lines examined here. This restriction of RS virus maturation to the apical domain suggests that the asymmetric release of infectious RS virus may be a factor in the generally local nature of RS virus infections in the host. Budding of virus to the lumen may also be important in the shedding of virus in respiratory secretions and spread of RS virus to other hosts.

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REFERENCES

1. Aroeti, B., and Y. I. Henis. 1991. Accumulation of Sendai virus glycoproteins in cell-cell contact regions and its role in cell fusion. *J. Biol. Chem.* **266**: 15845–15854.
2. Bächli, T. 1988. Direct observation of the budding and fusion of an enveloped virus by video microscopy of viable cells. *J. Cell Biol.* **107**:1689–1695.
3. Bächli, T., and C. Howe. 1973. Morphogenesis and ultrastructure of respiratory syncytial virus. *J. Virol.* **12**:1173–1180.
4. Ball, J. M., Z. Moldoveanu, L. R. Melsen, P. A. Kozlowski, S. Jackson, M. J. Mulligan, J. F. Mestecky, and R. W. Compans. 1995. A polarized human endometrial cell line that binds and transports polymeric IgA. *In Vitro* **31**:197–207.
5. Berthiaume, L., J. Joncas, and V. Pavlanis. 1974. Comparative structure, morphogenesis and biological characteristics of the respiratory syncytial (RS) virus and the pneumonia virus of mice (PVM). *Arch. Ges. Virusforsch.* **45**:39–51.
6. Bryson, D. G., S. McConnell, M. McAliskey, and M. S. McNulty. 1991. Ultrastructural features of alveolar lesions in induced respiratory syncytial virus pneumonia of calves. *Vet. Pathol.* **28**:286–292.
7. Bryson, D. G., M. F. Platten, S. McConnell, and M. S. McNulty. 1991. Ultrastructural features of lesions in bronchiolar epithelium in induced respiratory syncytial virus pneumonia of calves. *Vet. Pathol.* **28**:293–299.
8. Chen, S.-Y., Y. Matsuoka, and R. W. Compans. 1991. Assembly and polarized release of Punta Toro virus and effects of brefeldin A. *J. Virol.* **65**:1427–1439.
9. Clayson, E. T., L. V. Brando, and R. W. Compans. 1989. Release of simian virus 40 virions from epithelial cells is polarized and occurs without cell lysis. *J. Virol.* **63**:2278–2288.
10. Compans, R. W., D. H. Harter, and P. W. Choppin. 1967. Studies on pneumonia virus of mice (PVM) in cell culture. II. Structure and morphogenesis of the virus particle. *J. Exp. Med.* **126**:267–275.
11. Compans, R. W., and R. V. Srinivas. 1991. Protein sorting in polarized epithelial cells. *Curr. Top. Microbiol. Immunol.* **170**:141–181.
12. Faulkner, G. P., P. V. Shirodaria, E. A. C. Follett, and C. R. Pringle. 1976. Respiratory syncytial virus *ts* mutants and nuclear immunofluorescence. *J. Virol.* **20**:487–500.
13. Fuchs, H., and T. Bächli. 1975. Scanning electron microscopical demonstration of respiratory syncytial virus antigens by immunological markers. *J. Ultrastruct. Res.* **52**:114–119.
14. Fuller, S., C.-H. von Bonsdorff, and K. Simons. 1984. Vesicular stomatitis virus infects and matures only through the basolateral surface of the polarized epithelial cell line MDCK. *Cell* **38**:65–77.
15. García, J., B. García-Barreno, A. Viro, and J. A. Melero. 1993. Cytoplasmic inclusions of respiratory syncytial virus-infected cells: formation of inclusion

- bodies in transfected cells that coexpress the nucleoprotein, the phosphoprotein, and the 22K protein. *Virology* **195**:243–247.
16. **Gstraunthaler, G. J. A.** 1988. Epithelial cells in tissue culture. *Renal Physiol. Biochem.* **11**:1–42.
  17. **Heilman, C. A.** 1990. Respiratory syncytial and parainfluenza viruses. *J. Infect. Dis.* **161**:402–406.
  18. **Joncas, J., L. Berthiaume, and V. Pavilanis.** 1969. The structure of the respiratory syncytial virus. *Virology* **38**:493–496.
  19. **Kalica, A. R., P. F. Wright, F. M. Hetrick, and R. M. Chanock.** 1973. Electron microscopic studies of respiratory syncytial virus temperature-sensitive mutants. *Arch. Ges. Virusforsch.* **41**:248–258.
  20. **McIntosh, K., and R. M. Chanock.** 1990. Respiratory syncytial viruses, p. 1045–1072. *In* B. N. Fields and D. M. Knipe (ed.), *Field's virology*, vol. 1. Raven Press, New York.
  21. **Norrby, E., H. Marusyk, and C. Örvell.** 1970. Morphogenesis of respiratory syncytial virus in a green monkey kidney cell line (Vero). *J. Virol.* **6**:237–242.
  22. **Owens, R. J., and R. W. Compans.** 1989. Expression of the human immunodeficiency virus envelope glycoprotein is restricted to basolateral surfaces of polarized epithelial cells. *J. Virol.* **63**:978–982.
  23. **Owens, R. J., J. W. Dubay, E. Hunter, and R. W. Compans.** 1991. Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells. *Proc. Natl. Acad. Sci. USA* **88**:3987–3991.
  24. **Paccaud, M. F., and C. Jacquier.** 1970. A respiratory syncytial virus of bovine origin. *Arch. Ges. Virusforsch.* **30**:327–342.
  25. **Parry, J. E., P. V. Shirodaria, and C. R. Pringle.** 1979. Pneumoviruses: the cell surface of lytically and persistently infected cells. *J. Gen. Virol.* **44**:479–491.
  26. **Roberts, S. R., D. L. Lichtenstein, L. A. Ball, and G. W. Wertz.** 1994. The membrane-associated and secreted forms of the respiratory syncytial virus attachment glycoprotein G are synthesized from alternative initiation codons. *J. Virol.* **68**:4538–4546.
  27. **Rodriguez-Boulan, E., and W. J. Nelson.** 1989. Morphogenesis of the polarized epithelial cell phenotype. *Science* **245**:718–725.
  28. **Rodriguez-Boulan, E., and M. Pendergast.** 1980. Polarized distribution of viral envelope proteins in the plasma membrane of infected epithelial cells. *Cell* **20**:45–54.
  29. **Rodriguez-Boulan, E., and D. D. Sabatini.** 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for study of epithelial polarity. *Proc. Natl. Acad. Sci. USA* **75**:5071–5075.
  30. **Roth, M. G., R. V. Srinivas, and R. W. Compans.** 1983. Basolateral maturation of retroviruses in polarized epithelial cells. *J. Virol.* **45**:1065–1073.
  31. **Srinivas, R. V., N. Balachandran, F. V. Alonso-Caplen, and R. W. Compans.** 1986. Expression of herpes simplex virus glycoproteins in polarized epithelial cells. *J. Virol.* **58**:689–693.
  32. **Tashiro, M., J. T. Seto, S. Choosakul, M. Yamakawa, H.-D. Klenk, and R. Rott.** 1992. Budding site of Sendai virus in polarized epithelial cells is one of the determinants for tropism and pathogenicity in mice. *Virology* **187**:413–422.
  33. **Tashiro, M., M. Yamakawa, K. Tobita, H.-D. Klenk, R. Rott, and J. T. Seto.** 1990. Organ tropism of Sendai virus in mice: proteolytic activation of the fusion glycoprotein in mouse organs and budding site at the bronchial epithelium. *J. Virol.* **64**:3627–3634.
  34. **Tashiro, M., M. Yamakawa, K. Tobita, H.-D. Klenk, J. T. Seto, and R. Rott.** 1992. Significance of basolateral domain of polarized MDCK cells for Sendai virus-induced cell fusion. *Arch. Virol.* **125**:129–139.
  35. **Tashiro, M., M. Yamakawa, K. Tobita, J. T. Seto, H.-D. Klenk, and R. Rott.** 1990. Altered budding site of a pantropic mutant of Sendai virus, F1-R, in polarized epithelial cells. *J. Virol.* **64**:4672–4677.
  36. **Tucker, S. P., and R. W. Compans.** 1993. Virus infection of polarized epithelial cells. *Adv. Virus Res.* **42**:187–247.
  37. **Tucker, S. P., L. R. Melsen, and R. W. Compans.** 1992. Migration of polarized epithelial cells through permeable membranes of defined pore size. *Eur. J. Cell Biol.* **58**:280–290.