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Role for Influenza Virus Envelope Cholesterol in Virus Entry and Infection

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Enveloped viruses are highly dependent on their lipid envelopes for entry into and infection of host cells. Here, we have examined the role of cholesterol in the virus envelope, using methyl-β-cyclodextrin depletion. Pretreatment of virions with methyl-β-cyclodextrin efficiently depleted envelope cholesterol from influenza virus and significantly reduced virus infectivity in a dose-dependent manner. A nonenveloped virus, simian virus 40, was not affected by methyl-β-cyclodextrin treatment. In the case of influenza virus, infectivity could be partially rescued by the addition of exogenous cholesterol. Influenza virus morphology, binding, and internalization were not affected by methyl-β-cyclodextrin depletion, whereas envelope cholesterol depletion markedly affected influenza virus fusion, as measured by a specific reduction in the infectivity of viruses induced to fuse at the cell surface and by fluorescence-dequenching assays. These data suggest that envelope cholesterol is a critical factor in the fusion process of influenza virus.

Infection of host cells by enveloped viruses relies on the fusion of the viral envelope with either the endosomal or plasma membrane of the cell (12). As such, the protein and lipid compositions of both the viral envelope and the host cell membrane play crucial roles in virus infection. For orthomyxoviruses, such as influenza virus type A, it is well established that the virus is internalized in the cell prior to membrane fusion (27). Fusion occurs from within endosomes and depends on low pH. Exposure of the viral hemagglutinin (HA) protein to a pH of 5.0 causes extensive conformational changes, exposing the fusion peptide, which is responsible for initiating bilayer fusion (19, 43, 44).

For all enveloped viruses, the envelope is derived from the host cell during the process of virus budding. In the case of influenza virus, budding takes place at the apical plasma membrane and is heavily dependent on the presence of lipid microdomains, or "rafts," which are enriched in cholesterol and sphingomyelin (29, 39, 42, 51). Both of the major viral glycoproteins, HA and neuraminidase, associate with lipid microdomains (29). Purified influenza virus particles contain high levels of cholesterol: 44% of the total virus lipid is cholesterol, which represents ~11 to 12% of the total mass of the virion (10, 22, 29). In addition to influenza virus, many other viruses are known to utilize lipid microdomains during budding. Human immunodeficiency virus type 1 (HIV-1) is perhaps the best studied (30, 32), but other viruses that bud out of defined membrane microdomains include other retroviruses, as well as rhabdoviruses, e.g., vesicular stomatitis virus, and alphaviruses, e.g., Semliki Forest virus (5, 23, 24, 34, 40).

In addition to its involvement in virus budding, cholesterol has been shown to be important during virus entry. Semliki Forest virus fusion is absolutely dependent on the presence of cholesterol in the target (endosomal) membrane (1, 35, 48). HIV-1 and herpes simplex virus entry has also been shown to require cholesterol, both in the target (plasma) membrane and in the donor membrane (the viral envelope) (2, 6, 7, 16, 18, 47). Other viruses are dependent only on the cholesterol-containing lipid microdomains of the cell, e.g., the nonenveloped polyomavirus simian virus 40 (SV40), which enters cells via caveolae (8, 33).

It has recently been shown that depletion of cholesterol from cellular membranes has no effect on influenza virus entry (41). Here, we have examined the role of cholesterol in the virus envelope, using methyl-β-cyclodextrin depletion. Methyl-β-cyclodextrin efficiently depleted envelope cholesterol and significantly reduced influenza virus infectivity in a dose-dependent manner. Virus morphology, binding, and internalization were not affected, whereas envelope cholesterol depletion markedly affected virus fusion.

MATERIALS AND METHODS

Cells, viruses, and infections. MDBK cells (American Type Culture Collection) were maintained in Dulbecco's modified minimal essential medium (Cellgro) containing 10% calf serum, 100 U of penicillin/ml, and 10 μg of streptomycin/ml and passaged twice weekly. Cv-1 cells (American Type Culture Collection) were maintained in the alpha modification of minimal essential medium (Cellgro) containing 10% fetal calf serum, 100 U of penicillin/ml, and 10 μg of streptomycin/ml and passaged twice weekly.

Influenza virus A/WSN/33 (H1N1) was grown in 10-day-old embryonated eggs, (25). SV40 strain 377 was kindly provided by James Pipas, University of Pittsburg, and was grown in Cv-1 cells. The infections were performed essentially as described previously (38). Briefly, virus stocks were diluted in RPMI 1640 medium containing 0.2% bovine serum albumin (BSA) and buffered to pH 6.8 with HEPES. Unless otherwise described, the virus was adsorbed for 45 min at 37°C. The cells were then maintained in growth medium containing 2% serum at 37°C before analysis.

Methyl-β-cyclodextrin treatment of virions. Methyl-β-cyclodextrin was obtained from Sigma. Typically, 50 μl of influenza virus (1 mg/ml, or 0.36×10^9 hemagglutinating units/ml) was mixed with phosphate-buffered saline (PBS) or different concentrations of methyl-β-cyclodextrin and incubated at 37°C for 30 min. For binding and internalization assays, viruses with a multiplicity of infection (MOI) of ~25 focus-forming units/cell were used, and for infection studies, ~1 focus-forming unit/cell was used. Virus was treated with cyclodextrin and ultracentrifuged at 200,000 × *g* for 30 min to remove the cyclodextrin. Virus was

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resuspended in 200 μl of RPMI 1640 containing 0.2% BSA before infection of cells.

For competition experiments, 2 μl of exogenous cholesterol (Sigma) at a concentration of either 2 mg/ml or 200, 20, or 2 μg/ml was added to the virus-cyclodextrin mixture to obtain a final volume of 10 μl. The mixture of exogenous cholesterol, virus, and cyclodextrin was incubated at 37°C for 30 min before being used for infection.

Hemagglutination assay. Freshly isolated chicken blood was washed three times with PBS, and the red blood cells were resuspended in PBS at a concentration of 1%. Virus dilutions (50 μl) were made in PBS, and 50 μl of the red blood cell suspension was added to individual wells in a 96-well plate and allowed to settle for 45 min. The wells were judged to be HA negative (no agglutination of red cells) if a dot or pellet of red cells was present, and they were judged to be positive (red cells agglutinated by HA) if a smooth suspension of red cells was present.

Negative-stain electron microscopy. Samples were prepared for negative-stain electron microscopy by fixation with 2% glutaraldehyde–PBS. The samples were then placed onto a Formvar-coated hexagonal (300-hex) Cu grid, allowed to stand for 30 s, and immediately stained for 10 s with 2% aqueous uranyl acetate. After a brief drying period, the grid was viewed on a Philips Tecnai 12 electron microscope at 100 kV. Images were acquired with a digital Gatan Multiscan Camera (model 791) before transfer into Adobe Photoshop (version 7.0).

Binding assay. To monitor binding, methyl-β-cyclodextrin-treated or untreated influenza virions were bound to cells on ice for 90 min. The cells were washed, fixed, and processed as described below for immunofluorescence microscopy or flow cytometry.

Internalization assay. As a measure of virus internalization, cells were incubated with methyl-β-cyclodextrin-treated or untreated influenza virions on ice for 90 min and either fixed immediately or shifted to 37°C for 20 min. The cells were then washed with 0.1 M glycine–0.1 M NaCl, pH 3.0, buffer for 2 min, fixed, and permeabilized. Virion localization was assayed by immunofluorescence microscopy using anti-nucleoprotein (NP) antibodies.

Fusion assays. As a measure of virus fusion, we assayed the ability of virus fused at the cell surface to cause infection of cells (26). Briefly, influenza virus was bound to the surfaces of BHK cells on ice for 90 min, and fusion was triggered by lowering the pH of the medium to 5.0 for 5 min. Monensin (10 μM; Sigma) was added to prevent any entry of virus through endosomes, and the cells were analyzed for infection by immunofluorescence microscopy at 6 h postinfection.

For fluorescence-dequenching assays, we used the procedure of Stegmann et al. (45) as modified by Gilbert and coworkers (15). To label influenza virus, 10 μl of 1.7 mM octadecyl rhodamine B chloride (R18; Molecular Probes) was injected slowly into 1 ml of influenza virus (1 mg/ml), and the mixture was incubated in the dark on a rotary shaker at room temperature. For R18 fusion assays, 7 μg of labeled virus was bound to 2×10^6 MDBK cells at 4°C for 1 h in 600 μl of cell suspension in the presence of 10 μM monensin to block endocytosis. Unbound virus was removed by washing the cells with binding buffer (RPMI 1640 medium containing 0.2% BSA and 10 μM monensin, pH 6.8). The cells were resuspended in 5 mM HEPES–5 mM morpholineethanesulfonic acid–5 mM succinate buffer, pH 7.0, at 37°C. Fusion of virus on the cell membrane was triggered by adding a predetermined amount of 150 mM HCl to obtain a final pH of 4.9. Samples were analyzed on a Molecular Devices Spectramax Gemini XS fluorimeter. Fluorescence dequenching was measured during a 5-min time course at 3-s intervals with the fluorimeter set to excitation and emission wavelengths of 560 and 590 nm, respectively, with a 579-nm cutoff filter. Fusion efficiency was calculated following treatment with 1% Triton X-100 to obtain 100% dequenching.

Indirect immunofluorescence microscopy. Preparation of cells for indirect immunofluorescence microscopy was performed as described previously (50). Influenza virus NP was detected using the monoclonal antibody H16-L10-4R5 (American Type Culture Collection). SV40 was detected using an anti-T-antigen monoclonal antibody (clone 416; Research Diagnostics Inc.). The secondary antibodies used were Alexa 488-labeled (green) or Alexa 568-labeled (red) goat anti-mouse immunoglobulin G (Molecular Probes). The cells were mounted in MOWIOL 4-88 (Calbiochem) and viewed on a Nikon Eclipse E600 fluorescence microscope, using a 40× Plan Apo objective (NA 0.95) or a 20× Plan Apo objective (NA 0.75), and images were captured with a SPOT RT camera and SPOT software (version 3.5.4) before transfer into Adobe Photoshop (version 7.0). For quantitation, >100 cells were scored for each sample.

Flow cytometry. For flow cytometry preparation, cells were detached with 0.2% trypsin–EDTA (Cellgro) or cell dissociation buffer (Sigma), washed in PBS, fixed in 3% paraformaldehyde–PBS, and permeabilized in 0.075% saponin in 10% goat serum–PBS. The cells were incubated with the monoclonal antibody to influenza NP for 30 min, followed by incubation with Alexa 488-labeled goat anti-mouse immunoglobulin G for 30 min. The cells were analyzed on a FACSCalibur cytometer using CellQuest version 3.1f software (Becton Dickinson Immunocytometry Systems). At least 10,000 cells were analyzed for each sample.

Cholesterol determination. The cholesterol content was determined using an Amplex Red Cholesterol assay kit (Molecular Probes) according to the manufacturer's instructions. Typically, 50 μl of virus (1 mg/ml) was pelleted and resuspended in Amplex Red reaction buffer and analyzed on a Molecular Devices Spectramax Gemini XS fluorimeter, using an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

RESULTS

Treatment of influenza virions with methyl-β-cyclodextrin to deplete cholesterol significantly reduces virus infectivity. It was recently shown that depletion of cholesterol from cells has no effect on influenza virus entry (41). However, the previous experiments were designed to target the cellular membranes and not the viral envelope. As a first measure of the role of envelope cholesterol in influenza virus infection, we treated purified virions with various concentrations of methyl-β-cyclodextrin for 30 min to deplete the cholesterol. Exposure to methyl-β-cyclodextrin resulted in a dose-dependent inhibition of influenza virus infectivity, as measured by single-hit infection assays (Fig. 1). By immunofluorescence microscopy, ~70% of the cells were infected with influenza virus in the absence of methyl-β-cyclodextrin, as shown by the fluorescence signal of influenza NP in the nuclei of infected cells (Fig. 1A). Upon addition of 5 mM methyl-β-cyclodextrin, the level of infection dropped to ~55%, and with 10 mM methyl-β-cyclodextrin, it dropped to ~40%. Treatment of influenza virions with 20 mM methyl-β-cyclodextrin reduced infectivity to <20%, and with 50 mM methyl-β-cyclodextrin, infectivity was detectable in only ~5% of the cells. Figure 1B shows a graphical representation of these data, normalized to 100% for the untreated sample.

We next confirmed our immunofluorescence microscopy data by fluorescence-activated cell sorting (FACS) analysis to obtain more quantitative results (Fig. 1C and D). By flow cytometry, untreated virus showed an 87% infection level. Treatment with 5 mM methyl-β-cyclodextrin reduced the level of infection to 62%, 10 mM methyl-β-cyclodextrin gave a 45% infection level, and 20 mM cyclodextrin resulted in a 17% infection level, while 50 mM methyl-β-cyclodextrin allowed only 5% of the cells to be infected. In general, the flow cytometry data closely paralleled the results from immunofluorescence, with a dose-dependent effect of methyl-β-cyclodextrin that produced a roughly linear decrease in infectivity with increasing methyl-β-cyclodextrin concentrations.

It is important to note that our experimental protocol was designed to minimize the effects of methyl-β-cyclodextrin on cellular membranes. To control for nonspecific effects on the cell, we treated virions with methyl-β-cyclodextrin immediately before adding them to cells. Under these conditions, we retained essentially 100% infectivity, as measured by immunofluorescence microscopy (Fig. 2).

Methyl-β-cyclodextrin does not affect influenza virus structure. In order to examine the effects of methyl-β-cyclodextrin treatment of influenza virus particles, we first examined treated and untreated virions by negative-stain electron microscopy. As shown previously by other investigators (39), there were no obvious differences between influenza virus particles after

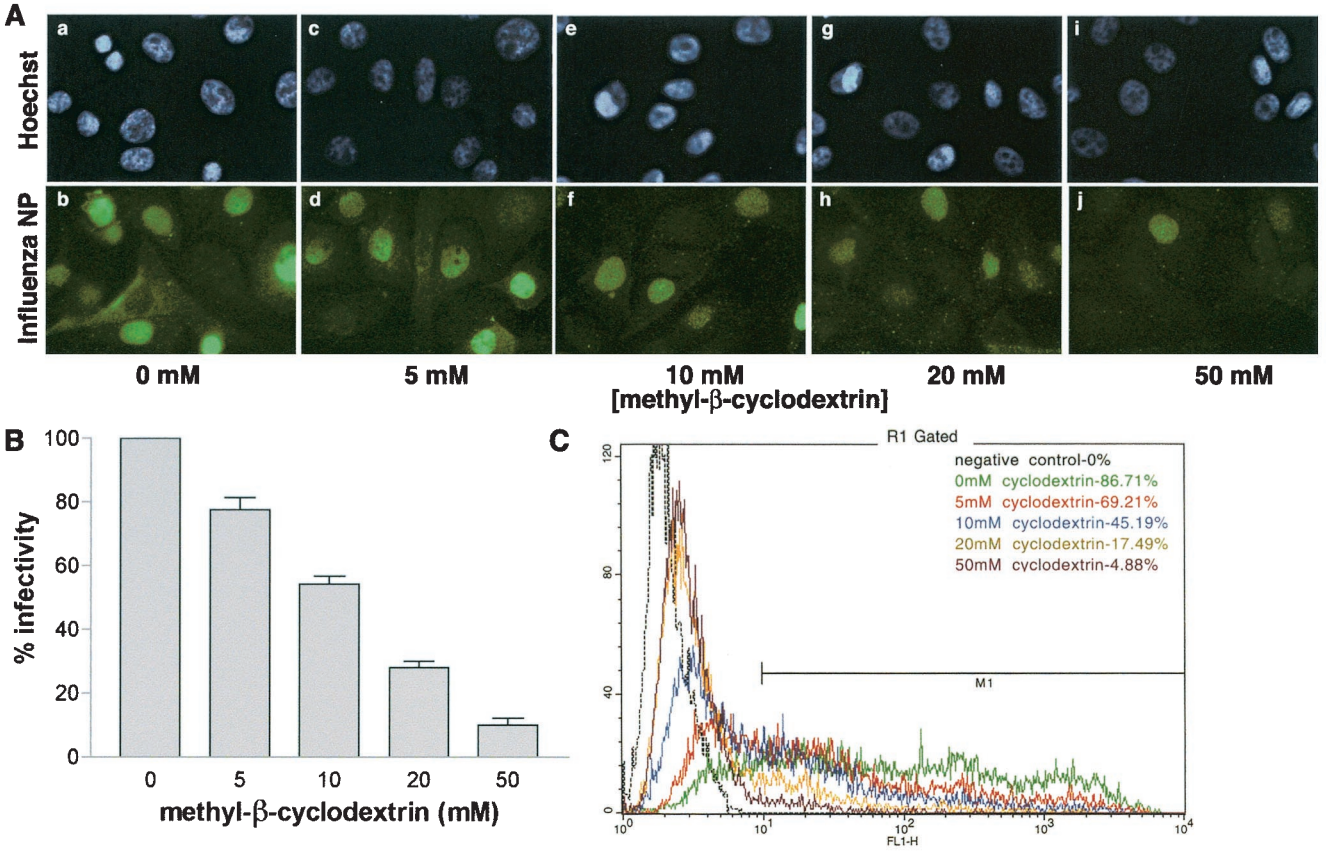

FIG. 1. Treatment of influenza virions with methyl-β-cyclodextrin to deplete cholesterol significantly reduces virus infectivity. (A) Influenza virus was pretreated with various concentrations of methyl-β-cyclodextrin, and MDBK cells were infected at an MOI of ~infectious unit per cell. The cells were analyzed by indirect immunofluorescence microscopy at 5 h postinfection using anti-NP antibodies (b, d, f, h, and j), or nuclei were stained with Hoechst 33258 (a, c, e, g, and i). (B) Quantitation of data in panel A. At least 100 cells were counted for each sample to determine the percentages of infectivity. The error bars represent the standard errors of the mean. (C) Influenza virus was pretreated with various concentrations of methyl-β-cyclodextrin, and MDBK cells were infected at an MOI of ~1 infectious unit per cell. The cells were analyzed at 5 h postinfection by FACS analysis using anti-NP antibodies. Negative control, uninfected cells.

methyl-β-cyclodextrin treatment (Fig. 3) and those that were untreated (not shown), confirming that the drug does not grossly affect virion morphology. As a more functional measure of virus structure, we performed hemagglutination assays of treated and untreated virions. The HA titer of the virus (1:1,024) did not change upon the addition of methyl-β-cyclodextrin (5, 10, 20, and 50 mM) to deplete envelope cholesterol, whereas the addition of 0.1% Triton X-100 reduced HA activity to background levels (1:10).

Treatment with methyl-β-cyclodextrin has no effect on SV40 infectivity. As a control virus for the effects of cholesterol depletion, we employed SV40. SV40 is a nonenveloped virus known to enter cells via lipid microdomains and to be very sensitive to cholesterol depletion of host cell membranes by methyl-β-cyclodextrin (37). SV40 was treated with various doses of methyl-β-cyclodextrin, and infection was monitored in a single-hit infection assay (Fig. 4). SV40 showed no significant drop in infectivity (Fig. 4), confirming that methyl-β-cyclodextrin treatment acted specifically on virus envelope cholesterol.

Measurement of cholesterol content of methyl-β-cyclodextrin-treated influenza viruses. We next assayed the relative depletion of envelope cholesterol mediated by methyl-β-cyclo-

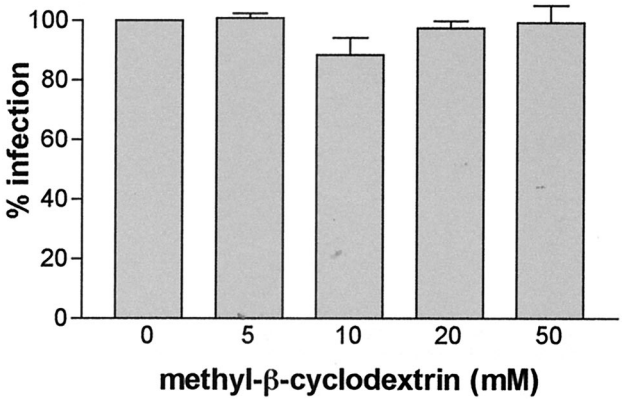

FIG. 2. Methyl-β-cyclodextrin has no effect on virus infectivity without pretreatment. Influenza virus was exposed to various concentrations of methyl-β-cyclodextrin, and MDBK cells were infected immediately at an MOI of ~1 infectious unit per cell. The cells were analyzed by indirect immunofluorescence microscopy using anti-NP antibodies, and the percentages of infectivity at 5 h postinfection were determined by scoring at least 100 cells for infection for each sample. The error bars represent the standard errors of the mean.

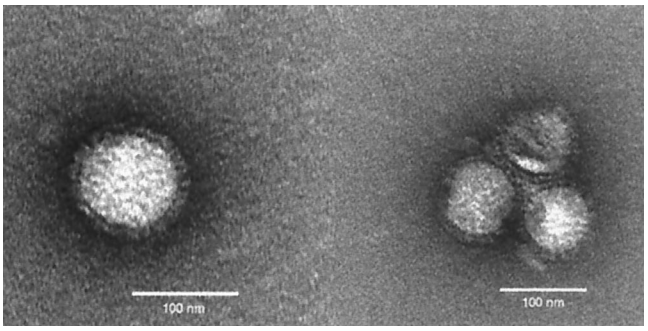

FIG. 3. Methyl-β-cyclodextrin does not affect influenza virus structure. Influenza virus was pretreated with 20 mM methyl-β-cyclodextrin, and virions were analyzed by negative-stain electron microscopy.

dextrin treatment. Purified influenza virus was treated with various concentrations of methyl-β-cyclodextrin for 30 min, and the cholesterol content was determined using a cholesterol assay kit (Fig. 5). Influenza virus showed a dose-dependent drop in the level of cholesterol, with ~90% depletion obtained with 20 mM methyl-β-cyclodextrin. These data confirm that methyl-β-cyclodextrin treatment produces specific and efficient depletion of virion envelope cholesterol.

Replenishment of envelope cholesterol can restore influenza virus infectivity. To assess whether the effect of methyl-β-cyclodextrin was permanent or reversible, and to show that the effects of the drug were due solely to cholesterol depletion, exogenous cholesterol was used to replenish the envelopes of methyl-β-cyclodextrin-treated influenza virions. We incubated the virions with various amounts of free cholesterol during the methyl-β-cyclodextrin treatment. This *trans*-supplementation approach partially restored influenza virus infectivity in a dose-dependent manner (Fig. 6). Addition of 2 mg of cholesterol/ml to virions treated with 20 mM methyl-β-cyclodextrin restored infectivity to a level of 42% in our single-hit infection assay. Due to technical difficulties, we were unable to use levels of cholesterol above 2 mg/ml, which might have restored infection more completely.

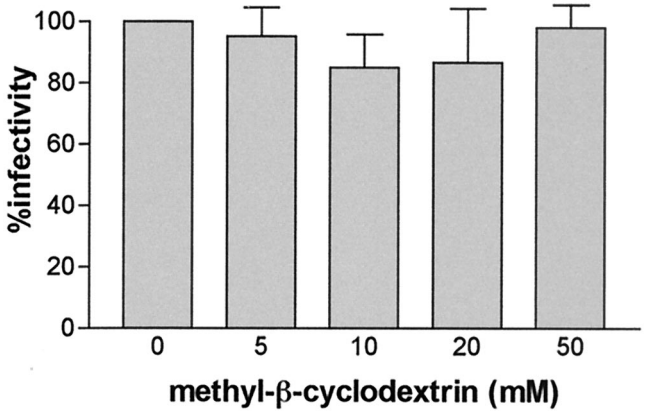

FIG. 4. Treatment with methyl-β-cyclodextrin does not reduce SV40 infectivity. SV40 was pretreated with various concentrations of methyl-β-cyclodextrin, and Cv-1 cells were infected at an MOI of ~1 infectious unit per cell. The cells were analyzed by indirect immunofluorescence microscopy with anti-T-antigen antibodies, and the percentages of infectivity at 24 h postinfection were determined by scoring at least 100 cells for infection for each sample. The error bars represent the standard errors of the mean.

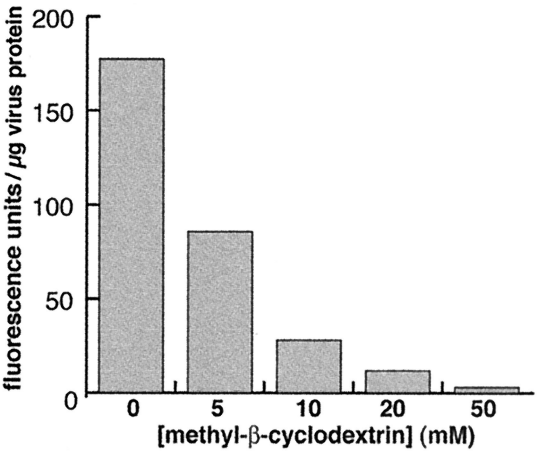

FIG. 5. Measurement of cholesterol content of methyl-β-cyclodextrin-treated viruses. Purified influenza virus was pretreated with various concentrations of methyl-β-cyclodextrin or was left untreated. The virions were repurified and analyzed using a cholesterol assay kit. The cholesterol contents were plotted based on fluorescence units.

Methyl-β-cyclodextrin does not affect influenza virus binding or internalization. We wished to address the point in the replication cycle where methyl-β-cyclodextrin-treated virions might be defective. Our first assay was to examine influenza virus binding. Cells were incubated on ice with a high MOI of virus that had been treated with 20 mM methyl-β-cyclodextrin or that was untreated. Virus binding was assayed by flow cytometry (Fig. 7A). We saw no significant differences between the abilities of methyl-β-cyclodextrin-treated and untreated

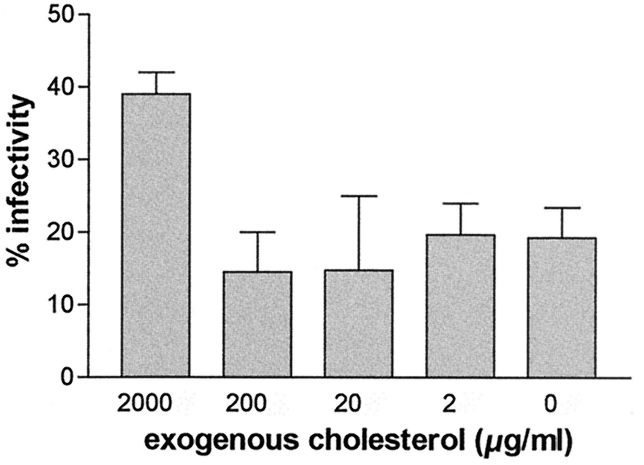

FIG. 6. Replenishment of envelope cholesterol can restore influenza virus infectivity. Influenza virus was pretreated with 20 mM methyl-β-cyclodextrin in the presence of various concentrations of exogenous cholesterol. MDBK cells were infected at an MOI of ~1 infectious unit per cell. The cells were analyzed by indirect immunofluorescence microscopy at 5 h postinfection using anti-NP antibodies, and the percentages of infectivity were determined by scoring at least 100 cells for infection for each sample. The error bars represent the standard errors of the mean.

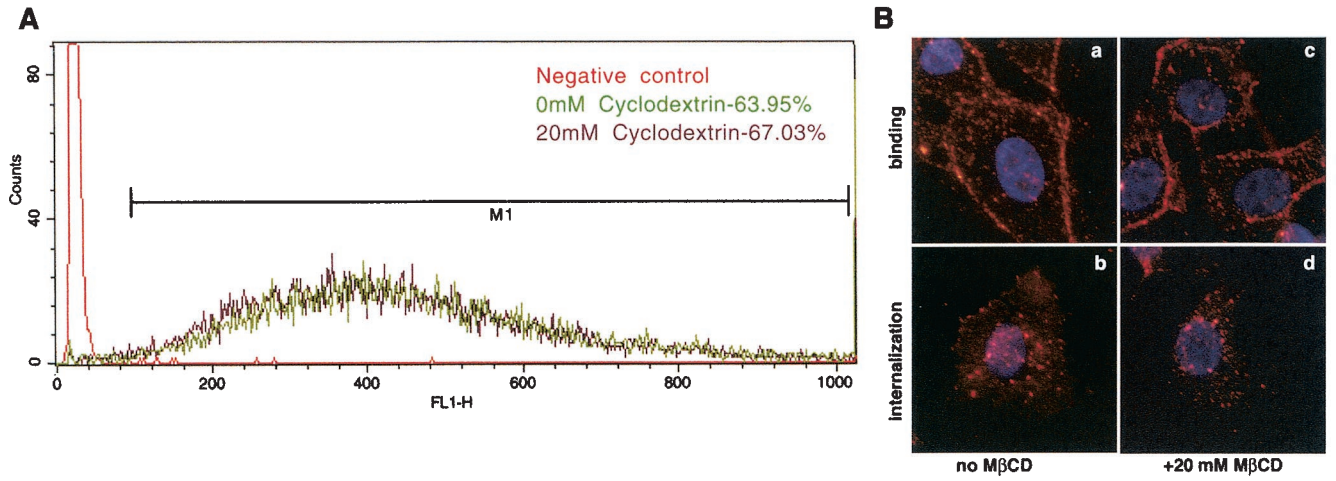

FIG. 7. Methyl-β-cyclodextrin does not affect influenza virus binding or internalization. (A) Influenza virus was treated with 20 mM methyl-β-cyclodextrin or was left untreated and was bound to the surfaces of MDBK cells on ice at a high MOI. The cells were analyzed by FACS analysis using anti-NP antibodies. Negative control, uninfected cells. (B) Influenza virus was treated with 20 mM methyl-β-cyclodextrin (c and d) or was left untreated (a and b) and was bound to the surfaces of MDBK cells on ice (binding) at a high MOI, followed by a shift to 37°C for 20 min (internalization). The cells were analyzed by indirect immunofluorescence microscopy with anti-NP antibodies, and the nuclei were stained with Hoechst 33258. Merged images of virus localization (red) and nuclei (blue) are shown.

viruses to bind to cells. In both cases, the levels of binding were virtually indistinguishable.

We next addressed whether methyl-β-cyclodextrin treatment affected virus internalization. Cells were incubated with a high MOI of methyl-β-cyclodextrin-treated or untreated influenza virions on ice and were shifted to 37°C for 20 min. Virion localization was then assayed by immunofluorescence microscopy (Fig. 7B). As with flow cytometry assays, microscopic analysis of cells at 4°C showed no significant differences in virus binding. With both methyl-β-cyclodextrin-treated and untreated virions, a 20-min temperature shift to 37°C resulted in the localization of virions to perinuclear vesicles. These data are consistent with equivalent internalizations of methyl-β-cyclodextrin treated and untreated viruses into endosomes.

Envelope cholesterol depletion inhibits influenza virus fusion. As an initial measure of influenza virus fusion, we assayed the ability of virus fused at the cell surface to cause infection of cells (26). Virions that were methyl-β-cyclodextrin treated or untreated were bound to cells on ice, and then fusion was induced with pH 5.0 buffer. Influenza virus infection was subsequently assayed by single-hit infection assays, with the inclusion of monensin to prevent virus entry through endosomes (Fig. 8). We saw a significant difference in the ability of methyl-β-cyclodextrin-treated viruses to fuse with cell membranes. We first carried out experiments by immunofluorescence microscopy. In the absence of methyl-β-cyclodextrin, we observed efficient infection of cells (~54%), albeit at a lower level than when the virus entered cells via endocytosis (compare Fig. 8A, a and d, with Fig. 1). In the presence of 20 mM methyl-β-cyclodextrin, virus infection was virtually undetectable when the viruses were fused at the cell surface with pH 5.0 buffer (Fig. 8A, b and e). Control experiments in which viruses were not fused at the cell surface produced almost no detectable infection in the presence of monensin to block endocytosis (Fig. 8A, c and f), confirming that the infection detected with pH 5.0 treatment was due solely to virions fused at the cell surface.

As with entry through endocytosis, low-pH-induced influenza virus fusion was sensitive to methyl-β-cyclodextrin treatment in a dose-dependent manner (Fig. 8B). Treatment with 5 mM methyl-β-cyclodextrin resulted in ~65% of cells being infected, relative to untreated virions. Methyl-β-cyclodextrin (10 mM) allowed an infection rate of only 25%, and 20 mM methyl-β-cyclodextrin allowed an infection rate of <10%. After treatment of virions with 50 mM methyl-β-cyclodextrin, virus infectivity was almost undetectable. These data show that influenza virus fusion is sensitive to cholesterol depletion with methyl-β-cyclodextrin.

To confirm these results, and to obtain more quantitative data, we carried out FACS analysis of our fusion assay. Under our flow cytometry conditions, untreated virus gave a 55% infection level (Fig. 8C and D). Treatment with 5 mM methyl-β-cyclodextrin reduced the level of infection to 20%, 10 mM methyl-β-cyclodextrin gave a 6% infection level, and 20 mM cyclodextrin resulted in a 3% infection level, with 50 mM methyl-β-cyclodextrin allowing only 1% of the cells to be infected. In general, the flow cytometry data closely paralleled results from immunofluorescence microscopy, with a dose-dependent effect of methyl-β-cyclodextrin that produced a roughly sigmoidal decrease in infectivity with increasing methyl-β-cyclodextrin concentrations.

To confirm that the reduced viral infection observed was due solely to inhibition of fusion, we employed a fluorescence-dequenching assay (20, 45). Influenza virus (WSN) was treated with methyl-β-cyclodextrin or remained untreated and was then labeled with R18. The virus was bound to the cell surface, and fusion was induced with low-pH buffer (Fig. 9). With untreated virions, we observed rapid dequenching of the probe as fusion occurred at low pH (Fig. 9). In contrast, at neutral pH, little or no fusion was observed in this assay (Fig. 9). When virions were treated with 20 mM methyl-β-cyclodextrin, we observed a significant drop in both the extent and initial rate of fusion (Fig. 9). This effect was dose dependent, as treatment with 50 mM methyl-β-cyclodextrin showed further reductions

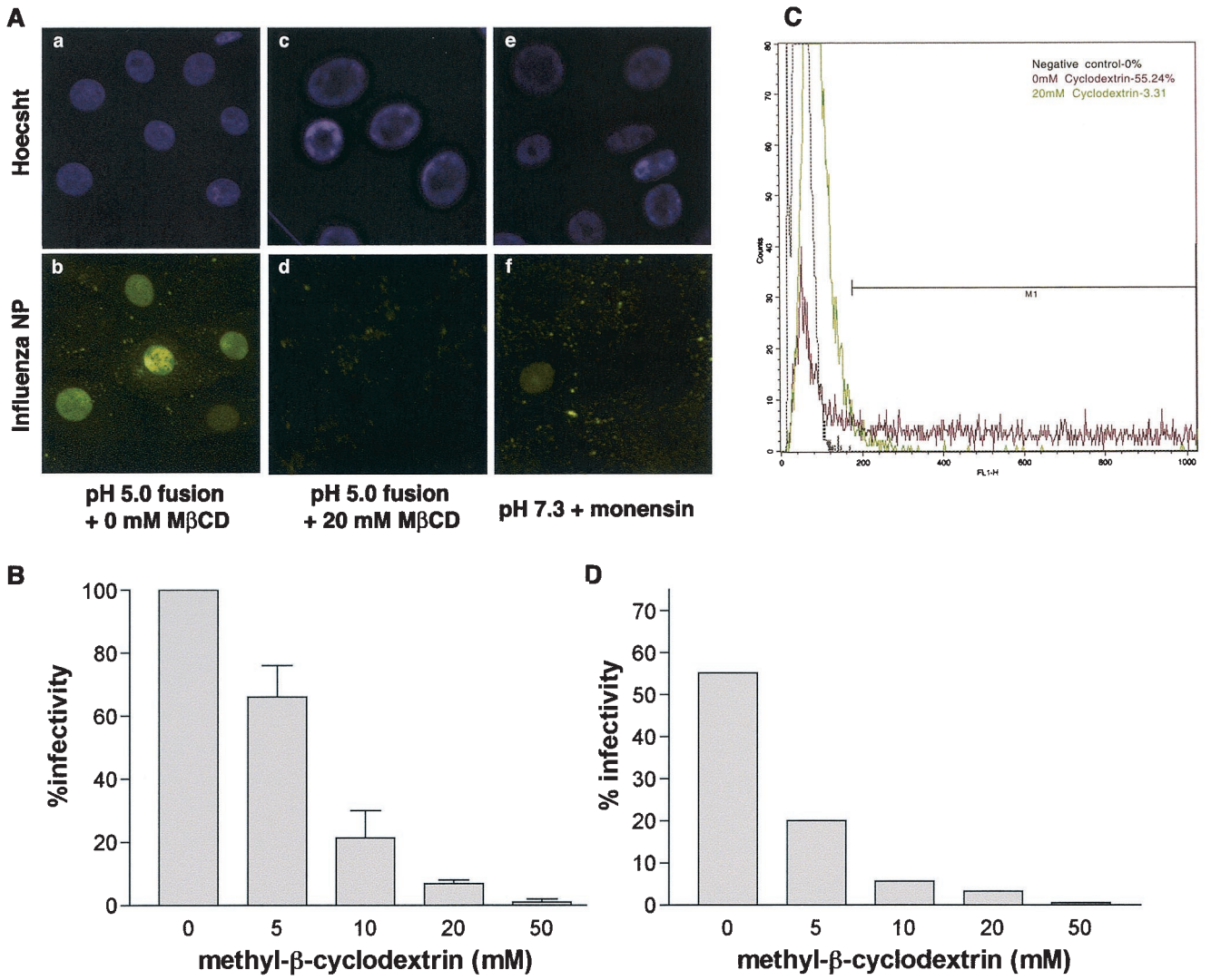

FIG. 8. Envelope cholesterol depletion inhibits influenza virus fusion. (A) Influenza virus was pretreated with 20 mM methyl-β-cyclodextrin or was left untreated and was bound to the surfaces of MDBK cells on ice. Fusion at the cell surface was induced by exposure to pH 5.0 buffer (a to d), or control samples were treated with pH 7.3 buffer (e and f). All samples were treated with monensin to block endocytosis. The cells were analyzed by indirect immunofluorescence microscopy at 5 h postinfection using anti-NP antibodies (b, d, and f), or the nuclei were stained with Hoechst 33258 (a, c, and e). (B) Quantitation of data in panel A with various concentrations of methyl-β-cyclodextrin. At least 100 cells were counted for each sample to determine the percentages of infectivity. The error bars represent the standard errors of the mean. (C) Influenza virus was pretreated with 20 mM methyl-β-cyclodextrin or was left untreated and was bound to the surfaces of MDBK cells on ice. Fusion at the cell surface was induced by exposure to pH 5.0 buffer. All samples were treated with monensin to block endocytosis. The cells were analyzed by FACS analysis using anti-NP antibodies. Negative control, uninfected cells. (D) Bar chart of FACS analysis as described for panel C with various concentrations of methyl-β-cyclodextrin.

in both the fusion rate and extent (Fig. 9). We estimate that a 45% reduction of fusion occurred with 20 mM methyl-β-cyclodextrin and a drop of ~60% occurred with 50 mM methyl-β-cyclodextrin. These data confirm that depletion of envelope cholesterol is important for influenza virus fusion with host cells.

DISCUSSION

In this paper, we examined the role of envelope cholesterol on influenza virus infection, with a focus on the events occurring during virus entry, namely, binding, internalization, and fusion. We showed a marked effect of influenza virus envelope cholesterol depletion on entry and infection, most likely due to an inhibition of virus fusion.

Several groups have studied the role of cholesterol in influenza HA-mediated fusion. For fusion between influenza virus and liposomes, the inclusion of cholesterol in the target membrane has been shown to have little effect on the extent or rate of fusion (46, 49). However, subsequent studies did show a strong effect of target membrane cholesterol on the growth of fusion pores when cells expressing HA were fused to planar phospholipid bilayers (36). In addition, cyclodextrin-mediated depletion of HA-expressing cells did not affect fusion to red blood cell ghosts (28). While allowing extensive manipulation

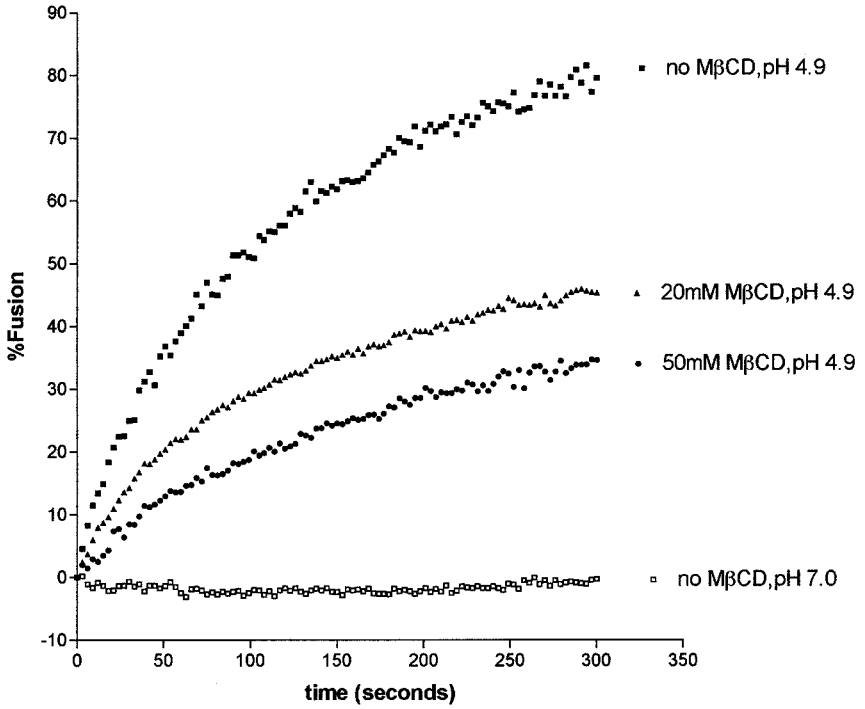

FIG. 9. Fluorescence-dequenching assays of virus fusion. Influenza virus was pretreated with methyl-β-cyclodextrin (MβCD) or left untreated, labeled with R18, and bound to the surfaces of MDBK cells on ice. The cells were warmed to 37°C, and fusion at the cell surface was induced by dropping the pH of the buffer to 4.9 (solid symbols), or control samples were maintained in pH 7.0 buffer (open squares).

of the experimental system, the use of HA-expressing cells, red blood cells, or planar bilayers for fusion studies suffers from the disadvantage that many biological parameters are not present, e.g., membrane curvature, density of HA, and presence of other proteins.

Our present data clearly show an effect of envelope cholesterol on influenza virus fusion with cell membranes; however, our data have all been obtained with the WSN (H1N1) strain of influenza virus. It remains possible that differences may occur with other virus strains, and it is important to note that the majority of studies of influenza virus fusion have been performed with H3 serotypes (e.g., X:31). Our fluorescence-dequenching assays show values for the initial rate and extent similar to those observed for the PR/8/34 strain of the virus (31), and they are clearly pH dependent; however, some differences from fusion of the X:31 strain of the virus may be apparent.

What, then, is the role of cholesterol in the virion envelope? Although very limited numbers of HA molecules seem to be required to actually trigger the fusion event (3, 17), it is clear that there must be cooperativity among HA trimers in order for fusion to occur (3, 4, 11, 13, 14). Based on the known role of cholesterol-containing lipid microdomains in the formation of influenza virions (29, 39, 51), we propose that virion cholesterol is important for the organization of influenza virus HA trimers into fusion-competent domains and that depletion of cholesterol inhibits virus infectivity due to inefficient fusion.

Our data show that pH-mediated fusion of virions with the plasma membrane is sensitive to envelope cholesterol. In our infectivity assays, we found that virus fusion (Fig. 8) is consistently more sensitive than overall infection to methyl-β-cyclodextrin depletion (Fig. 1). We observed a sudden decrease in infectivity between viruses treated with 5 mM methyl-β-cyclodextrin and those treated with 10 mM methyl-β-cyclodextrin (Fig. 8)—a decrease to the same degree was not observed following entry through endosomes (Fig. 1). It is possible that this represents differences in the local environment between fusion from within late endosomes and low-pH-induced fusion at the plasma membrane. We have yet to address the effect of envelope cholesterol on fusion within the endosome. Also notable is the fact that measurement of virus fusion by fluorescence-dequenching assays indicated that the virus was somewhat less sensitive to cholesterol depletion than measurement by infectivity assays. It is possible that the different MOIs employed in the different assays contribute to differences in the apparent sensitivity of methyl-β-cyclodextrin (45). Cholesterol depletion of HIV-1 envelopes has been shown to affect virus infectivity, based on inhibition of virus internalization (7, 18). HIV-1 fuses with the plasma membrane, so cholesterol was suggested to play a role in the conformational changes accompanying gp41-mediated fusion (18, 24). In general terms, HIV-1 entry appears to be more sensitive to cholesterol depletion than influenza virus entry. It is possible that the complex receptor-primed conformational changes accompanying HIV-1 fusion are more dependent on the lipid architecture of the viral envelope than those occurring during the conceptually simpler pH-triggered fusion of influenza virus.

Influenza virus is known to selectively incorporate both proteins and lipids into its envelope (21, 22, 29), and influenza virus HA association with lipid microdomains, or rafts, is well

established (39). It remains to be seen whether the effect of methyl-β-cyclodextrin shown here is specific to the presence of lipid microdomains in the virus envelope or whether the cholesterol itself is the key component.

Previous studies have shown that HA-mediated hemifusion is dependent on the lipid composition of the contacting membrane monolayers (9), although cholesterol itself was not analyzed. In this paper, we show a specific dependence on cholesterol in the viral envelope (the donor membrane for fusion); however, the identification of the precise stage in influenza virus envelope fusion for which cholesterol is required awaits further investigation.

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