Basolateral Maturation of Retroviruses in Polarized Epithelial Cells

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We have investigated the maturation sites of avian and mammalian C-type retroviruses in polarized epithelial cells. Examination of thin sections of Madin Darby canine kidney cells infected with RD114 or avian reticuloendotheliosis virus revealed that these viruses mature from the basolateral membrane domains. Similar results were obtained with a continuous line of mouse mammary epithelial cells infected with Friend, Moloney, Rauscher, or Kirsten murine leukemia viruses, or Friend virus-related or Moloney virus-related mink cell focus-forming viruses. Immunofluorescence observations indicate that viral glycoproteins are inserted only at the basolateral membranes in these cells. Because of the availability of DNA and protein sequence data, and of molecularly cloned viruses, these virus systems offer advantages for molecular studies on directional transport of plasma membrane glycoproteins.

Epithelia which serve as selective barriers are composed of cells with two distinct surface domains, designated apical and basolateral, which are separated by a continuous belt of tight junctions, the *Zonula occludens*. Each surface domain contains its own complement of proteins, indicating that epithelial cells maintain mechanisms for sorting plasma membrane proteins to their proper locations (24). In certain epithelial cell cultures, some enveloped viruses bud in a polarized manner (3, 16, 20). Influenza and parainfluenza viruses were reported to mature selectively from the apical surfaces of Madin Darby canine kidney (MDCK) cell cultures, whereas vesicular stomatitis virus (VSV) buds exclusively from the basolateral membranes (20). The polarized maturation of enveloped viruses suggests that the host cell recognizes at least one structural protein of each virus, since host cell mechanisms are responsible for the synthesis and transport of viral polypeptides (7). Recently, we have obtained evidence that the cloned hemagglutinin glycoprotein of influenza A virus is expressed in a polarized manner in the absence of other viral components in primary cultures of African green monkey kidney cells (M. G. Roth et al., submitted for publication). These results indicate that host cell sorting mechanisms recognize some features of the viral glycoprotein molecule. Thus, it is likely that the site of virus maturation is determined by the site of membrane insertion of viral glycoproteins, which are localized in the same membrane domains from which the viruses bud (12, 19).

Studies on the polarized maturation of envel-

oped viruses in epithelial cells have been confined so far to cytolytic viruses which progressively disrupt tight junctions during infection. For this report, we investigated the maturation sites in MDCK cells of another virus family, the type C retroviruses, which establish nonlytic, persistent infections. We also observed that a continuous line of mouse mammary epithelial (MME) cells showed polarity of virus maturation and glycoprotein insertion similar to that observed in MDCK cells.

MATERIALS AND METHODS

Viruses and cells. MDCK cells were obtained from the American Type Culture Collection and were maintained as previously described (21). Primary chicken embryo fibroblasts made from 13-day-old SPAFAS chick helper factor negative chicken embryos were kindly supplied by E. Hunter. A cloned subline of MME cells originally established from a spontaneous BALB/c murine mammary tumor (8) was obtained from Clayton Buck. JLS-V5 cells persistently infected with Rauscher murine leukemia virus (MuLV) were obtained from the Cell Culture Department of the Naval Biosciences Laboratory, Oakland, Calif. Eve-line cells persistently infected with Friend MuLV were obtained from D. P. Bolognesi and A. J. Langlois. NIH 3T3 cells and NIH 3T3 cells persistently infected with Moloney MuLV and Kirsten MuLV were supplied by Stuart Aaronson. NIH 3T3 cells persistently infected with a Friend virus-related and a Moloney virus-related mink cell focus-forming virus were supplied by Sandra Ruscetti. The culture fluids from confluent monolayers of MuLV-infected cells were clarified by centrifugation and used as a source of infectious virus. Avian reticuloendotheliosis virus

(REV-A) and RD 114 were supplied by E. Hunter and S. Chatterjee.

Reagents and antisera. Anti-REV hyperimmune rabbit serum was kindly provided by E. Hunter. Goat anti-gp70 (Rauscher) serum was prepared in this laboratory. Goat anti-p30 (Rauscher) serum was obtained from the Office of Program Resources and Logistics of the National Cancer Institute through the courtesy of R. Wilsnack, Huntington Research Center, Brooklandville, Md. Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G and rabbit anti-goat IgG were obtained from Miles Laboratories, Inc., Elkhart, Ind.

Electron microscopy. Monolayers of cells grown on plastic petri dishes were washed three times with phosphate-buffered saline and fixed *in situ* with 1% glutaraldehyde for 30 min. The monolayers were post-fixed with 1% osmium tetroxide at 4°C for 1 h and dehydrated with 70, 95, and 100% ethanol. Cell monolayers lifted intact off the plastic substrate by the addition of propylene oxide were embedded in an epoxy-resin mixture. Thin sections were cut on a Sorvall MT 2B ultramicrotome (Du Pont Co., Wilmington, Del.), mounted on a 300-mesh copper grid, stained with uranyl acetate and lead citrate, and examined in a Philips 301 electron microscope.

Immunofluorescence. Indirect immunofluorescence was carried out on fixed or unfixed, intact or ethyleneglycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid (EGTA)-treated monolayers according to Alonso and Compans (2).

RESULTS

Maturation sites of retroviruses in MDCK cells. Subconfluent monolayers of MDCK cells were infected with two unrelated retroviruses known to replicate in canine cells, REV or RD 114, in the presence of polybrene (2 μ g/ml). After the monolayers had achieved confluence, they were maintained by serial passage to establish persistently infected cell lines. Recently infected as well as persistently infected cells were examined by electron microscopy for sites of virus maturation and were found to contain approximately one to five virions, respectively, per cell cross section. More than 80% of the virions were associated with basolateral membranes (Fig. 1 and 2), and the remaining 20% of the virions were found near the apical cell surface. Virions found near the apical membranes appeared to be released particles, since immature or budding forms were not observed. Similar results were obtained with MDCK cells infected with REV or RD 114 and fixed for electron microscopy after several passages, suggesting that persistent infection of MDCK cells by these retroviruses does not change the pattern of virus maturation. When virions associated with either apical or basolateral membranes were quantitated in MDCK cells infected with influenza viruses, 90 to 99% of the virions were found to be associated with the apical surfaces. MDCK cells infect-

ed with VSV were found to contain 99% of the virions at the basolateral membrane domain during early periods of infection when cytopathic effects were not evident, although during late periods of infection with either influenza virus or VSV in MDCK cells, polarity of virus maturation was progressively lost as cells rounded and died. Therefore, the location of cell-associated type C retroviruses in recently or persistently infected MDCK cells was distinctly different from that previously reported for orthomyxo- or paramyxoviruses and resembled more closely that of the rhabdovirus VSV. As discussed below, we believe that the RD 114 or REV virions associated with apical membranes of MDCK cells are likely to have been released from growing cells in subconfluent monolayers, where tight junctions may not have completely separated the two membrane domains so that virus particles formed at basal membranes could diffuse into the culture medium.

Maturation sites of MuLVs in epithelial cells. A cloned subline of MME cells described by Damsky et al. (8) shows ultrastructural and morphological features characteristic of polarized epithelial cells with well-developed tight junctions. To investigate whether this cell line exhibits polarized virus maturation, we examined the pattern of maturation of VSV and A/WSN influenza virus in this cell line. As observed with MDCK cells, VSV budded from the basolateral membranes of MME cells, whereas influenza virus budded from the apical surface (data not shown). Thus, MME cells were found to exhibit polarity of virus maturation and appeared suitable for studies of the maturation pattern of murine retroviruses in a polarized epithelial cell line.

Persistent infections with the Friend, Rauscher, Moloney, or Kirsten strains of MuLV, as well as F-MCF or M-MCF viruses, were established in MME cells according to the procedures described for infection of MDCK cells with REV. When the cells were uniformly infected with MuLV, they were examined for sites of virus maturation by electron microscopy. The degree of virus expression in MME cells was significantly lower than the virus expression found in mouse fibroblast cells, as evidenced by the number of virus particles observed per cell cross section. As seen for REV-infected MDCK cells, nearly all of the virus particles were associated with the basolateral membranes; only occasional virions were found near the apical surface (Fig. 3a to c). Thus, basolateral maturation appears to be a common pattern for retroviruses in polarized epithelial cells.

MME cells infected with different MuLVs occasionally contained intracellular vesicles filled with intact virions, as well as material

FIG. 1. Basolateral maturation of REV in MDCK cells. A thin section from a primary infection shows virus particles (arrow) at the basolateral surface (left) but not the apical membranes (right). The inset shows a high-magnification view of a virus particle at a late stage of budding from the basal membrane. The basolateral surfaces exhibit an electron dense layer of adherent protein, which is seen at high magnification in the inset. Magnification: $\times 22,000$; inset, $\times 78,000$.

which resembled degraded viral particles. These vesicles were seen primarily near the basolateral membranes (Fig. 3c) and may represent secondary lysosomes incorporating virus particles inserted by endocytosis from areas of basolateral plasma membrane. Occasionally, aberrant elongated particles and budding forms with double

cores (Fig. 3c, 4c and d), in addition to morphologically typical C-type viruses, were observed in these cells. The culture supernatants from these cells, however, contained infectious virus that displayed typical morphology when cultivated in NIH 3T3 cells (data not shown). Occasionally, we could observe C-type viruses bud-

FIG. 2. Basal portion of an MDCK cell persistently infected with RD 114, showing several virus particles along the basal membrane. A virus particle budding into a smooth membrane vesicle is indicated by the arrow. Magnification: $\times 30,000$.

ding from the cytoplasm into the perinuclear space (Fig. 4b) and, more frequently, into smooth membrane vesicles (Fig. 4a), indicating the occurrence of intracisternal budding in these cells. Although the intracytoplasmic virus-containing vesicles were devoid of ribosomes, the outer membrane of the nucleus was contiguous with the rough endoplasmic reticulum, suggesting that MuLV can occasionally mature at rough or smooth endoplasmic reticular membrane cisternae.

Site of insertion of viral glycoproteins. Previous studies have shown that viral glycoproteins are inserted into the plasma membrane domain from which the viruses bud, and that they probably determine the site of virus maturation (12, 19). The requirement and the precise role of envelope glycoproteins in the maturation of retroviruses have not been well established. We therefore examined the sites of insertion of viral glycoproteins in REV-infected MDCK cells and Rauscher MuLV-infected MME cells by indirect immunofluorescence.

Monolayers fixed with ethanol-acetic acid (95:5, vol/vol) were examined to visualize cell-associated viral proteins. Intact, unfixed monolayers were examined to localize viral glycoproteins on the apical membranes. To visualize viral glycoproteins localized on basolateral membranes, monolayers were pretreated with EGTA to disrupt the tight junctions (6) and expose basolateral membranes for interaction with antibody (19). Under these conditions, anti-REV serum reacted specifically with REV-infected but not uninfected MDCK cells fixed with ethanol-acetic acid. Also, nearly all of the cells in the monolayer were infected with REV, as shown

by the uniform pattern of fluorescence. Anti-REV serum failed to react with intact, unfixed monolayers (Fig. 5a). However, when cells pretreated with EGTA were reacted with anti-REV, nearly all of the cells showed surface immunofluorescence (Fig. 5b), indicating that viral glycoproteins are inserted into basolateral but not apical membranes in these cells. MuLV-infected cells express on their cell surface a glycosylated form of the *gag* gene-encoded precursor polyprotein in addition to the *env* gene-encoded viral glycoproteins. Unlike the envelope proteins, the glycosylated *gag* precursors are not incorporated into the virions and behave like integral membrane proteins (10, 26). We examined the cell surface localization of both envelope glycoproteins and glycosylated *gag* precursors in Rauscher MuLV infected MME cells. Neither anti-gp70 serum nor anti-p30 serum showed surface immunofluorescence on intact, unfixed monolayers (Fig. 5c and e), whereas both of the antisera showed surface immunofluorescence on unfixed, EGTA-treated monolayers (Fig. 5d and f), indicating that both *env* and *gag* gene-encoded viral glycoproteins are located on the basolateral and not on the apical membrane domains of Rauscher MuLV-infected MME cells.

DISCUSSION

In polarized epithelial cells infected with type C retroviruses, extracellular virus was associated predominately with basolateral surfaces, and budding virions were observed exclusively at the basolateral membrane domain. Thus, maturation of type C retroviruses resembles that of VSV and is clearly different from that of ortho-

FIG. 3. Maturation sites of MuLV in MME cells. (a) Electron micrograph of a thin section from Kirsten MuLV/MME cells, showing virus particles at basal but not apical membranes. (b) MME cells recently infected with Rauscher MuLV, showing basolateral maturation of virus particles (arrows). (c) MME cells persistently infected with Rauscher MuLV, showing a large number of virus particles along the basolateral membranes. A vacuole containing several virus particles is shown by the arrow. The arrowhead points to an elongated particle with a double core. Virus particles were not associated with the apical membranes (not shown). Magnification: a, $\times 30,000$; b, $\times 22,000$; c, $\times 65,000$.

FIG. 4. Features of MuLV maturation in MME cells. (a) A thin section from Friend MCF/MME cells, showing the characteristic pattern of basolateral maturation, as well as intracisternal budding into smooth membrane vesicles (arrow). (b) Rauscher MuLV/

myxoviruses and paramyxoviruses. Available models for the maturation of retroviruses suggest a role for viral glycoproteins in the initiation of the budding process (5, 15). Our studies, using the monovalent ionophore monensin to inhibit the transport of viral glycoproteins to the cell surface, also suggest a requirement for glycoproteins in virus maturation (27). However, several reports indicate that retroviruses can bud in the absence of viral glycoproteins (1, 9, 17, 23, 28). The present studies suggest that retrovirus maturation in polarized epithelial cells resembles that of other virus families in that maturation appears to occur only in those plasma membrane domains which incorporate viral glycoproteins. This supports the conclusion that the retrovirus glycoproteins play a role in determining the viral maturation site. Previous reports have suggested an intracisternal mode of virus maturation, as well as budding at the plasma membranes, in cell lines producing high amounts of MuLV (14). We have observed intracisternal maturation into smooth membrane vesicles, and occasionally into perinuclear spaces, in MME cells. It is not clear whether the observed pattern of virus maturation is a consequence of the directional transport of viral glycoproteins to the basolateral membranes, where cell-cell and cell-substratum interaction offer a constraint on virus budding, which might result in an intracellular accumulation of viral glycoproteins.

All viral glycoproteins previously studied exhibit polarity of surface expression in polarized epithelial cells. The apical and basolateral membrane domains of polarized epithelial cells contain distinct sets of proteins (11, 13, 18), although some of the cellular proteins may be inserted into both apical and basolateral membranes (18). In this context, it is interesting to note that the glycosylated *gag* precursors of MuLV appeared to be transported in a directional manner to the basolateral membrane domains. The nature of the membrane association of glycosylated *gag* precursors has not been clearly understood. These molecules share the carboxy terminus with the Pr65^{gag} but differ at the amino terminal end of the molecule, where they contain a long, hydrophobic leader peptide (25), possibly added to the *gag* message by an unusual splicing event. Recent studies with a monoclonal antibody that specifically recognizes the

MME cells, showing virus particles budding into the perinuclear space. (c) Friend MuLV/MME cells, showing an elongated particle with a double core budding from lateral membrane processes. (d) A low-magnification view of Kirsten MuLV/MME cells grown in suspension cultures, showing several elongated virus particles. Magnification: a, $\times 63,000$; b, $\times 63,000$; c, $\times 63,000$; d, $\times 30,000$.

FIG. 5. Indirect immunofluorescence of REV/MDCK and Rauscher MuLV/MME cells. Indirect immunofluorescence of intact (a) or EGTA-treated (b) REV/MDCK cells stained with anti-REV serum. The arrow in Fig. 5a indicates a hole in the monolayer where the basolateral membranes of the peripheral cells were exposed and showed surface fluorescence. Indirect immunofluorescence of intact (c and e) or EGTA-treated Rauscher MuLV/MME cells stained with anti-gp70 (c and d) or anti-p30 serum, showing surface immunofluorescence of EGTA-treated cells but not intact monolayers. Bottom photos show intracellular staining of REV/MDCK (g) and Rauscher MuLV/MMEC (h) cells with anti-REV serum and anti-p30 serum, respectively.

leader peptide of the glycosylated *gag* precursors indicate that these molecules may be anchored to the membrane by means of the amino terminal leader peptide (E. Pillemar, D. Kooistra, O. Witte, and I. Weissman, personal communication), analogous to the neuraminidase glycoprotein of influenza A viruses (4). Thus, the glycosylated *gag* precursors of MuLV may provide a system for the study of directional transport of plasma membrane glycoproteins that are inserted into the plasma membrane by their amino terminus. The mechanisms involved in directional transport of surface proteins are not presently understood. Recently, we have observed that the expression of influenza virus hemagglutinin is polarized in primary African green monkey kidney cells infected with a recombinant simian virus 40 carrying hemagglutinin DNA, suggesting that cellular sorting mechanisms directly recognize viral glycoproteins (Roth et al., submitted for publication). Previous studies have established that glycosylation is not required for the polarized expression of viral glycoproteins or maturation of viruses (12, 22). Taken together, these results indicate that a feature of the polypeptide portion of a viral glycoprotein is probably recognized by host cell sorting mechanisms. The viruses used previously for studies on the mechanism of directional transport of glycoproteins cause extensive cytopathic effects, confining most investigations to early periods of infection. Disruption of intercellular junctions followed by cell rounding is a particularly severe problem with VSV, the only virus previously shown to mature at basolateral membranes. Because retroviruses establish persistent, nonlytic infections of epithelial cultures which can be passaged for extended periods without apparent cellular transformation or loss of polarity of virus maturation, retrovirus-infected epithelial cells should prove useful for studies on the polarized surface expression of viral glycoproteins. In addition, extensive nucleic acid and amino acid sequence data now exist for retroviral glycoproteins, and many *env* genes have been cloned. Further studies of these viruses may provide information on the molecular features of envelope proteins that are recognized by host cell sorting mechanisms.

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