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TRANSPORT OF VIRAL GLYCOPROTEINS AND ITS MODULATION BY MONENSIN

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We have obtained evidence (1) that distinct pathways of membrane glycoprotein transport exist in polarized epithelial cell monolayers, differing in their sensitivity to monensin, a sodium ionophore. Monensin blocks the transport of secretory and membrane glycoproteins at the level of the Golgi complex, which appears dilated as an effect of the drug (2). The replication of vesicular stomatitis virus (VSV), which forms at basolateral membranes of Madin-Darby canine kidney (MDCK) cells, was markedly inhibited by monensin, with a block occurring in the transport of the membrane glycoprotein, G, to the cell surface. In contrast, influenza virus maturation, which occurs at the free apical surfaces of MDCK cells, was not affected significantly using similar concentrations of the ionophore. We have recently demonstrated that the influenza hemagglutinin (HA) protein continues to be transported to the surface of monensin-treated cells, is cleaved into HA1 and HA2, and is incorporated into budding influenza virus particles (3). On the other hand, VSV G protein appears to accumulate in membranes of the dilated Golgi apparatus of monensin-treated cells. In this report, we have investigated the effects of monensin on the kinetics of transport of viral membrane glycoproteins to the cell surface. We have also studied the effect of the ionophore on the directional transport of viral glycoproteins in MDCK cells doubly-infected with influenza virus and VSV, and on the replication of viruses that are assembled at intracellular membranes.

APPEARANCE OF NEWLY-SYNTHESIZED HEMAGGLUTININ AT THE APICAL SURFACE OF INFLUENZA VIRUS-INFECTED CELLS

We used a surface immune precipitation procedure (4) to examine the effect of monensin on the kinetics of transport of

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influenza virus HA protein to cell surfaces. The HA protein was detected on surfaces of untreated or monensin-treated cells infected with influenza virus (A/WSN strain) as early as 20 min after synthesis (Fig. 1). However, there was a dramatic difference in the rate of cleavage of HA since the HA₁ protein, a cleavage product, was present on the surface of monensin-treated cells only after 60 min, whereas the HA₁ protein in controls was detected after a 20-min chase period. In addition, the incorporation of ³H-mannose into HA protein in monensin-treated cells was markedly higher than in controls, suggesting an inefficient trimming of mannose residues during processing of the HA protein in the presence of the ionophore. With another virus strain (A/USSR), HA had a faster electrophoretic mobility in monensin-treated cells (Fig. 2), but was detected at surfaces of control or monensin-treated cells after a 20-min chase. The incorporation of ³H-mannose into HA protein in monensin-treated cells was also significantly higher when compared to untreated cells. These results indicate that the HA protein is transported to the apical surfaces of monensin-treated MDCK cells at a rate similar to that of controls.

FIGURE 1. Cell surface appearance of influenza virus (A/WSN) HA protein. Monolayers of influenza virus-infected MDCK cells (MOI = 10) were untreated (lanes a, c, e, g, i, and k) or were treated with 10⁻⁵ M monensin (lanes b, d, f, h, j, and l) after adsorption. Cells were pulsed at 7 h postinfection for 15 min with ³H-mannose (a and b), and chased with Eagle's medium containing 20 mM cold mannose for 10 min (c and d), 20 min (e and f), 30 min (g and h), 40 min (i and j), or 60 min (k and l). Cell surface glycoproteins were analyzed by exposure of intact monolayers to anti-HA monoclonal antibody, followed by lysis and immune precipitation. A. Lysates prior to immune precipitation. B. Immune precipitates.
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FIGURE 2. Cell surface appearance of influenza virus (A/USSR) HA protein. MDCK cell monolayers infected with influenza virus \(^{5}(\text{MOI} = 10)\) were maintained in the presence or absence of \(10^{-5}\) M monensin. Labelling conditions for each lane correspond to those in Fig. 1.

TRANSPORT OF VIRAL MEMBRANE GLYCOPROTEINS IN DOUBLY-INFECTED CELLS

Previous reports indicated that the directional transport of viral glycoproteins is a characteristic not only of singly- but also of doubly-infected MDCK cells (5). We therefore wished to determine the effect of monensin on transport of HA and G proteins in MDCK cells simultaneously infected with influenza virus and VSV. No significant differences were observed in the levels of HA expression on surfaces of untreated or monensin-treated doubly-infected MDCK cells (Fig. 3A). The level of expression of neuraminidase (NA) protein on surfaces of control or monensin-treated cells was also found to be similar (Fig. 3B). In contrast, the amount of G protein present in the plasma membrane of controls was markedly greater than on surfaces of cells cultured in the presence of the ionophore (Fig. 3C). The level of fluorescence with antibody to G protein in monensin-treated doubly-infected cells was higher than in VSV-infected cells treated with the ionophore; however, similar fluorescence intensities were observed when cells singly infected with influenza virus were stained with anti-G antibody, indicating that the observed fluorescence represents a higher background level.
FIGURE 3. Surface expression of viral membrane glycoproteins in doubly-infected cells. MDCK cell monolayers were infected with influenza virus (A/WSN) at an MOI of 2. After 1 h adsorption, cells were superinfected for 1 h with VSV (MOI = 5), and incubated in the presence (- - -) or absence (---) of 10^{-9} M monensin. At 9 h postinfection with influenza virus, cells were suspended with EGTA and processed for indirect surface fluorescence (3) by first adding anti-HA, anti-NA, or anti-G monoclonal antibodies to aliquots in suspension, followed by a fluorescein-conjugated second antibody. Cell surface expression of influenza virus HA protein (A), and NA protein (B), and of VSV G protein (C) is shown. From Alonso-Caplen et al. (6).
We also examined the effect of monensin on infectious virus yields from doubly-infected MDCK monolayers (Table 1). Infectivity titers of influenza virus were not significantly affected even at a $10^{-5}$ M concentration of monensin. In contrast, yields of VSV showed greater than a 99% decrease at $10^{-6}$ M or $10^{-5}$ M monensin when compared to controls. These results indicate that the differential effect of monensin on influenza and VSV replication is similar in singly- and doubly-infected MDCK cells.

| Monensin concentration | Influenza virus titers (p.f.u./ml) | VSV titers (p.f.u./ml) |
|------------------------|----------------------------------|-----------------------|
| Control                | $3.8 \times 10^7$                | $2.0 \times 10^8$     |
| $10^{-6}$ M            | $2.5 \times 10^7$                | $2.3 \times 10^6$     |
| $10^{-5}$ M            | $1.5 \times 10^7$                | $9.0 \times 10^5$     |

*aConfluent monolayers of MDCK cells were infected with influenza virus (MOI=10) and after 1 h, with VSV (MOI=10). Virus yields were measured at 24 h postinfection using MDCK cells for influenza virus plaque assays or BHK21 cells for VSV plaque assays.

EFFECT OF MONENSIN ON ENVELOPED VIRUSES THAT FORM AT INTRACELLULAR MEMBRANES

Avian infectious bronchitis virus (IBV) is a coronavirus which matures at the rough endoplasmic reticulum (7) and snowshoe hare virus (SSH) is a bunyavirus reported to assemble at membranes of the Golgi complex (8). We found that release of infectious IBV particles grown in African Green monkey kidney (Vero) cells was inhibited by 90% or more at $10^{-6}$ or $10^{-5}$ M monensin (Fig. 4A). Similarly, yields of cell-associated IBV virions were significantly reduced, indicating that monensin is not merely blocking exocytosis of infectious particles but the production of virus particles as well. As shown in Fig. 4B, viral protein synthesis was observed at $10^{-6}$ M monensin, where infectious virus yields were reduced by over 90% as compared to controls. This suggests that monensin inhibits the assembly and release of infectious IBV particles under conditions where no effect on viral protein synthesis is observed. At $10^{-5}$ M monensin, there was a significant decrease in viral protein synthesis, but this concentration may be toxic to the host cell. In a recent report (9), it was shown that monensin blocked the release of a murine coronavirus from infected cells. However, virus assembly at
the membranes of the rough endoplasmic reticulum was not inhibited by monensin, and there was an apparent accumulation of cell-associated virions. It will be of interest to determine whether the differences in these results may be related to the differences in glycosylation processes of murine and avian coronaviruses (9, 12).

**FIGURE 4.** A. Effect of monensin on yields of IBV from Vero cells. Cells were infected with IBV (MOI = 0.5) or VSV (MOI = 2), monensin was added post-adsorption, and infectivity titers determined by plaque assays. Yields of both released and cell-associated IBV, obtained after freeze-thawing, were measured. Released IBV titers (●), cell-associated IBV titers (○), VSV infectivity titers (■). The control (100%) titer was 4.5 x 10⁷ pfu/ml for released IBV, 1.1 x 10⁷ pfu/ml for cell-associated IBV, and 2.7 x 10⁷ pfu/ml for VSV. From Alonso-Caplen et al. (12).

B. Viral protein synthesis in monensin-treated Vero cells. At 19 h postinfection, cells were pulse-labeled for 1 h with H-leucine. IBV-infected, untreated (A); 10⁻⁸ M (B), 10⁻⁷ M (C), 10⁻⁶ M (D), and 10⁻⁵ M monensin (E); lane F, VSV viral proteins used as molecular weight standards. From Alonso-Caplen et al. (12).

When yields of released and cell-associated SSH virions were examined (Fig. 5A), it was found that monensin did not block virus assembly or the release of SSH particles into culture supernatants. Polyacrylamide gel electrophoretic analysis of SSH-infected BHK21 cells treated with the ionophore (Fig. 5B) revealed the synthesis of all viral proteins. In contrast, other investigators (10, 11) have reported that monensin inhibits the replication of LaCrosse (LAC)
virus, another bunyavirus. Infectious virus yields were greatly reduced but the synthesis of viral proteins was not affected (10); in addition, there was an accumulation in monensin-treated cells of an incompletely processed form of Gl, one of the two envelope glycoproteins of LAC (11). The difference between these results may be related to the slower replication cycle of LAC in BHK21 cells as compared to SSH.

![Graph showing the relationship between monensin concentration and infectious titers of SSH.](image)

**FIGURE 5.** A. Yields of SSH from monensin-treated BHK21 cells. Monensin was added to cells after infection with SSH (MOI = 10). Yields of both released and cell-associated virions, obtained by exposure of intact cells to neutralizing antibody followed by one cycle of freeze-thawing, were measured at 24 h postinfection. Released SSH titers (○), intracellular SSH titers (●). The control (100%) titer was 7.6 x 10^6 pfu/ml for released SSH, and 6.0 x 10^4 pfu/ml for cell-associated SSH.

B. Viral protein synthesis in monensin-treated BHK21 cells. SSH-infected BHK21 cells were pulse-labeled with ^3H-leucine for 1 h at 16 h postinfection. Purified SSH virions (A); SSH-infected, untreated cells (B); SSH-infected cells treated with 10^-5 M monensin (C).

**CONCLUSIONS**

Our results indicate that systems for the intracellular transport of viral membrane glycoproteins in MDCK cell monolayers differ in sensitivity to monensin. Influenza virus glycoproteins, HA...
and NA, are transported to the free apical surfaces via a monensin-resistant pathway, whereas the targeting of VSV G protein to the basolateral membrane domain involves a monensin-sensitive route. These distinct transport pathways are functional not only in singly-infected but also in doubly-infected MDCK monolayers. Our findings suggest that sorting of viral glycoproteins occurs at the stage when they exit from the Golgi apparatus enroute to the plasma membrane, since monensin is thought to block glycoprotein transport at the level of the Golgi complex. We have also observed a differential effect of monensin on enveloped viruses which assemble at intracellular membranes. The replication of IBV and its release from the membranes of the rough endoplasmic reticulum are inhibited, whereas monensin treatment of host cells has no effect on the formation of SSH virions. Possibly, the actual budding process of IBV may be more easily affected than that of SSH by changes in ionic gradients within the host cell that may occur as an effect of monensin treatment.

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