Modulation of Glycosylation and Transport of Viral Membrane Glycoproteins by a Sodium Ionophore

FIRELLI V. ALONSO-CAPLEN and RICHARD W. COMPANS
Department of Microbiology, University of Alabama in Birmingham, Birmingham, Alabama 35294

ABSTRACT Analysis of viral glycoprotein expression on surfaces of monensin-treated cells using a fluorescence-activated cell sorter (FACS) demonstrated that the sodium ionophore completely inhibited the appearance of the vesicular stomatitis virus (VSV) G protein on (Madin-Darby canine kidney) MDCK cell surfaces. In contrast, the expression of the influenza virus hemagglutinin (HA) glycoprotein on the surfaces of MDCK cells was observed to occur at high levels, and the time course of its appearance was not altered by the ionophore. Viral protein synthesis was not inhibited by monensin in either VSV- or influenza virus-infected cells. However, the electrophoretic mobilities of viral glycoproteins were altered, and analysis of pronase-derived glycopeptides by gel filtration indicated that the addition of sialic acid residues to the VSV G protein was impaired in monensin-treated cells. Reduced incorporation of fucose and galactose into influenza virus HA was observed in the presence of the ionophore, but the incompletely processed HA protein was cleaved, transported to the cell surface, and incorporated into budding virus particles. In contrast to the differential effects of monensin on VSV and influenza virus replication previously observed in monolayer cultures of MDCK cells, yields of both viruses were found to be significantly reduced by high concentrations of monensin in suspension cultures, indicating that cellular architecture may play a role in determining the sensitivity of virus replication to the drug. Nigericin, an ionophore that facilitates transport of potassium ions across membranes, blocked the replication of both influenza virus and VSV in MDCK cell monolayers, indicating that the ion specificity of ionophores influences their effect on the replication of enveloped viruses.

Cells infected with enveloped RNA viruses provide valuable systems for elucidating the pathways involved in subcellular transport of membrane glycoproteins (8, 20, 44). As a result of viral inhibition of host protein synthesis, only one or a few viral membrane glycoproteins are synthesized in infected cells. These viruses possess limited genetic capacity, and the glycosylation and transport of their membrane glycoproteins to the cell surface are probably carried out by the same systems used by the host cell for biogenesis of its own membrane glycoproteins.

Monovalent ionophores such as monensin, which is reported to interfere with the translocation of secretory as well as most membrane glycoproteins, have been used to characterize the pathways of intracellular glycoprotein transport in eucaryotic cells (18, 37–41, 43). In addition, the effects of monensin on IgM and H2 glycosylation in lymphoid cells and fibronectin glycosylation in cultured human fibroblasts, as well as its influence on oligosaccharide maturation of viral glycoproteins have also been reported (14, 19, 24, 38, 39). These effects are thought to be a consequence of inhibition of glycoprotein transport by the ionophore. We have recently provided evidence that, in polarized epithelial cells, systems for transport of glycoproteins of two enveloped viruses to the cell surface differ in their sensitivity to monensin (1). These results were obtained with enveloped viruses that insert their membrane glycoproteins into different plasma membrane domains in Madin-Darby canine kidney (MDCK)[1] cell monolayers: vesicular stomatitis virus (VSV), which forms at basolateral membranes, and influenza virus, which is assembled at the free apical surface (27). Replication of VSV in MDCK cells was inhibited markedly by monensin, with a block occurring in the transport of the G protein to the cell

[1] *Abbreviations used in this paper:* FACS, fluorescence-activated cell sorter; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; PBS-BSA, PBS def containing 1% BSA; PBS def, PBS deficient in calcium and magnesium; VSV, vesicular stomatitis virus.

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surface. In contrast, influenza virus maturation was not affected significantly, even at high concentrations of the ionophore.

To further analyze the effects of monensin on replication of these enveloped viruses, we investigated the effects of the ionophore on viral protein synthesis and the glycosylation of viral membrane glycoproteins. In addition, we studied the transport of viral membrane glycoproteins to the cell surface and the maturation of virions under conditions of monensin inhibition. We also compared the effect of monensin on virus assembly in monolayers vs. suspension cultures of MDCK cells, and determined the effect on virus replication of another ionophore that differs from monensin in ion specificity.

MATERIALS AND METHODS

Cells and Viruses: MDCK and Madin-Darby bovine kidney (MDBK) cells were grown by described procedures (32). BHK21 cells were grown as previously described (13). All cells were grown at 37°C in an atmosphere of 5% CO_2 in air. Stocks of influenza virus, A/WSN(H_1N_1) strain, were prepared in MDBK cells (7), and stocks of the Indiana strain of VSV were propagated in BHK21 cells (32). Infectivity titers of influenza virus were assayed by plaquing on MDCK cells (42) and of VSV by plaque assay on BHK21 cells (32).

Antibody Production: Two monoclonal antibodies prepared in our laboratory were used throughout this study: CB3-4, specific for the hemagglutinin (HA) protein of A/WSN influenza virus and DC3-22, specific for the G protein of the Indiana strain of VSV. The antigen used for immunization was prepared as follows: confluent cultures of MDCK cells were infected with either influenza virus or VSV, harvested at 8 h postinfection, and processed for membrane fractionation by described procedures (5). The fraction containing smooth membrane vesicles was mixed with complete Freund's adjuvant and used as antigen for the initial immunization of mice; in subsequent immunizations, smooth membrane vesicles in PBS were used in the absence of adjuvant. The immunization schedule and cell fusion procedure were carried out as described by Kearney et al. (15, 16). Enzyme-linked immunoadsorbent assay (ELISA) with smooth membrane fractions from either influenza virus- or VSV-infected MDCK cells as antigen was used for screening positive hybrids, and were then recloned by procedures detailed elsewhere (15, 16). Additional screening of recloned hybrids, e.g. indirect immunofluorescence as well as immunoprecipitation of polypeptides from virus-infected cells, was done to determine the specificity of the clones. The monoclonal antibodies were also found to show positive reaction with G or HA proteins from monensin-treated virus-infected cells by indirect immunofluorescence or immunoprecipitation. Monoclonal antibodies were obtained as ascites fluids of hybridoma-bearing mice.

Immunofluorescence and FACS Analysis: Confluent MDCK cell monolayers in 60-mm plastic petri dishes were washed twice with PBS and infected with either influenza virus at a multiplicity of infection (m.o.i.) of 1.0 or VSV (m.o.i. = 2.0). After a 2-h adsorption period, unadsorbed virus was aspirated and replaced with serum-free Dulbecco's medium with or without a 10^{-5} M final concentration of monensin. At intervals postinfection, virus-infected cells were washed twice with PBS deficient in calcium and magnesium (PBS def). A 30 mM solution of EGTA in calcium-free Eagle's minimal essential medium was then added and cells were incubated at 37°C from 30 min to 1 h. In the case of monensin-treated cells, the 30 mM EGTA solution also contained a final 10^{-5} M concentration of the ionophore. Rounded-up cells were transferred to conical centrifuge tubes and washed once with PBS def. Cells were fixed with 1% formaldehyde, washed twice, and reacted with 0.1 M lysine in PBS def. After washing, cells were resuspended in 100 μl of antiserum diluted with PBS def containing 1% BSA (PBS-BSA). Incubation with antiviral antibody was carried out for 20 min at room temperature. Cells were washed twice with PBS-BSA and resuspended in 100 μl of fluorescein-conjugated anti-mouse immunoglobulin. After a 20-min incubation at room temperature, cells were washed twice, fixed with 1% formaldehyde, and reacted with 0.1 M lysine. A suspension of $\sim 2 \times 10^6$ cells/ml was filtered through glass wool to obtain a single cell suspension before analysis in a FACS IV fluorescence-activated cell sorter.

Radiolabeling of Virus-infected Cells: Confluent MDCK or BHK21 cell monolayers in 35-mm or 60-mm dishes were washed twice with PBS and infected with either influenza virus or VSV (m.o.i. = 20). After adsorption at 37°C for 2 h, monensin was added to a final 10^{-5} M concentration. At times indicated in the text, cells were labeled with L-[4,5-^{3}H]leucine (59.2 Ci/mmol), D-[6-^{3}H]glucosamine (20.2 Ci/mmol), D-[2-^{3}H]mannose (18 Ci/mmol), L-[6-^{3}H]fucose (84 Ci/mmol), D-[1-^{3}H]galactose (8.2 Ci/mmol), or D-[1-^{14}C]glucosamine (57.9 mCi/mmol). At the end of the labeling period, cells were lysed in sample reducing buffer (0.0625 M Tris-HCl, pH 6.8 containing 0.5% 2-mercaptoethanol, 10% glycerol, and 2.3% SDS).

Polyacrylamide Gel Electrophoresis: Samples for gel electrophoresis were boiled for 2 min before loading. Either 7.5% or 10% polyacrylamide slab gels (17) were run for 16–18 h at an 8 mA-constant current per slab gel. Gels were fixed in 10% acetic acid-40% methanol in distilled water for 30 min, and prepared for fluorography (2). Gels were dried under vacuum and exposed on Kodak X-Omat AR films at −70°C.

Isolation of Viral Glycoproteins: [^{3}H]Glucosamine-labeled or [^{3}H]-mannose-labeled purified virions or virus-infected cell lysates were loaded on preparative 10% polyacrylamide slab gels with a single well that covered the width of the gel. At the end of the run, a 1-cm wide strip obtained by cutting the slab gel longitudinally at the center was sliced manually into 1-mm slices. Gel slices were soaked overnight at 37°C in 0.3 ml of 90% Protosol (New England Nuclear, Boston, MA); 4.0 ml of a toluene-Liquifluor (New England Nuclear) scintillation cocktail mixture was then added. Gel slices were counted in a Searle liquid scintillation counter, and the location of glycoprotein peaks was determined. The glycoproteins were eluted electrophoretically from the remaining gel using an ISCO protein concentrator (ISCO, Inc., Lincoln, NE). The glycoprotein samples were either lyophilized overnight or immediately digested with glycosidases.

Pronase Digestion and Gel Filtration of Viral Glycopeptides: Glycoprotein samples were incubated with pronase for 72 h at 60°C (10). Viral glycopeptides were then chromatographed on a Bio-Gel P6 gel filtration column (200–400 mesh, 1.0 cm × 115 cm) with a flow rate of 3.2 ml/h at 4°C. The columns were calibrated using [^{14}C]glucosamine-labeled Sindbis virus glycopeptides as molecular weight markers. 1-ml fractions were collected and 0.3-ml aliquots, to which 8.0 ml of Scintiverse (Fisher Scientific Co., Pittsburgh, PA) scintillation cocktail was added, were counted. Glycopeptide peaks of interest were pooled, lyophilized, and desalted on Bio-Gel P2 columns (50–100 mesh, 1.5 cm × 25 cm).

Neuraminidase Digestion: Desalted glycopeptide samples were dissolved in 0.5 ml of 0.1 M sodium acetate buffer (pH 5.5), digested with 100 μl of neuraminidase (500 U/ml) for 24 h at 37°C (22), and analyzed on Bio-Gel P6 columns.

Endo-β-N-acetylglucosaminidase-H (endo-H) Digestion: After desalting, glycopeptide fractions were pooled, lyophilized, and dissolved in 100 μl of 0.05 M citrate-phosphate buffer (pH 6.5) and digested with 0.02 U of endo-H for 24 h at 37°C (22), before chromatography on Bio-Gel P6 columns.

Chemicals and Isotopes: Monensin, nigericin, pronase, and neuraminidase were purchased from Calbiochem-Behring Corp, La Jolla, CA. Endo-H was obtained from Miles Laboratories, Inc, Elkhart, IN. [^{3}H]Leucine (59.2 Ci/mmol), [^{3}H]glucosamine (20.2 Ci/mmol) and [^{3}H]fucose (84 Ci/mmol) were purchased from New England Nuclear, Boston, MA, and [^{3}H]mannose (18 Ci/mmol), [^{3}H]galactose (8.2 Ci/mmol), and [^{14}C]glucosamine (57.9 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, IL.

RESULTS

Quantitative Analysis of Glycoprotein Expression on Surfaces of Virus-infected MDCK Cells Treated with Monensin

The expression of influenza virus HA glycoprotein and VSV G protein on surfaces of control or monensin-treated MDCK cell monolayers was quantitated with a FACS. The FACS plots of VSV-infected MDCK cells in the presence or absence of 10^{-5} M monensin (Fig. 1 *A–D*) show that the expression of G protein on surfaces of untreated cells increased progressively during the course of infection. In contrast, monensin-treated cells showed no fluorescence above background levels even at 10 h postinfection. Thus, monensin completely inhibited the transport of G protein to the surfaces of MDCK cells. On the other hand, the FACS profiles of MDCK cells infected with influenza virus (Fig. 1 *E–H*) revealed increasing levels of HA on cell surfaces as infection progressed, in the presence or absence of the ionophore. The time course of appearance of influenza virus HA protein from 6–10 h postinfection was apparently unaltered by the iono-

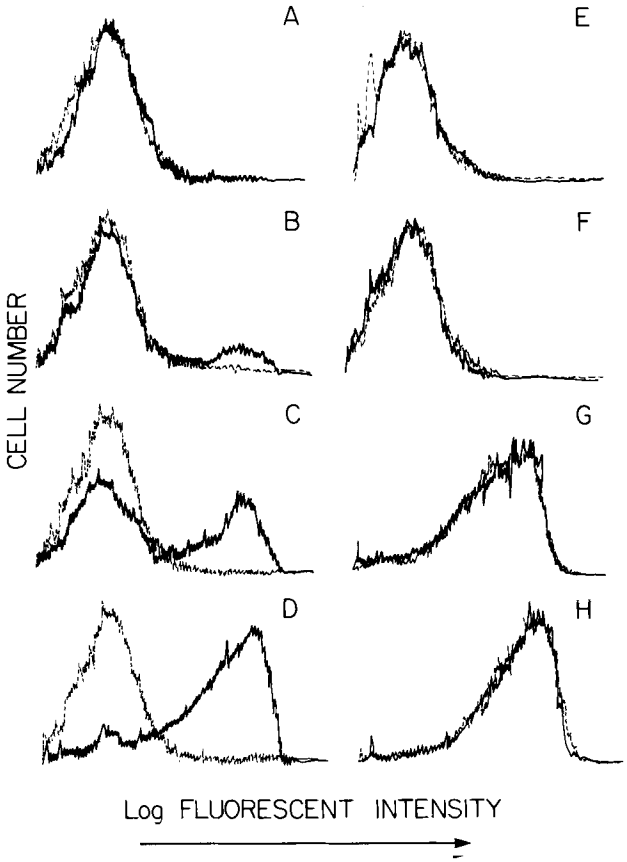

FIGURE 1 Effect of monensin on cell surface expression of viral membrane glycoproteins. (*A–D*) FACS profiles of VSV-infected MDCK cells (m.o.i. = 2.0) in the presence (- - -) or absence (——) of 10^{-5} M monensin. Cells were harvested at 6, 8, and 10 h postinfection and processed for indirect surface fluorescence as described in Materials and Methods. (*A*) Background fluorescence of uninfected MDCK cells (——) and VSV-infected cells at 8 h postinfection in which addition of the first antibody was omitted (- - -). (*B–D*) VSV-infected cells at 6 h (*B*), 8 h (*C*), and 10 h (*D*) postinfection; control (——); monensin-treated (- - -). (*E–H*) FACS profiles of influenza virus–infected MDCK cells (m.o.i. = 1.0) at 6, 8, and 10 h postinfection in the presence (- - -) or absence (——) of 10^{-5} M monensin. (*E*) Background fluorescence of uninfected MDCK cells (——) and influenza virus-infected cells at 8 h postinfection in which addition of the first antibody was omitted (- - -). (*F–H*) Influenza virus-infected cells at 6 h (*F*), 8 h (*G*), and 10 h (*H*) postinfection; control (——); monensin-treated (- - -).

phore, since the fluorescence intensity profiles of control and monensin-treated cells were virtually indistinguishable at each time point examined.

Even after short intervals following virus labeling, e.g., 20–60 min, influenza virus HA protein was already detected on the surface of monensin-treated MDCK cells. Influenza virus-infected cells were pulsed with [^{3}H]mannose and chased with 20 mM cold mannose in the presence or absence of the ionophore, and cell surface glycoproteins were analyzed by exposure of intact cells to anti-HA monoclonal antibody, followed by lysis and immune precipitation. No significant differences were found in the kinetics of appearance of HA on surfaces of control vs. monensin-treated cells (not shown).

Viral Protein Synthesis in Monensin-Treated Cells

To further analyze the effects of monensin on influenza virus and VSV replication, we examined the synthesis of viral proteins in monolayers of MDCK or BHK21 cells. In contrast to MDCK, BHK21 cells are fibroblastic and do not exhibit polarity of virus maturation; i.e. influenza virus or VSV assemble over most of the cell surface. As shown in Fig. 2, there was a slight increase in the electrophoretic mobility of G protein in VSV-infected MDCK cells treated with 10^{-6} M monensin; this shift was more pronounced in BHK21 lysates. No alteration was detected in any of the nonglycosylated viral proteins. In parallel experiments, a decrease in the molecular weight of influenza virus HA was also detected in monensin-treated BHK21 cell lysates. In influenza virus-infected MDCK cells, the control protein pattern showed two bands corresponding to HA, which we have designated as upper and lower HA bands. In monensin-treated MDCK cells, only the lower HA band was detected and it appeared to be more intense than the lower HA band of controls. These results suggest that changes in the glycosylation of viral glycoproteins occur in monensin-treated cells. As observed with VSV, synthesis of the nonglycosylated viral proteins appeared to be unaffected by the ionophore.

Pulse-chase analysis of influenza virus-infected MDCK cells in the presence or absence of 10^{-6} M monensin was used to further investigate the two forms of influenza virus HA glycoprotein shown in Fig. 2. After a 10-min pulse, only the lower HA band was detected (Fig. 3). Some of the lower form appeared to be converted into the upper HA band in a 30-min chase; after a 60-min chase period, most of the HA

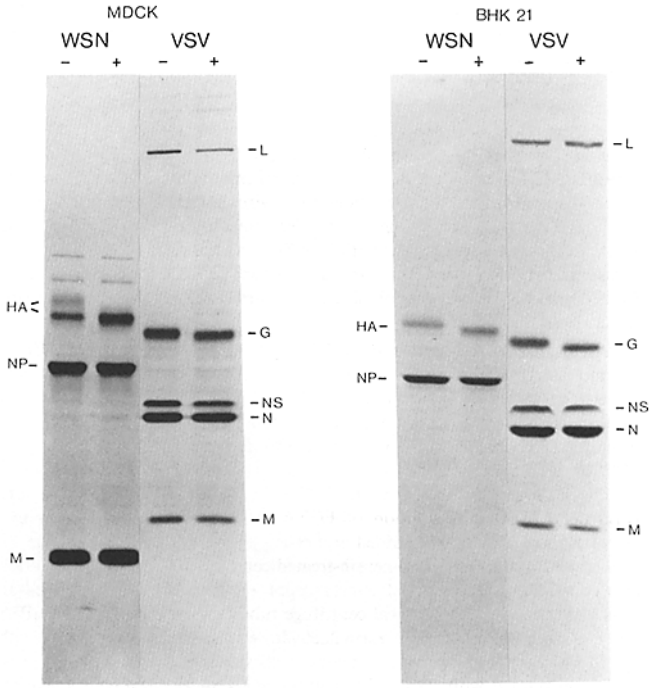

FIGURE 2 Synthesis of viral proteins in MDCK or BHK21 cells in the presence or absence of 10^{-6} M monensin. Control (−) or monensin-treated (+) cells were infected with either influenza virus (WSN strain) or VSV at an m.o.i. of 20. Infected MDCK cells were pulse-labeled for 1 h with [^{3}H]leucine at 7 h postinfection for WSN or at 9 h postinfection for VSV. BHK21 cells were labeled for 1 h with [^{3}H]leucine at 8 h postinfection for WSN or at 6 h postinfection for VSV. Cells were then lysed in sample reducing buffer described in Materials and Methods and analyzed on 7.5% polyacrylamide gels. N.B. Influenza M protein, present in both untreated and monensin-treated BHK21 cell lysates, had run off together with the dye marker in this gel.

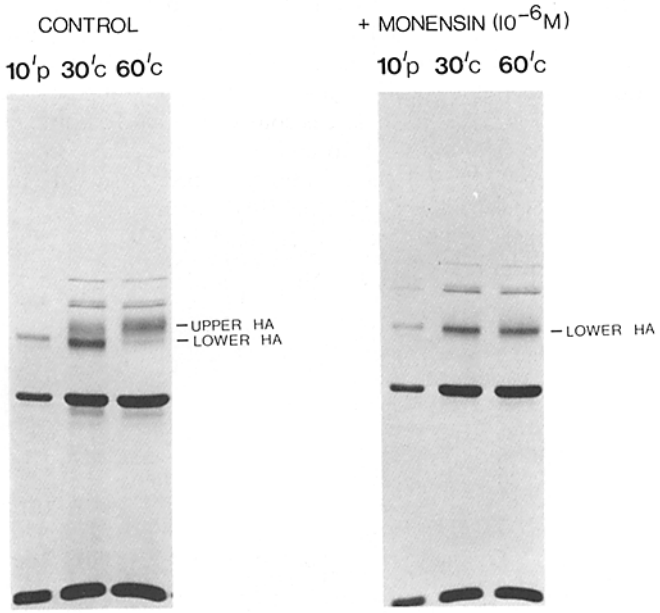

FIGURE 3 SDS PAGE analysis of processing of influenza virus HA in MDCK cells. Cells were pulsed for 10 min at 8 h postinfection (*10'p*) with [^{3}H]leucine in leucine-deficient Eagle's minimal essential medium in the presence or absence of 10^{-6} M monensin. Media containing 10-fold excess leucine with or without monensin was then added for a 30-min (*30'c*) or 60-min (*60'c*) chase period. Roughly 100,000 cpm of each cell lysate was analyzed on 7.5% polyacrylamide gels.

molecules were found in the upper band. These results suggest that the upper HA band is the mature form derived by processing of the lower HA band. In the presence of 10^{-6} M monensin, the lower band was not chased into the upper HA band even after 60 min.

Glycosylation of VSV G in MDCK and BHK21 Cells

The faster electrophoretic mobility of VSV G protein in monensin-treated cells suggested possible alterations in its oligosaccharide processing. To further investigate this phenomenon, we analyzed the oligosaccharides of G protein isolated from VSV-infected MDCK or BHK21 cells radiolabeled with [^{3}H]- or [^{14}C]glucosamine sugar precursors for 4 h in the presence or absence of 10^{-5} M monensin. Chromatography of glycopeptides obtained after extensive pronase digestion of G protein from untreated VSV-infected MDCK cells (Fig. 4*A*) revealed a major glycopeptide size class of ~2,800–4,200 mol wt (fractions 45–53) with several shoulders of lower mol wt components that probably differ in sialic acid content, as reported by other investigators (4, 9). In the case of G from monensin-treated cells, two major glycopeptide size classes were resolved with molecular weights of 2,400–4,000 (fractions 46–55) and 1,600–2,200 (fractions 57–63), respectively. Glycopeptides in G protein from control or monensin-treated cells were found to be predominantly resistant to endo-H, indicating that they contain complex sugars (data not shown). The larger glycopeptide size classes were sensitive to digestion

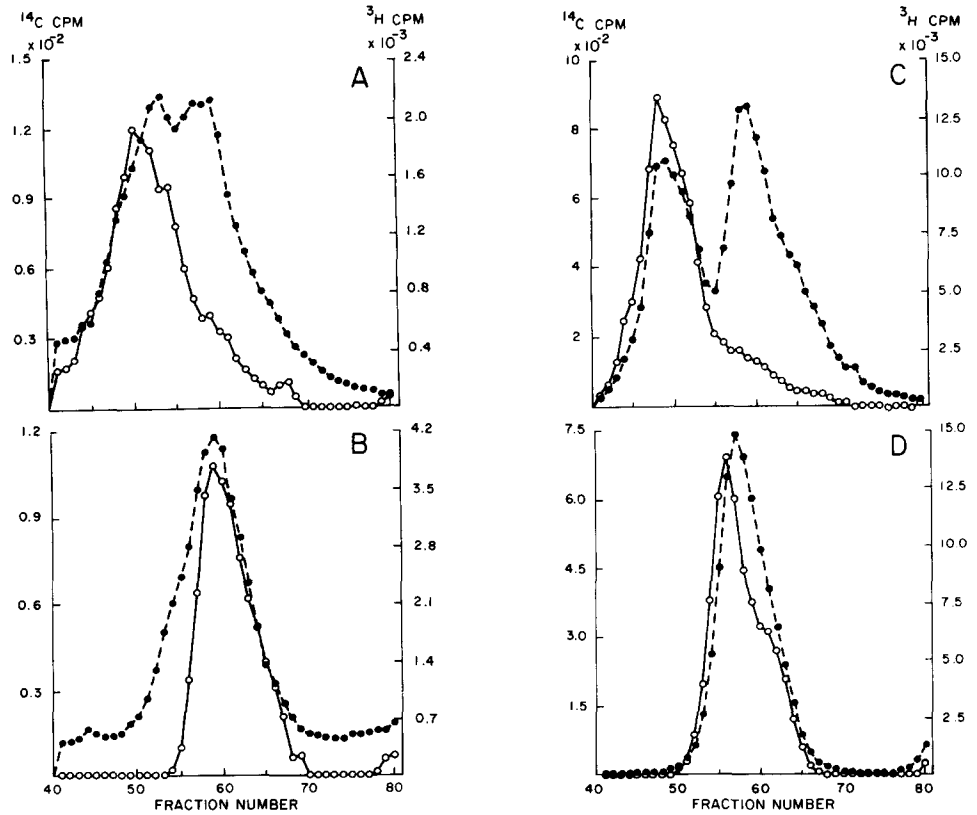

FIGURE 4 Glycopeptides of VSV G protein from untreated or monensin-treated MDCK or BHK21 cells. Cells were infected with VSV at an m.o.i. of 20 and labeled for 4 h at 6 h postinfection (MDCK) or at 4 h postinfection (BHK21) with either [^{14}C]glucosamine (control, O) or [^{3}H]glucosamine (+ 10^{-5} M monensin, ●). VSV G protein was isolated as described in Materials and Methods. The control and monensin-treated glycoproteins were co-digested with pronase and analyzed by gel filtration on Bio-Gel P6. One-third of the volume from each fraction was used for radioactivity determination, and the remainder was pooled, lyophilized, and desalted before digestion with neuraminidase. The neuraminidase-treated G glycopeptides were rechromatographed on Bio-Gel P6 columns, and the entire fractions were counted. (*A*) Pronase digests from control and monensin-treated MDCK cells. (*B*) Neuraminidase digests of glycopeptides from control and monensin-treated MDCK cells. (*C*) Pronase digests from control and monensin-treated BHK21 cells. (*D*) Neuraminidase digests of glycopeptides from control and monensin-treated BHK21 cells.

with neuraminidase (Fig. 4*B*), resulting in lower mol wt species. However the smaller glycopeptides present in G protein from monensin-treated cells were apparently not affected by neuraminidase, indicating that they do not contain sialic acid residues. Similar observations were made with G protein isolated from VSV-infected BHK cells (Fig. 4*C–D*). The control G glycoprotein contained a major glycopeptide peak (fractions 45–54) of ~2,600–4,200 mol wt (Fig. 4*C*) while G protein from monensin-treated cells yielded two major size classes with apparent mol wts of 2,800–4,000 (fractions 46–53) and 1,600–2,300 (fractions 56–63). The larger glycopeptides contained sialic acid residues as evidenced by their sensitivity to digestion with neuraminidase, whereas the smaller glycopeptides in G from monensin-treated cells were again found to be neuraminidase-resistant (Fig. 4*D*). These results indicate that monensin impairs the addition of sialic acid to a significant fraction of oligosaccharides on the G glycoprotein in infected MDCK or BHK21 cells.

Glycosylation of Influenza Virus HA in MDCK and BHK21 Cells

The absence of the upper HA band in monensin-treated MDCK cells and the faster electrophoretic mobility of HA protein in BHK21 cells exposed to monensin suggested alterations in glycosylation of HA in the presence of the ionophore. To investigate this possibility, we analyzed the glycopeptides obtained by pronase digestion of HA glycoproteins. It has been previously reported that influenza HA contains two types of oligosaccharide chains: type I (complex) oligosaccharides contain glucosamine, galactose, mannose, and fucose, whereas type II oligosaccharides consist of a high amount of mannose in addition to glucosamine, but lack galactose and fucose (23). Type II glycopeptides have been resolved into two size classes, designated IIa and IIb. In the upper HA band from untreated influenza virus-infected MDCK cells (Fig. 5*A*), we observed a major glycopeptide size class with an apparent mol wt of ~2,800–3,500 corresponding to type I glycopeptides (fractions 48–53), two additional peaks in the type II region with mol wts of 2,100–2,200 (fractions 55–58) and 1,500–1,900 (fractions 60–64) and a shoulder in the 1,300–1,500 region (fractions 66–68). The lower HA band from monensin-treated cells contained glycopeptides of ~2,100–3,000 daltons (fractions 51–58) and shoulders of glycopeptides with mol wts of ~1,700–2,000 (fractions 59–62) and 1,300–1,600 (fractions 63–66). Endo-H treatment of HA glycopeptides from control or monensin-treated cells (Fig. 5*B*) indicated that the larger glycopeptide size classes were of the complex type while the smaller glycopeptides were mannose-rich. The minor peak in fractions 68–75 in Fig. 5*B* presumably represents mannose-rich side chains that are cleaved from the glycopeptides by endo-H digestion. The molecular weights of the cleavage products from untreated cells were found to be slightly larger than those of HA from monensin-treated cells.

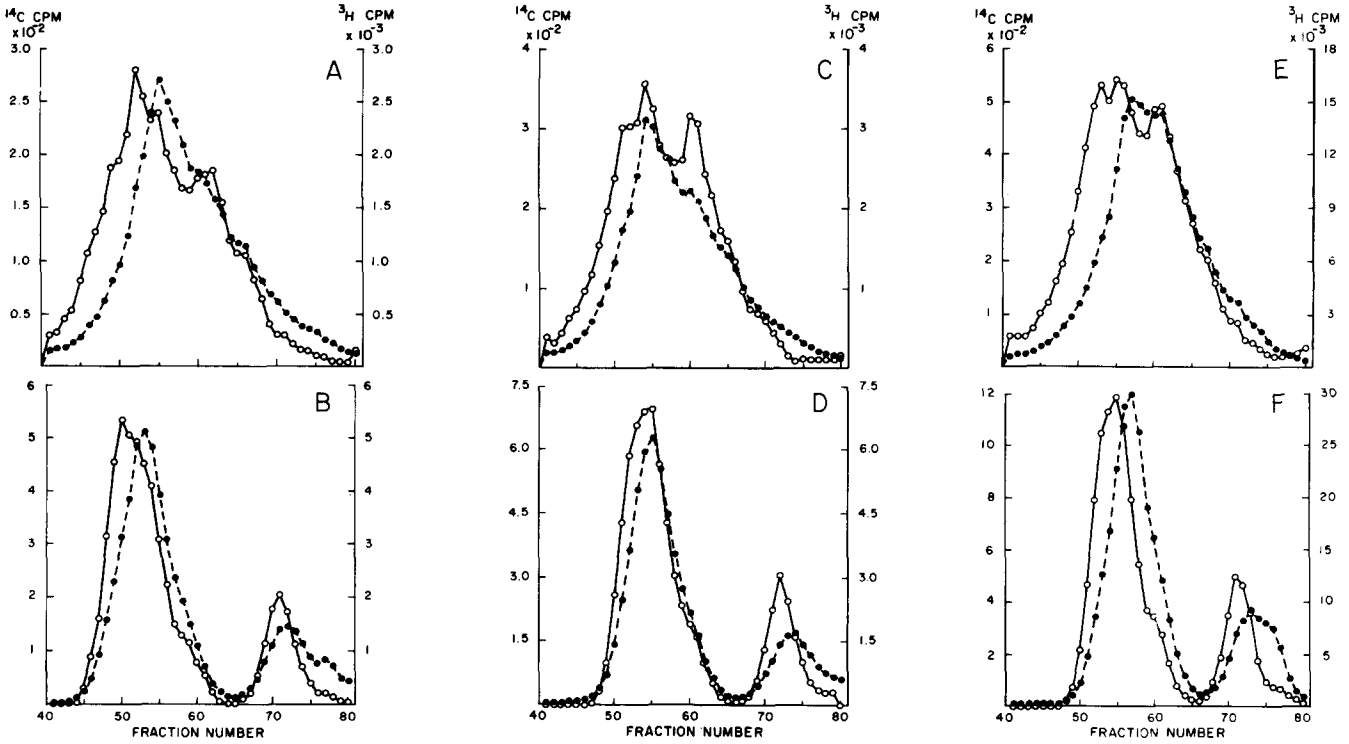

FIGURE 5 Glycopeptides of influenza virus HA from untreated or monensin-treated MDCK or BHK21 cells. Cells were infected with influenza virus (m.o.i. = 20) and labeled for 4 h at 4 h postinfection (MDCK and BHK21) with either [^{14}C]glucosamine (control, O) or [^{3}H]glucosamine (+10^{-5} M monensin, ●). Influenza virus HA glycoproteins isolated from untreated and monensin-treated cells were co-digested with pronase and analyzed as described in the legend to Fig. 4. Aliquots were digested with endo-H and rechromatographed on Bio-Gel P6 (*A*) Pronase digest of upper HA from untreated and lower HA from monensin-treated MDCK cells. (*B*) Endo-H digests of upper HA (control) and lower HA (monensin-treated) glycopeptides. (*C*) Pronase digests of lower HA from control and monensin-treated MDCK cells. (*D*) Endo-H digests of lower HA glycopeptides from control and monensin-treated MDCK cells. (*E*) Pronase digests of HA from control and monensin-treated BHK21 cells. (*F*) Endo-H digests of HA glycopeptides from control and monensin-treated BHK21 cells.

Gel filtration of glycopeptides derived from the lower HA band isolated from untreated MDCK monolayers (Fig. 5*C*) revealed type I glycopeptides of 2,200–3,500 mol wt (fractions 48–57) and type II glycopeptides with mol wt of 1,400–1,900 (fractions 60–65). A major peak with an apparent mol wt of 2,100–3,300 (fractions 49–58) and a shoulder of roughly 1,400–1,900 (fractions 60–66) was observed in the lower HA band from monensin-treated cells. The smaller glycopeptides were endo-H sensitive, i.e. mannose-rich, as shown in Fig. 5*D*, while the major glycopeptide size class was endo-H resistant. These data, in conjunction with the results of the pulse-chase experiments, suggest that the lower HA band is an incompletely processed form of the upper HA band.

Glycopeptides with mol wt of 2,200–3,500 (fractions 48–57) and 1,500–2,000 (fractions 59–64) were resolved from the HA glycoprotein isolated from untreated BHK21 cells infected with influenza virus (Fig. 5*E*). Gel filtration of HA glycopeptides from monensin-treated cells revealed a peak with mol wt of 2,100–2,300 (fractions 51–58) and a shoulder of 1,300–1,900 (fractions 60–66). Endo-H digestion of these glycopeptides (Fig. 5*F*) indicated that only the smaller glycopeptides contained mannose-rich oligosaccharides.

These results indicate that in monensin-treated cells, HA glycoproteins contain truncated oligosaccharide chains that remain endo-H resistant. To investigate the changes in oligosaccharide composition, we pulse-labeled influenza virus-infected MDCK cells with various sugar precursors in the presence or absence of 10^{-5} M monensin. The results (Fig. 6) show that the addition of the terminal sugars fucose and galactose was significantly impaired in the presence of monensin. Densitometer scans of the fluorograph (not shown) indicated about a twofold inhibition in incorporation of [^{3}H]fucose into HA, and more than a 10-fold inhibition in incorporation of [^{3}H]galactose. In contrast, labeling with [^{3}H]-glucosamine was not markedly inhibited in the presence of the ionophore. We also observed that incorporation of [^{3}H]mannose into the lower HA band either in control or monensin-treated cells was two- to fourfold higher than into the upper HA band, suggesting that trimming of mannose residues from mannose-rich oligosaccharides is involved in conversion of the lower to the upper form.

Assembly of Virions in Monensin-Treated MDCK Cells

We investigated whether the lower HA band observed as the major species in monensin-treated MDCK cells was the form of HA that was transported to the cell surface and subsequently incorporated into budding virus particles. We purified influenza virions grown in MDCK cells in the presence of absence of 10^{-5} M monensin. Fig. 7 shows that influenza virions obtained from monensin-treated cells contained the lower HA band; in contrast, virions from untreated cells contained the upper HA band. Cleavage of the HA glycoprotein into HA_1 and HA_2 continued to occur in the presence of the ionophore, and a shift in the electrophoretic mobility of HA_1 was observed in virions from monensin-treated cells; no alteration in molecular weight was detected in HA_2. The HA_2 glycoprotein is known to contain only one oligosaccharide chain and therefore, a block in the addition of one or two terminal sugars by monensin would not be easily detected. Analysis of the glycopeptides indicated that virions from monensin-treated cells contained HA with incompletely processed complex oligosaccharides (Fig. 8*A–B*).

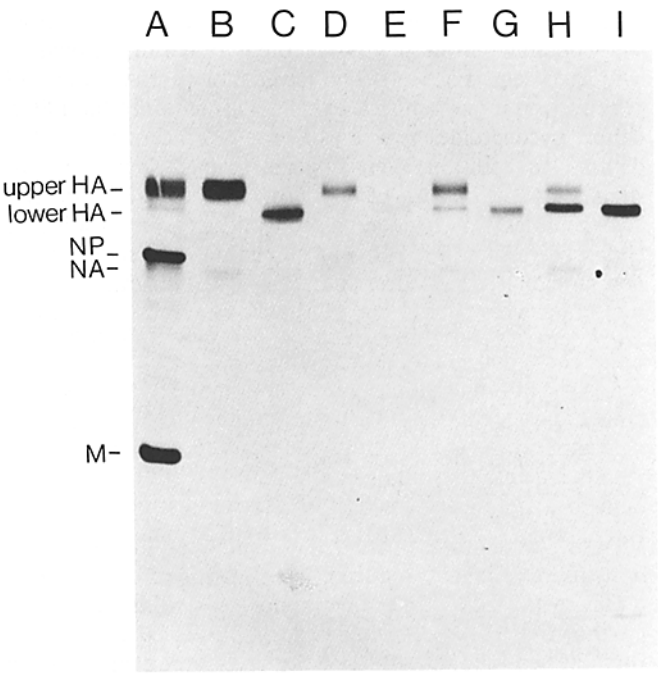

FIGURE 6 Glycosylation of influenza virus HA in untreated or monensin-treated MDCK cells. Influenza virus-infected cells (m.o.i. = 20) were pulse-labeled for 1 h at 7 h postinfection with 100 μCi/dish of various ^{3}H-sugars, with or without 10^{-5} M monensin. Equal volumes of cell lysates were analyzed on 10% polyacrylamide gels Lane *A*, [^{3}H]leucine-labeled influenza virus-infected cell lysate used as marker; lanes *B* (– monensin) and C (+monensin), [^{3}H]fucose-labeled; lanes *D* (–) and *E* (+), [^{3}H]galactose-labeled; lanes *F* (–) and *G* (+), [^{3}H]glucosamine-labeled; and lanes *H* (–) and *I* (+), [^{3}H]mannose-labeled.

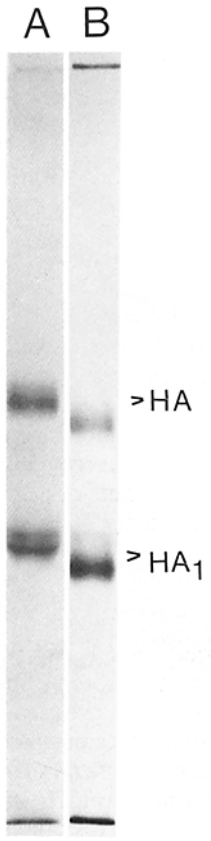

FIGURE 7 Comparison of the envelope glycoproteins of influenza virions from control and monensin-treated MDCK cells. Influenza virus-infected cells (m.o.i. = 10) were incubated in Eagle's minimal essential medium containing 10 μCi/ml of [^{3}H]glucosamine, with or without 10^{-5} M monensin. Virus was harvested after 24 h, purified and analyzed on a 7.5% polyacrylamide gel; ~40,000 cpm of each virus preparation was used. Control, lane *A*; monensin-treated, lane *B*. The HA_2 protein migrated together with the dye front in this gel.

Two glycopeptide size classes of ~2,900 and 1,800 mol wt were obtained from the HA protein of virions grown in untreated cells (Fig. 8*A*). The lower mol wt HA protein of virions from monensin-treated cells also contained the 1,800-dalton glycopeptides as well as a major size class of ~2,400 daltons. In addition, there appears to be a lower mol wt shoulder (fractions 61–65) in both upper and lower HA glycopeptides. The smaller glycopeptides were sensitive to endo-H digestion (Fig. 8*B*), indicating that they contain mannose-rich oligosaccharides. These results demonstrate that HA molecules with altered complex oligosaccharides are transported to the cell surface and incorporated into influenza virions in monensin-treated cells.

Although VSV virions were not detected at surfaces of monensin-treated MDCK cells by electron microscopy (1), we investigated whether low levels of virons could be obtained from such cells. As shown in Fig. 9, virus particles were purified from monensin-treated cells and they contained all VSV structural proteins, but the amounts of radiolabeled viral proteins were greatly reduced. These particles contained G protein with slightly faster electrophoretic mobility, similar to the incompletely glycosylated G seen in infected cells treated with the ionophore. Although the protein content of virus preparations from monensin-treated cells was only reduced by ~50%, a large amount of this represented contaminating cellular material. A number of cellular protein bands were evident in stained gels (not shown) or fluorographed protein gels of VSV particles purified from monensin-treated cells; the specific infectivity (plaque-forming units per μg of protein) of these particles was only 3% of the control value. Since monensin blocks the appearance of G protein on the cell surface, and virus budding at cell surfaces was not observed, it is possible that some VSV virions may have been formed intracellularly in monensin-treated cells.

Comparison of the Effect of Monensin on Replication of Influenza Virus and VSV in Monolayer vs. Suspension Cultures of MDCK Cells

We determined the effects of monensin on virus yields from MDCK cells in a suspension culture system. As shown in Fig. 10, yields of both influenza virus and VSV grown in suspension cultures of MDCK cells were significantly reduced at high concentrations of the ionophore. It was also observed that in the controls, infectivity titers of both viruses propagated in MDCK suspension cultures were ~10-fold lower than virus yields obtained from MDCK monolayers. In parallel experiments, yields of infectious VSV obtained from MDCK monolayers were significantly inhibited by monensin, whereas influenza virus replication was unaffected, as reported previously (1). These results indicate a significant role of cellular architecture in determining the sensitivity of enveloped virus replication to monensin.

Effect of Nigericin on Virus Yields

Another monovalent ionophore, nigericin, has been reported to inhibit the secretion of procollagen and fibronectin from cultured human fibroblasts (43), as well as to preferentially block secretion of acetylcholinesterase in chick embryo skeletal muscle cultures, but not the appearance of the acetylcholine receptor, an integral membrane protein (37). In another report, however, it was found that nigericin disrupted

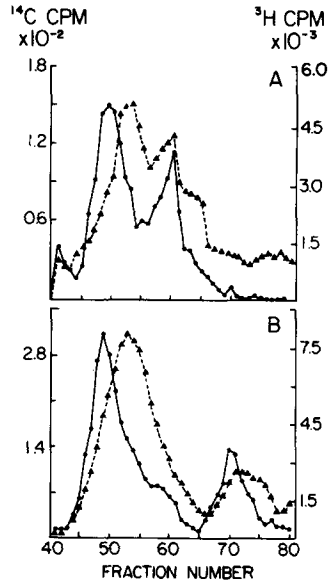

FIGURE 8 Analysis of HA glycopeptide profiles from purified influenza virions grown in untreated or monensin-treated cells. Purified influenza virus particles were prepared as described in the legend to Fig. 6. However, virions from untreated cells were labeled with [^{14}C]glucosamine and virions from monensin-treated cells were labeled with [^{3}H]glucosamine. HA glycoproteins isolated from influenza virus particles grown in control (●) or monensin-treated (▲) cells were co-digested with pronase and processed for gel filtration as described in the legend to Fig. 4 (*A*) Pronase digests of HA glycoproteins of influenza virions from control or monensin-treated cells. (*B*) Endo-H digests of HA glycopeptides of influenza virions from control or monensin-treated cells.

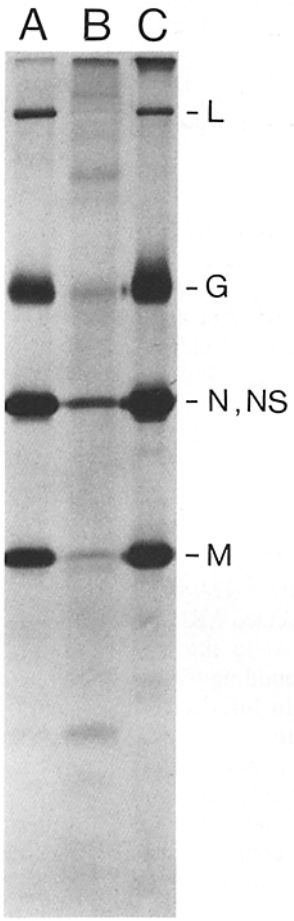

FIGURE 9 Comparison of protein profiles of purified VSV virions from untreated or monensin-treated MDCK cells. Cells were infected with VSV at an m.o.i. of 10. After a 1 h adsorption period at 37°C, unadsorbed virus was aspirated and replaced with 40% leucine-deficient Eagle's minimal essential medium containing 5 μC/ml of [^{3}H]leucine, with or without 10^{-5} M monensin. Virus was harvested at 24 h postinfection; an aliquot of the culture supernatant was used for determination of the infectivity titer and the remainder was purified on a 5–40% linear potassium tartrate gradient. About 40,000 cpm each of virions from control (lane *A*) or monensin-treated cells (lane *B*) were analyzed on 10% polyacrylamide gels. The structural proteins of [^{3}H]leucine-labeled VSV grown in BHK21 cells are shown as standards (lane *C*).

the transport of both the acetylcholine receptor and acetylcholinesterase enzyme (35). Nigericin is selective for potassium ions, whereas monensin facilitates sodium ion transport across membranes (25). We observed that treatment of infected MDCK cells with concentrations of nigericin between 10^{-8} and 10^{-6} M reduced the yields of VSV by about twofold

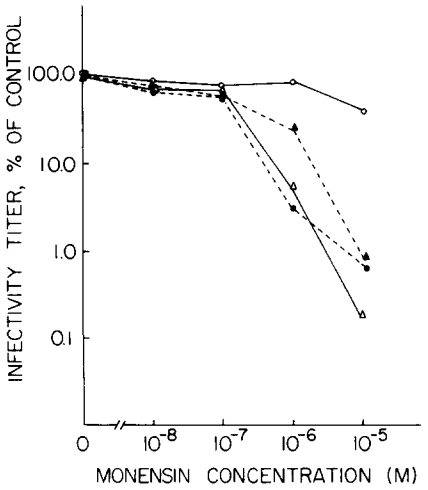

FIGURE 10 Comparison of the effect of monensin on virus yields from MDCK monolayer and suspension cultures. Confluent MDCK cell monolayers were infected with either influenza virus or VSV (m.o.i. = 10). MDCK suspension cultures were prepared by treating monolayers with 0.1% EDTA in PBS def for 30 min at 37°C. Cells were washed twice in PBS def and resuspended in Eagle's minimal essential medium modified for suspension cultures. Cells were continuously rotated in a "Labquake" rotator at 37°C. Monensin was added postadsorption to monolayers or after final pelleting to suspension cultures. After 24 h, virus yields were measured by plaque assays. Influenza virus titers, monolayers (○); VSV titers, monolayers (△); influenza virus titers, suspension (●); VSV titers, suspension (▲). At the 100% point, the influenza virus titers were 3.9×10^7 plaque-forming unit (pfu)/ml (monolayer), 8.7×10^6 pfu/ml (suspension), and the VSV titers were 1.3×10^8 pfu/ml (monolayer), 9.8×10^7 pfu/ml (suspension). Note that treatment of MDCK cells for up to 24 h with low concentrations of monensin, e.g., 10^{-8} to 10^{-6} M ,does not significantly affect cell viability. Prolonged treatment of cells with 10^{-5} M monensin results in a 60–70% cell viability, however, the monensin-treated MDCK cells remain as a monolayer with two well-defined surface domains.

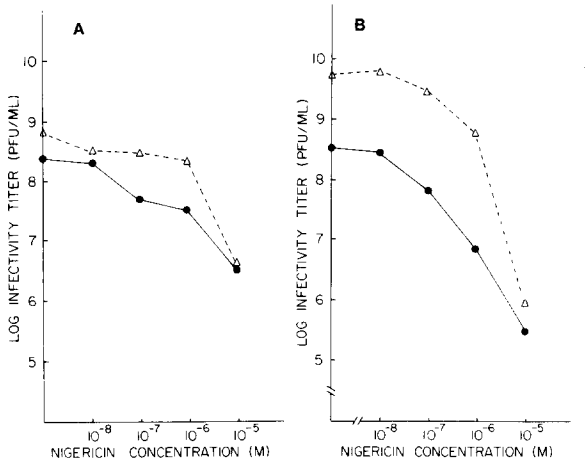

FIGURE 11 Effect of nigericin on yields of infectious influenza virus and VSV in MDCK or BHK21 cells. Nigericin was added postadsorption to either MDCK (*A*) or BHK21 (*B*) cells infected with influenza virus or VSV (m.o.i. = 10). Culture fluids were harvested at 24 h postinfection and infectivity titers were determined by plaque assays. Influenza virus titers (●); VSV titers (△).

and the yields of influenza virus by two- to tenfold (Fig. 11*A*). Both influenza virus and VSV replication in MDCK cells were found to be significantly inhibited by 10^{-5} M nigericin, in contrast to results obtained with monensin. The infectivity titers of both viruses were also reduced in parallel experiments performed on BHK21 cells (Fig. 11*B*).

DISCUSSION

We have used ionophores as probes to investigate the intracellular transport of viral glycoproteins. Our previous finding that VSV G protein transport to the cell surface is blocked by monensin, whereas influenza virus assembly and glycoprotein transport are not, suggested that distinct pathways exist for transport of glycoproteins to the plasma membranes of MDCK cells (1). We also found that influenza virions assembled only at the apical surface of monensin-treated MDCK cells, suggesting that HA glycoprotein is localized in this membrane domain. The present results indicate that although the movement of influenza virus HA to the apical surfaces of MDCK monolayers is unaffected by monensin, glycosylation of HA is altered, i.e., the addition of fucose and galactose is impaired. Evidence has been obtained that the inhibitory effects of monensin are at the point of exit of proteins from the Golgi complex en route to the plasma membrane (41, 43). Recently, it was reported that monensin inhibited the transport of Semliki Forest virus glycoproteins from the medial to trans Golgi cisternae (11). Sugar transferases involved in the addition of terminal sugars to glycoproteins, e.g., galactosyl transferase, are located in the distal compartments of the Golgi apparatus (30); the possibility that some sugar transferases are inactive in monensin-treated cells seems unlikely since VSV oligosaccharides appear to be complete except for the addition of sialic acid. Our data therefore strongly suggest that the pathway used for the transport of HA to the surfaces of monensin-treated cells bypasses at least some elements of the Golgi complex, thus accounting for the virtual absence of galactose. The incompletely glycosylated HA proteins are cleaved into HA_1 and HA_2, and subsequently incorporated into budding influenza virions. The alterations in glycosylation of G protein by monensin are a consequence, rather than a cause of the block in its transport to the cell surface, since it was previously shown that complete inhibition of glycosylation by tunicamycin does not prevent VSV maturation (31). Our results indicate that the pathway for transport of G protein to the cell surface is different from that used by influenza virus HA protein, and is inhibited by monensin at the distal compartments of the Golgi complex. Although sialyl transferases have been observed to be coisopycnic with galactosyl transferase, a marker enzyme of the distal Golgi complex (3), it is possible that additional sialylation may occur after the glycoproteins exit from the Golgi apparatus, which would account for the partial inhibition of glycosylation of the G protein.

Many cell lines of diverse epithelial origin, with morphologically well differentiated apical and basolateral surfaces and intercellular junctions, exhibit properties of natural transporting epithelia in culture (12). When MDCK cells were grown on collagen-coated nylon nets, they developed a transepithelial electrical resistance, a transepithelial electrical potential, and a preferred selectivity for sodium over chloride ions (21). The presence of intact tight junctions correlated well with the ability to develop transepithelial electrical resistance and to form "domes" or hemicysts (6). Experiments involving infection of dissociated MDCK cells with either influenza virus or VSV (28, 29) have indicated that attachment to a substrate or to adjacent cells is important for expression of epithelial

polarity. To investigate the role of epithelial polarity in the differential effects of monensin on the yields of viruses, we grew influenza virus and VSV in suspension cultures of MDCK cells. The results reveal a significant influence of cellular architecture in determining the resistance of influenza virus replication to monensin. It is possible that disruption of the normal transepithelial flow of ions as a result of suspension of MDCK cells may alter the sensitivity of enveloped viruses to monensin. In addition, a reduction in both virus yields was observed in untreated suspension cells, suggesting that the interaction of the epithelial cells with a substratum is important for efficient virus replication.

Reports that noninfectious VSV particles lacking viral glycoprotein are produced by cells infected with *ts* 045 mutants of VSV at restrictive temperature (36) raise questions about the requirement for the viral glycoprotein in virus maturation at the cell surface. Our evidence indicates that G protein and all the other nonglycosylated proteins of VSV continue to be synthesized in the presence of monensin; however, transport of G protein to the cell surface is effectively blocked by the ionophore, and the production of VSV particles in MDCK cells is significantly reduced. The G protein probably accumulates in the Golgi apparatus as a result of the block in its transport to the cell surface. VSV particles released from drug-treated cells contained G protein with a slightly faster electrophoretic mobility, like the form that was seen in total lysates of monensin-treated cells; virions lacking G were not detected, nor was virus budding observed at the cell surface. These data suggest that G protein at least facilitates the maturation of VSV virions, and that virions released from monensin-treated cells may have formed intracellularly, perhaps at membranes of the Golgi complex.

A central question is where in the cell does sorting of viral glycoproteins take place. Immunoelectron microscopy studies with ultrathin frozen sections of MDCK cells doubly infected with influenza virus and VSV have been reported to show that HA and G proteins share a similar intracellular pathway of transport at least to the Golgi complex (26). Our data are consistent with the idea that sorting of glycoproteins destined for transport to either the apical or basolateral membrane domains of MDCK cells occurs within the Golgi apparatus. The fact that polarized maturation of viruses was maintained in MDCK cells doubly infected with influenza virus and VSV also suggested that viral membrane glycoproteins were segregated into different sets of transport vesicles before their insertion into distinct plasma membrane domains (33). There is considerable evidence that the Golgi complex plays an important role in the segregation of various kinds of proteins destined for different organelles (34). The possibility that apical and basolateral glycoproteins are inserted over the entire surface of the MDCK cell, and later are sorted out to the "correct" membrane domain by specific retrieval mechanisms, can not yet be excluded, although we have not been able to detect newly synthesized viral glycoproteins at the membrane domain other than that where budding occurs. We suggest that different populations of transport vesicles that contain different sets of viral glycoproteins are formed at the membranes of the Golgi, and that such vesicles are targeted to the apical or basolateral regions of the plasma membrane. Further information on the nature of these vesicles should provide direct evidence for the existence of at least two distinct pathways of intracellular transport of membrane glycoproteins to surfaces of polarized epithelial cells.

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