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Polarity of influenza and vesicular stomatitis virus maturation in MDCK cells: Lack of a requirement for glycosylation of viral glycoproteins*

(enveloped viruses/virus assembly/glycosylation inhibitors/membrane biogenesis)

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ABSTRACT We have investigated whether glycosylation of membrane glycoproteins is a determinant of the site of maturation of enveloped viruses in Madin–Darby canine kidney (MDCK) cells. In MDCK cell monolayers, vesicular stomatitis virus buds exclusively from the basal or lateral plasma membranes and contains a sialylated glycoprotein, whereas influenza virus buds exclusively from the apical plasma membrane and lacks neuraminic acid. In order to study the possible relationship between glycosylation of viral glycoproteins and the budding site, infected MDCK cells were treated with tunicamycin at a concentration that completely inhibits glycosylation of viral glycoproteins and the site of virus maturation was examined by electron microscopy. When tunicamycin-treated monolayers were compared to controls, the polarity in the maturation sites of both viruses was maintained. These results indicate that glycosylation of viral glycoproteins is not required for the determination of the cellular maturation site of these enveloped viruses.

Enveloped RNA viruses, which mature by budding from host cell plasma membranes, provide excellent systems for the investigation of processes by which cells synthesize and transport membrane-associated proteins. These viruses code for only a small number of proteins, and host cell mechanisms presumably are involved in transport of viral components to the plasma membrane during the assembly process (1, 2). Many such viruses also inhibit host cell synthesis, thus facilitating the study of intracellular migration processes and insertion of viral proteins into cellular membranes.

In early electron microscopic studies of the maturation of influenza virus in the chorioallantoic membrane of infected chicken embryos (3), it was observed that virus budding occurred at the free luminal cell surfaces. Recently, similar polarity in enveloped virus maturation has been observed in epithelial cells grown *in vitro* (4). Monolayers of the epithelial Madin–Darby canine kidney (MDCK) line form tight junctions between adjacent cells and exhibit an electrical potential between the upper and lower surfaces (5–7). Further, the junctional complexes form a barrier between the apical and the basolateral cell surfaces. It has recently been reported (4) that vesicular stomatitis virus (VSV) buds exclusively from the basolateral membranes in these cells, whereas influenza and parainfluenza viruses bud exclusively from the free apical surface. MDCK cell monolayers also exhibit fluid transport phenomena and contact inhibition characteristic of normal renal tubular epithelia (5–8). The functional polarity of such epithelial surfaces requires restriction of certain enzymes to specific regions of the plasma membranes (9), and it is likely that the polarity of enveloped virus maturation in MDCK cells involves the same mechanisms by which normal epithelial cells direct different proteins to specific regions of the plasma membrane.

A significant difference exists in the carbohydrate components of the viruses that bud from the apical in contrast to the basolateral surfaces of MDCK cells. Influenza and parainfluenza viruses contain a neuraminidase and, therefore, lack sialic acid (10, 11). In contrast, VSV, which buds from the basolateral membranes, lacks a neuraminidase and contains a sialylated glycoprotein (10, 12). Therefore, features of the carbohydrate side chains of viral glycoproteins could play a role in determining their destinations (4). We have investigated this possibility by treatment of infected MDCK cells with tunicamycin, a glucosamine-containing antibiotic that inhibits the formation of *N*-linked oligosaccharides in glycoproteins by blocking the earliest step in the transfer of sugars to the nascent polypeptide (13, 14). Previous studies in our laboratory have demonstrated that, at a concentration of tunicamycin that completely inhibits glycosylation, the hemagglutinin glycoprotein of influenza virus is inserted into cellular membranes and undergoes migration to the cell surface (15). In addition, virion formation can be observed in tunicamycin-treated cells infected with either influenza virus or VSV (15, 16). These previous observations indicate that tunicamycin can be used to examine the role of glycosylation in determining the cellular maturation sites of these viruses.

MATERIALS AND METHODS

Cells and Viruses. MDCK cells were obtained from Flow Laboratories (McLean, VA) and grown as monolayers in Eagle's minimal essential medium supplemented with 2% newborn calf serum (Biocell, Carson, CA). Cells were grown at 37°C in an atmosphere of 5% CO_2 in air. BHK 21-F cells were grown as described (17).

Plaque-purified VSV of the prototype Indiana serotype was obtained from D. H. L. Bishop. VSV stock virus was prepared by infecting confluent monolayers of BHK 21-F cells with a 1/1000 dilution of the original virus and collecting the supernatant fluid after 24 hr. Stocks of the Ao/WSN (H_0N_1) strain of influenza virus were grown in Madin–Darby bovine kidney (MDBK) cells as described (18). Titers of both virus stocks were assayed by plaquing on MDCK cells by a modification of the method of Tobita *et al.* (19). Confluent monolayers in 6-cm dishes were washed twice with phosphate-buffered saline and inoculated with 0.5 ml of virus dilutions. After a 2-hr adsorption

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Abbreviations: VSV, vesicular stomatitis virus; $NaDodSO_4$, sodium dodecyl sulfate.

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period at 37°C, plates were overlayed with 5 ml of 0.95% agar in Dulbecco's modified minimal essential medium. For plates containing influenza virus, 2.5 μg of trypsin per ml was included in the overlay. After 2 days at 37°C for VSV, and 3 days for influenza virus, plates were stained with agar containing 0.01% neutral red.

For infection of cells, nearly confluent MDCK monolayers were washed twice with phosphate-buffered saline and inoculated with either virus at a multiplicity of 10–15 plaque-forming units per cell. After 2 hr the inoculum was replaced with serum-free Eagle's minimal medium. Monolayers to be processed for electron microscopic observation were washed twice with phosphate-buffered saline prior to addition of minimal medium.

Addition of Tunicamycin and Isotopes. Tunicamycin (from Robert Hamill, Eli Lilly) was diluted to various concentrations in minimal medium and added 2 hr after infection. For radiolabeling of cells, the medium was replaced with minimal medium containing isotopes and tunicamycin at the appropriate concentration. [^{3}H]Glucosamine was obtained from ICN Pharmaceuticals (Irvine, CA); [^{3}H]leucine and ^{14}C-labeled amino acids were from Schwarz/Mann.

Electrophoresis. Samples were prepared for electrophoresis by boiling in dissociation buffer [62.5 mM Tris·HCl, pH 6.8/2% sodium dodecyl sulfate ($NaDodSO_4$)/2% 2-mercaptoethanol/20% glycerol] and electrophoresed for 18 hr at 10 V in 12% polyacrylamide slab gels (3 mm) with a Tris glycine running buffer (pH 8.3) (20). Gels were prepared for fluorography by 3.5 hr of fixation in 10% methanol/10% acetic acid, followed by three 30-min washes in dimethyl sulfoxide, 2 hr in 20% 2,5-diphenyloxazole in dimethyl sulfoxide and 12 hr in tap water, and drying under reduced pressure (21). Radiolabeled bands were identified by a 1-week exposure at −70°C of Kodak X-Omat R film to the dried gel.

Electron Microscopy. Virus-infected MDCK cell monolayers grown on plastic films (SaranWrap, Dow) were fixed *in situ* with 1% glutaraldehyde in phosphate-buffered saline for 5 min followed by 2% osmium tetroxide for 20 min. The plastic

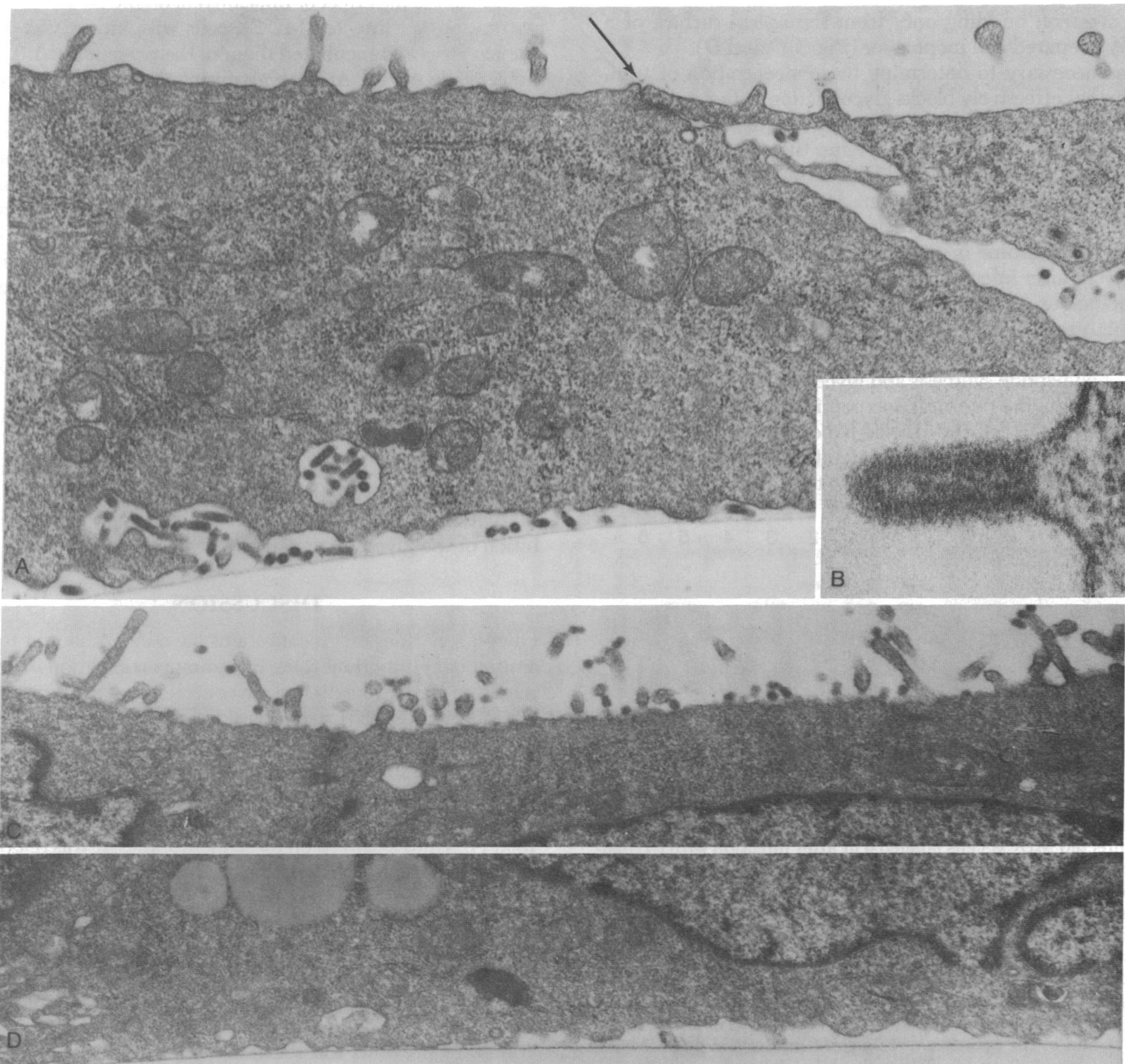

FIG. 1. Polarity of enveloped virus maturation in MDCK cells. (*A*) VSV virions associated with the basal and lateral membranes of MDCK cells 11 hr after infection. A tight junction (arrow) separates the apical from the basolateral membrane domains. (×25,000.) (*B*) Higher magnification of a VSV virion budding at a lateral membrane. (×200,000.) (*C*) Influenza virions budding from the apical surface of a MDCK cell 20 hr after infection. (×13,000.). (*D*) Basal surface of the same cell, attached to a plastic substrate, showing no virions. (×13,000.)

films with cells attached were rolled up and dehydrated sequentially with 70, 95, and 100% acetone washes prior to embedding in an epoxy resin mixture. Thin sections were cut on a Sorvall ultramicrotome, mounted on 300 mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips 301 electron microscope.

RESULTS

In order to preserve the morphological features of MDCK cell monolayers during preparation for electron microscopy, monolayers were grown on plastic films and fixed and embedded *in situ* (22). In cross sections of these cell monolayers, the orientation of the cells on the substrate and the tight junctions between cells were intact. Fig. 1*A* shows two VSV-infected MDCK cells connected by a tight junction. As previously reported (4), numerous VSV virions can be seen associated with the basal and lateral membranes beneath the tight junctions. Virions were only occasionally found on the apical surfaces and were never found in the process of budding from apical membranes as long as cell junctions remained intact. A budding virion is shown at high magnification in Fig. 1*B*. Influenza virions are seen budding only from the apical surface of a similarly prepared cell monolayer (Fig. 1 *C* and *D*).

It was necessary to determine the concentration of tunicamycin that effectively blocks glycosylation in these cells. We infected MDCK monolayers with ≈10 plaque-forming units of influenza virus or VSV per cell. After the adsorption period, the viral inoculum was replaced with serum-free maintenance medium containing tunicamycin. At each concentration of tunicamycin tested, virus-infected monolayers were labeled with [^{3}H]glucosamine or with [^{3}H]leucine for 1-hr pulses beginning 6 hr after infection. Cells were then solubilized and the [^{3}H]leucine- or [^{3}H]glucosamine-labeled viral polypeptides were analyzed on NaDodSO$_4$/polyacrylamide slab gels by fluorography. Fig. 2 (*Left*) shows the effects of tunicamycin on VSV polypeptide synthesis and glycosylation. In the absence of tunicamycin, the five viral polypeptides L, G, N, NS, and M are resolved as [^{3}H]leucine-labeled bands (lane 1). As expected, a [^{3}H]glucosamine-labeled band migrates at the position of the viral glycoprotein, G (lane 2), and is the only [^{3}H]glucosamine-labeled band comigrating with a leucine-labeled viral polypeptide. In the presence of 0.5 μg of tunicamycin per ml, the [^{3}H]leucine-labeled G polypeptide shows increased electrophoretic mobility (lane 3), indicating a decrease in molecular weight, and contains no detectable glucosamine label (lane 4). In Fig. 2 *Right*, polypeptides in influenza virus-infected cells are analyzed in the same manner. In the absence of tunicamycin, the major [^{3}H]leucine bands indicate the positions of the viral HA, NP, and M plus NS polypeptides (lane 1); the HA band is seen to be labeled with [^{3}H]glucosamine (lane 2). In the presence of 0.5 μg of tunicamycin per ml, little, if any, glucosamine label can be detected in viral glycoproteins (lane 4), and the [^{3}H]leucine-labeled HA band is also not detected (lane 3). It has been previously shown that unglycosylated HA (designated HA_0) is difficult to resolve from the NP polypeptide (15), which probably accounts for the inability to resolve this band with [^{3}H]leucine. At a concentration of 1.0 μg of tunicamycin per ml, glycosylation is completely inhibited, as indicated by the absence of glucosamine labeling of the influenza viral glycoproteins (lane 6).

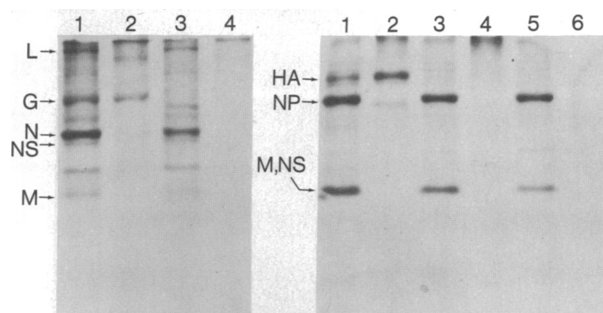

FIG. 2. Synthesis of viral polypeptides in VSV- or influenza virus-infected MDCK cells labeled 6 hr after infection with [^{3}H]leucine or [^{3}H]glucosamine. Radiolabeled polypeptides were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and identified by fluorography. (*Left*) VSV polypeptides labeled with [^{3}H]leucine (lanes 1 and 3) or [^{3}H]glucosamine (lanes 2 and 4). Lanes 1 and 2, polypeptides labeled in the absence of tunicamycin; lanes 3 and 4, samples labeled in the presence of 0.5 μg of tunicamycin per ml. (*Right*) Influenza viral polypeptides synthesized in the absence of tunicamycin (lanes 1 and 2), in the presence of 0.5 μg of tunicamycin per ml (lanes 3 and 4), and in the presence of 1.0 μg of tunicamycin per ml (lanes 5 and 6). Lanes 1, 3, and 5, [^{3}H]leucine label; lanes 2, 4, and 6, [^{3}H]-glucosamine label. In lane 2, the hemagglutinin (HA) is the major [^{3}H]glucosamine-labeled band, and the minor ^{3}H-labeled band in the region in front of the NP protein corresponds to the neuraminidase (NA) glycoprotein.

To examine the effect of tunicamycin on the polarity of virus maturation, we infected MDCK cells with either VSV or influenza virus and incubated them in the presence of 1.0 μg of tunicamycin per ml. At intervals during the growth cycle, these monolayers were fixed *in situ*, embedded, and examined by electron microscopy. Influenza virions maturing in the presence of tunicamycin budded exclusively from the apical membranes of MDCK monolayers (Fig. 3 *A* and *B*). The virions were never observed to bud from the basal or from the lateral membranes, but were restricted to the apical surface above the tight junctions for as long as the monolayer remained intact. VSV virions budding in the presence of tunicamycin were observed along the basal membranes or lateral membranes beneath the tight junctions (Fig. 3 *C–E*). Occasionally VSV virions were seen budding inside structures that could be either invaginations of the basal membrane or cytoplasmic vacuoles; this was also true of cells infected in the absence of tunicamycin. VSV virions were not observed budding from the apical surface of intact monolayers in either the presence or absence of tunicamycin. These results indicate that the polarity of virus maturation in MDCK cells is maintained under conditions of complete inhibition of the glycosylation of viral glycoproteins.

DISCUSSION

Oligosaccharides on glycoproteins have been thought to play a number of important roles, including participation in protein secretion (23), protection of glycoproteins against proteolytic degradation (24, 25), cell adhesion and morphology (26, 27), and membrane transport processes (28). Because viruses maturing at the apical in contrast to the basolateral surfaces of MDCK cells show a qualitative difference in sialic acid content which parallels a difference in sialic acid content in some cellular enzymes localized on these membranes, it was reasonable to suggest (4) that glycosylation of viral glycoproteins might be involved as a determinant of the cellular maturation site. Important information on the role of carbohydrates in membrane glycoproteins has been obtained by using inhibitors of glycosylation, such as 2-deoxy-D-glucose, D-glucosamine (29, 30), and tunicamycin (15, 16, 24, 31, 32). Tunicamycin inhibits the synthesis of *N*-acetylglucosaminylpyrophosphorylisoprenol and therefore prevents the synthesis of asparagine-linked oligosaccharides (13, 14). The role of glycosylation in intracellular migration of membrane glycoproteins has been studied by using tunicamycin in a number of distinct systems, and the results indicate that proteins with different amino acid sequences have

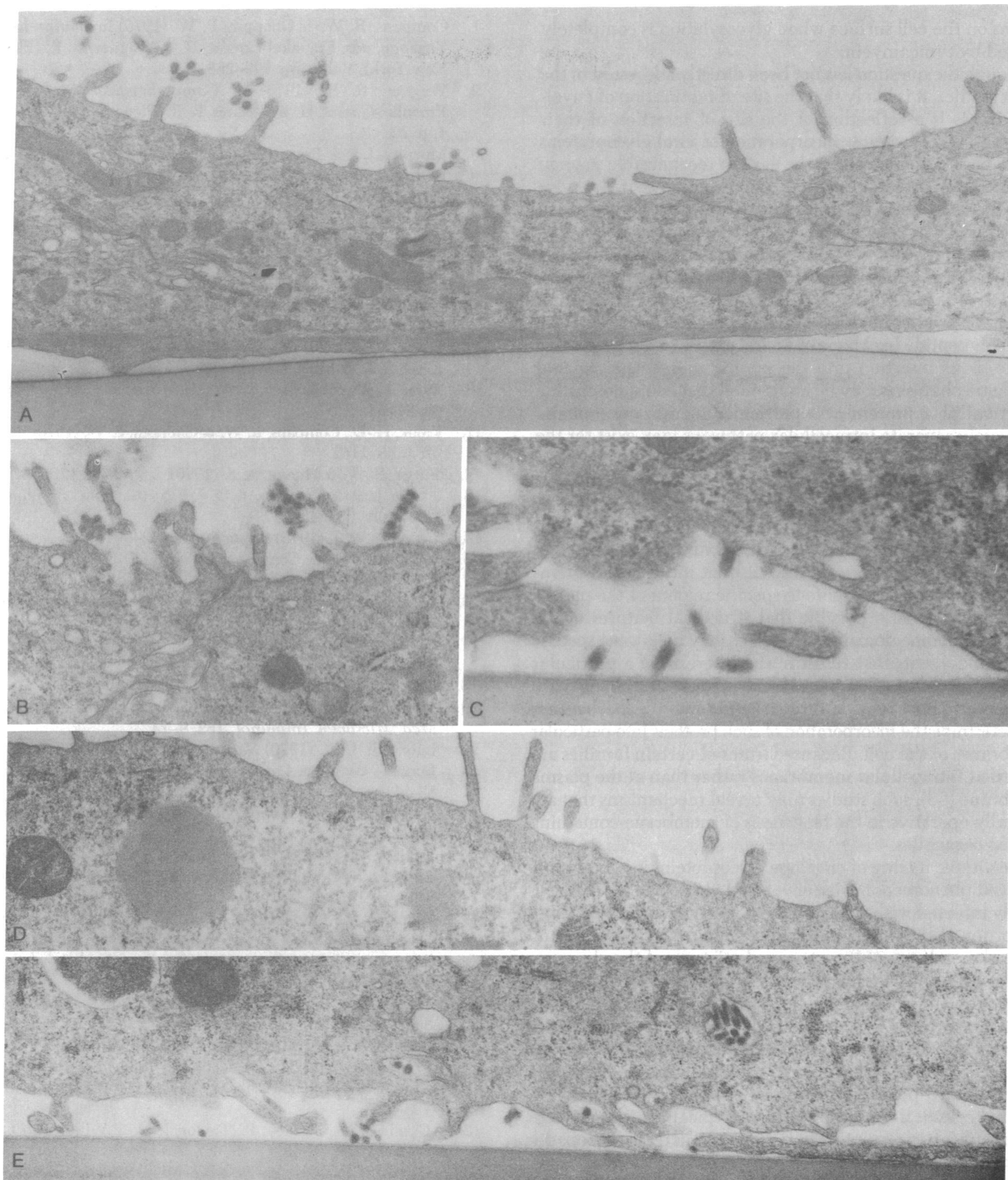

FIG. 3. Polarized maturation of influenza and VSV virions in MDCK monolayers in the presence of 1.0 μg of tunicamycin per ml. (*A*) Influenza virions associated with the apical surface of a cell 18 hr after infection. The basal surface is free of virions. (×23,000.) (*B*) Junction of two cells 18 hr after infection, showing influenza virions associated with the apical surface. (×23,000.) (*C*) VSV virions budding from the basal membrane in the presence of tunicamycin. (×40,000.) (*D*) Apical surface of an MDCK cell 10 hr after infection with VSV, free of virions. (×20,000.) (*E*) VSV virions associated with the basal surface of the same cell as in (*D*). (×20,000.)

different requirements for glycosylation in order to assume a conformation that allows migration to the cell surface (15, 16, 31, 32). Previous studies (15, 16) have indicated that glycoproteins of enveloped viruses, including influenza and VSV, can undergo migration to the plasma membrane in cells treated with tunicamycin and that virus maturation can occur under conditions where glycosylation of glycoproteins is completely inhibited. For VSV, fully infectious virions have been obtained containing nonglycosylated G proteins, indicating that glycosylation is not required for viral biological activity (17), whereas glycosylation appears to be important for biological activities of influenza virus glycoproteins (15, 29). The present results demonstrate that polarity in maturation of influenza virus and VSV, which mature at different domains on the surface of MDCK cells, does not require glycosylation of the viral glycoproteins; budding of these viruses is restricted to particular

domains on the cell surface when glycosylation is completely inhibited by tunicamycin.

Although the question has not been directly addressed in the present studies, it is likely that the site of maturation of enveloped viruses is a reflection of the site of insertion of their membrane glycoproteins. Incorporation of viral glycoproteins into the plasma membrane is the earliest recognizable stage in virus assembly (1, 33), and it has been reported that viral glycoproteins are selectively incorporated into the same surface domain in MDCK cells where virus maturation occurs (34). The junctional complexes on MDCK cells probably restrict the lateral diffusion of viral glycoproteins, which normally occurs in the plane of the membrane (35). Because glycosylation is not essential for polarity in virus maturation, it is likely that features of the polypeptide backbone of a viral glycoprotein determine the site of its insertion into the plasma membrane. Several possible mechanisms may be envisioned that could determine the arrival of a protein at a particular membrane domain. Completely separate intracellular pathways may exist for the synthesis and intracellular migration of proteins destined for the apical in contrast to the basal membrane, and a structural feature of the nascent peptide may direct it to a particular pathway. Alternatively, the initial site of synthesis may be unimportant, but completed polypeptides may exhibit features that cause them to segregate into distinct intracellular membrane compartments en route to specific regions of the plasma membrane. It is also possible that structural features of the plasma membrane domains themselves may determine specific recognition events that result in the insertion of a particular subset of proteins. Comparative structural studies on viral glycoproteins may provide further insight into the mechanisms that determine the incorporation of such proteins into particular membranes of the cell. Because viruses of certain families are formed at intracellular membranes rather than at the plasma membrane (33), such studies may reveal mechanisms that are generally operative in the biogenesis of membrane-containing cellular organelles.

Phenotypic mixing of envelope glycoproteins is a commonly observed phenomenon with enveloped viruses. In MDBK cells doubly infected with VSV and the paramyxovirus SV5, a high proportion of the progeny particles contain VSV genomes and internal membrane (M) protein and mixtures of the glycoproteins of VSV and SV5 (36, 37). These cells also form epithelial monolayers in which maturation of SV5 was observed only at the apical surface (4), which would suggest that glycoproteins of VSV and SV5 might normally be segregated into separate domains on the MDBK cell surface. It is therefore of interest to determine if polarity of virus maturation is altered in doubly infected cells or if formation of phenotypically mixed particles occurs only after disruption of tight junctions by the cytopathic effects of virus infection, which may permit the lateral diffusion and intermixing of viral glycoproteins that is needed for formation of particles with mixed envelope glycoproteins. In preliminary observations on MDBK cells doubly infected with VSV and SV5, we have observed some examples of VSV virions budding at apical cell surfaces in cells exhibiting morphologically intact tight junctions. Further studies on doubly infected cells may aid in clarifying the mechanisms that determine the polarity in virus maturation in epithelial cell monolayers.

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