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Asymmetric Requirement for Cholesterol in Receptor-Bearing but Not Envelope-Bearing Membranes for Fusion Mediated by Ecotropic Murine Leukemia Virus

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We show that fusion mediated by ecotropic murine leukemia virus envelope is dependent on cholesterol in receptor-bearing membranes. The effect is >10 times larger in insect cells than mammalian cells, probably because the former can be more extensively depleted of cholesterol. The fact that cholesterol is apparently not needed in envelope-bearing membranes suggests that it plays a role in an asymmetric step in membrane fusion and argues against a class of models in which cholesterol is important in symmetric fusion intermediates. The insect cell system has promise for clarifying the role of membrane rafts in other aspects of cell physiology.

When enveloped viruses leave or enter cells, lipid bilayers break and reform in a controlled fashion. Despite considerable progress in determining the structures of some of the proteins involved, a detailed understanding of this process is lacking, especially as it concerns changes in the configuration of lipids. For many—but not all—enveloped viruses, the lipid composition of the virus or target membrane is known to affect fission or fusion (21). For example, alphaviruses usually require cholesterol in target membranes for fusion, although point mutations in one of their envelope genes can obviate this need (7, 26). Cholesterol has many effects on membranes that could affect fusion, such as increasing the packing density of lipids (46), increasing resistance to bending (50), altering the conformations and functions of membrane proteins (14), and promoting phase separation of certain lipids into membrane domains called "rafts" (5, 33, 44, 52).

Rafts laterally organize proteins and lipids within the plasma membrane (4); they are involved in cholesterol transport and cell signaling (9, 18, 27). Some plasma membrane proteins are constitutively raft associated (e.g., influenza virus envelope hemagglutinin [13, 22, 51]), while others move into rafts in response to environmental signals (e.g., the T-cell receptor after exposure to antigen [32] or the human immunodeficiency virus (HIV) coreceptor CXCR4 after exposure to HIV envelope gp120 [28]). The membranes of viruses of several classes, including orthomyxoviruses, paramyxoviruses, alphaviruses, and retroviruses, are richer in cholesterol and sphingolipids than bulk plasma membrane and similar in composition to the rafts from which they are presumed to bud (3, 36, 38, 41, 45, 51). Disruption of rafts by cholesterol or sphingolipid depletion reportedly blocks an early step in HIV infection (17, 28).

We recently reported that the receptor for ecotropic murine leukemia virus (MLV) is a raft protein and that cholesterol depletion inhibited envelope receptor-mediated fusion (25). The degree of inhibition we observed, ~10-fold, could have been limited by the fact that extraction of more than about 70% of cholesterol from mammalian cells is toxic (20). Insect cells, in contrast, do not synthesize cholesterol, tolerate growth in cholesterol-depleted medium, and can be extensively cholesterol depleted (10, 11, 35, 37, 43). Therefore, we were interested to see if greater inhibition of MLV fusion by cholesterol depletion could be achieved using insect cells, as this could facilitate understanding the role cholesterol and/or rafts play in virus fusion. Here, we describe an insect cell system for studying fusion mediated by MLV envelope and its receptor, CAT1, in which fusion is inhibited more than 100-fold by cholesterol depletion. Our main finding is that cholesterol is essential in the receptor-bearing membrane but not in the envelope-bearing membrane. The results are discussed in terms of models of the role of cholesterol in virus fusion.

MATERIALS AND METHODS

Plasmids. Sindbis virus stable expression vectors for CAT1 fused to green fluorescent protein (GFP) and vesicular stomatitis virus glycoprotein (VSV-G) fused to GFP at its C terminus have been described (25). A related transient expression vector for CAT1gfp was made by amplifying CAT1gfp with oligonucleotides containing artificial 5′ *Xba*I sites and cloning it into the *Xba*I site in pSINrep5 (catalog no. K750-01; Invitrogen). A similar transient vector for fusogenic Moloney MLV envelope was made by transferring MLVenv12 (19) to the *Xba*I site of pSINrep5. A double-subgenomic-promoter Sindbis virus vector encoding GFP after the second subgenomic promoter was made by amplifying a segment of pSINrep19gfp (1) containing GFP and its upstream subgenomic promoter and inserting this into the *Sph*I site of pSINrep5. This vector was then modified to express MLVenv12 from the upstream subgenomic promoter by transferring MLVenv12 as a restriction fragment into upstream sites in the polylinker region; the final vector was designated pSINrep5MLVenv12-gfp. Details of the constructions are available on request.

Cell culture. Baby hamster kidney (BHK) cells were cultured at 37°C. in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS). Mosquito C6/36 cells (catalog no. CRL-1660; American Type Culture Collection) were cultured at 28°C in DMEM with 10% heat-inactivated FBS (30). Cholesterol-depleted C6/36 cells were cultured in DMEM containing 10% FBS delipidated with Cab-O-Sil as previously described (48).

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Cholesterol assay. The AmplexTM Red cholesterol assay kit from Molecular Probes (catalog no. A-12216) was used to measure cholesterol extracted from cells with isopropyl alcohol as described previously (16).

Vector RNA and electroporation. Plasmids were linearized with *Not*I and transcribed in vitro using SP6 RNA polymerase (Promega) and 7-methyl-G (New England Biolabs). The transcription mixture was added to cells in 0.4 ml of ice-cold phosphate-buffered saline (PBS) and pulsed twice using a Bio-Rad electroporation device set to 0.8 kV and 25 μF (19).

Preparation of infectious vesicles and treatment with methyl-β-cyclodextrin. About 10^7 BHK cells were electroporated with RNA from pSINrep5MLVenv12 or pSINrep5CAT1gfp. One day after electroporation, the cells were harvested by scraping with a rubber policeman, sonicated in 2 ml of DMEM with an Ultrasonic Cell Disruptor (model no. 36810; TORBEO) at the maximum setting for 30 s, and then filtered through 0.45-μm-pore-size filters. Mammalian cells were treated with 10 mM methyl-β-cyclodextrin (Sigma) in DMEM for 30 min at 37°C. Vesicles were treated with methyl-β-cyclodextrin by incubating 0.4 ml of vesicles with 0.4 ml of 20 mM methyl-β-cyclodextrin in DMEM for 30 min at room temperature. Then 5 ml of fresh DMEM was added, and the mixture was centrifuged at 50,000 rpm in a Beckman Sw55 rotor for 2 h. The pelleted vesicles were resuspended in 0.4 ml of DMEM containing 1% FBS.

Western blot analysis. Cells grown in T-75 flasks were lysed in 1 ml of RIPA (1× PBS, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate) containing 0.1 mg of phenylmethylsulfonyl fluoride/ml, 30 μl of aprotinin/ml, and 1 mM sodium orthovanadate. The lysates were pipetted through 200-μl pipette tips several times and clarified by brief centrifugation at 4°C. Aliquots were heated to 70°C or boiled in an equal volume of Laemmli sample buffer, electrophoresed in 10% SDS-polyacrylamide gel electrophoresis gels, and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were probed with monoclonal mouse anti-GFP antibody (catalogue no. 33-2600; Zymed), polyclonal rabbit anti-caveolin antibody (catalogue no. C13630; Transduction Laboratories), or goat anti-Rauscher MLVgp70 (Quality Biotech, Camden, N.J.), followed by horseradish peroxidase-labeled anti-mouse, anti-rabbit, or anti-goat IgG and a horseradish peroxidase substrate (catalogue no. 34075; Pierce).

Detergent solubility assay. BHK cells electroporated 1 day previously were washed twice with PBS and extracted with 1 ml of ice-cold NTE (100 mM NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA) containing 1% Triton X-100 for 30 min on ice. The extract was centrifuged at 120,000 × *g* for 20 min at 4°C to separate soluble and insoluble material. The pellet was resuspended in 1 ml of NTE, and equal portions of soluble or pelleted material were analyzed by Western blotting.

Surface biotinylation. Cell surface proteins were labeled with biotin using sulfosuccinimidyl-6-(biotinamido)hexanoate (catalogue no. 21335; Pierce) as described previously (25). Cell lysates were precipitated with streptavidin-agarose for 1 h at 4°C. The agarose beads were washed three or four times with cell lysis buffer and eluted with SDS-polyacrylamide gel electrophoresis loading buffer, and the eluate was analyzed by Western blotting.

Triton X-100 treatment and confocal microscopy. C6/36-CATgfp cells grown in Lab-Tek eight-well chambered coverglass devices (catalogue no.155411; Nalge Nunc International) were treated with 0.5 to 1.0% Triton X-100 in CO_2-independent medium (catalogue no. 18055-088; Gibco BRL) on ice for 5 to 10 min and then gently washed. Samples were observed with a Leica TCS-NT/SP confocal fluorescence microscope.

Fusion assay after transient transfection. C6/36 cells grown in normal or delipidated serum were electroporated with RNA from pSINrep5CATgfp or pSINrep5MLVenv12-gfp and seeded at 4×10^5/35-mm-diameter gridded tissue culture dish (catalogue no. 174926; Nalge Nunc). One day later, the number of GFP-positive cells in each dish was determined by counting with a fluorescence microscope with a 10× objective. XC or BHK cells (1.5×10^6) expressing MLV *env* were then added to each well. The number of syncytia per dish was determined with the same microscope 1 or 2 days later.

RESULTS

Effect of cholesterol depletion on syncytium formation in mammalian cells. We began by examining the effect of cholesterol depletion on fusion between mammalian (BHK) cells expressing a fusogenic form of the ecotropic MLV envelope truncated at the p2 cleavage site of TM (19) and cells expressing the MLV receptor CAT1. Treatment of XC cells, which naturally express CAT1, with the cholesterol-depleting drug cyclodextrin inhibited syncytium formation following the addition of envelope-expressing BHK cells (Fig. 1C versus B), whereas treatment of the BHKenv cells had no effect (Fig. 1F versus E). XC cells do not form syncytia when cocultivated with BHK cells that do not express MLVenv (data not shown). The inhibitory effect of cholesterol depletion in XC cells was modest but consistent with the facts that CAT1 is raft associated and cholesterol depletion inhibits MLV infection (25). The lack of effect of cholesterol depletion on envelope-expressing cells was somewhat surprising in view of previous studies showing that MLV particles have the cholesterol-rich lipid composition of rafts (45).

Detergent solubility assays. To see if MLV envelope is raft associated, we treated BHK cells expressing MLV envelope with 1% Trition X-100 at 4°C. This treatment solubilizes nonraft proteins but leaves raft proteins associated with pelletable cell membranes (2, 6). Following centrifugation, we analyzed the supernatant and pellet fractions by Western blot analysis. As positive controls, we used caveolin, an ~20-kDa prototypical raft protein, and ~100-kDa CATgfp, which we previously showed was raft associated by using the more precise sucrose gradient analysis method (25); both proteins were predominantly pelletable (Fig. 2, lanes 1 and 3 versus lanes 2 and 4). In these experiments, GFP provided an epitope tag for CAT. CATgfp was previously shown to be fully functional as an MLV receptor (24, 25, 31). In contrast to CATgfp and caveolin, MLVenv was predominantly solubilized, like VSV-G–GFP, a known nonraft protein (12), indicating that MLVenv is mostly not associated with rafts (Fig. 2, lanes 6 and 8 versus lanes 5 and 7). The fact that small portions of MLVenv and VSV-V–GFP were pelletable in this assay could be due to aggregation rather than association with cholesterol-rich membranes. Lack of raft association of MLVenv is consistent with cholesterol depletion having little effect on the ability of MLVenv-bearing membranes to fuse with membranes expressing CAT1.

Cyclodextrin sensitivity of infectious vesicles. To test the effect of cholesterol depletion in another way, we used a previously described infectious-vesicle assay (19, 23) in which BHK cells expressing MLVenv or CAT1 via Sindbis virus vectors are sonicated to produce vesicles that contain MLVenv or CAT1 on the outer surface and vector RNA inside. When these vesicles are added to cells expressing the cognate protein (CAT1 or MLVenv, respectively), they fuse to the plasma membrane and release vector RNA into the cytoplasm, where it replicates as a Sindbis virus replicon. The amplified vector directs synthesis of more encoded protein (MLVenv or CAT1), which induces fusion with neighboring cells. The syncytia expand rapidly and in 2 days produce macroscopically visible plaques. An advantage of this assay over infection with virus is that the envelope and receptor are treated symmetrically—either can be expressed in the vesicle via the Sindbis virus vector, and the other can be expressed in the target cell. Treatment of CAT infectious vesicles with cyclodextrin reduced their titer 12- to 15-fold, whereas treatment of MLVenv vesicles reduced their titer only about 2-fold (Table 1). These results support the conclusion from the cell-cell fusion data shown in Table 1 that cholesterol is more important in the receptor-bearing membrane than in the envelope-bearing membrane.

Lack of effect of cholesterol depletion on surface expression

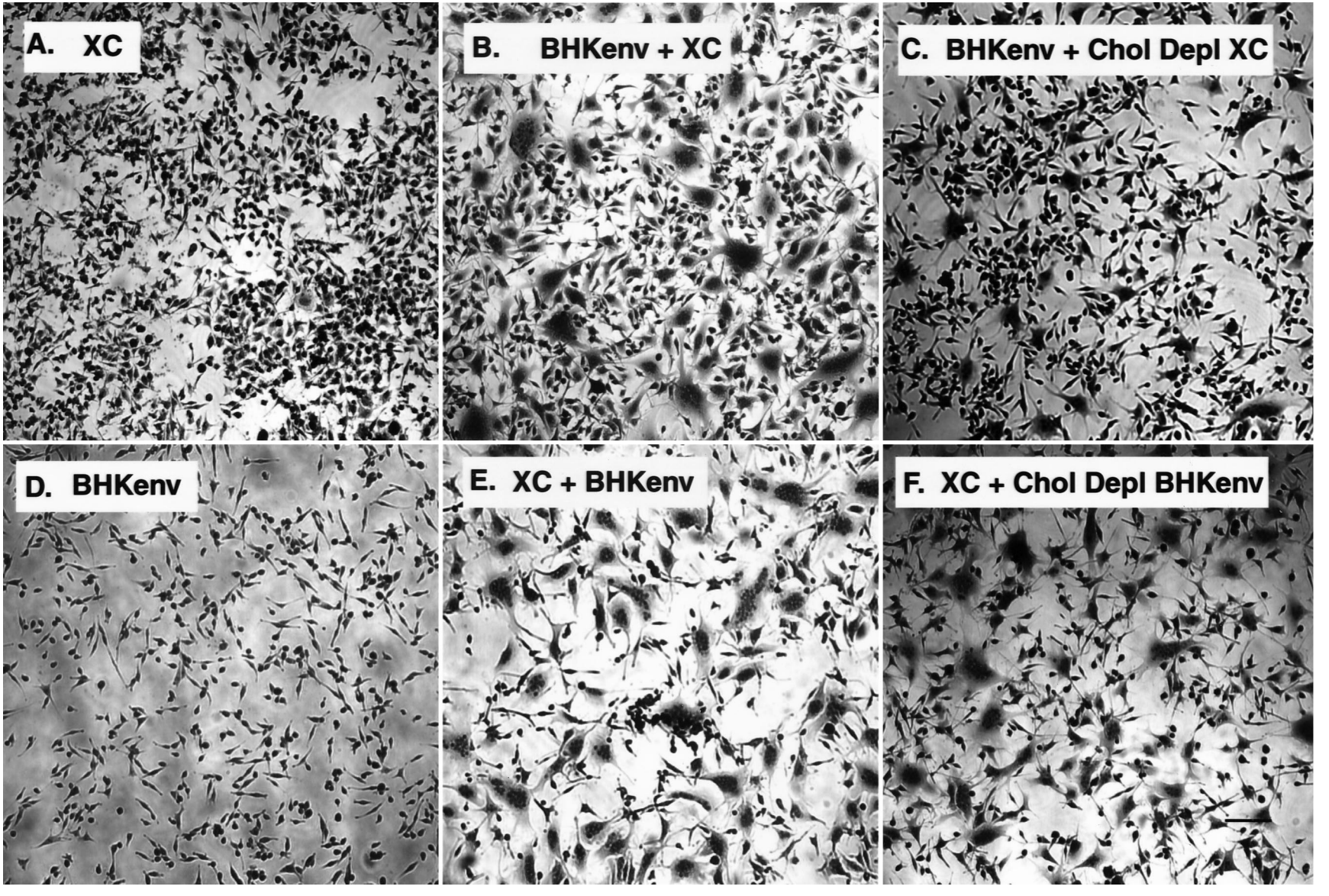

FIG. 1. Cholesterol depletion of CAT-expressing XC cells, but not MLVenv-expressing BHK cells, inhibits syncytium formation. XC cells were seeded in wells A, B, and C, and BHKenv cells were seeded in wells D, E, and F. One day later, the cells in wells C and F were treated with 10 mM cyclodextrin for 30 min and washed. BHKenv cells were added to wells B and C, and XC cells were added to wells E and F. The next day, the cells were stained with 0.17% methylene blue–carbol fuchsin in methanol to reveal syncytia. Chol Depl, cholesterol depleted.

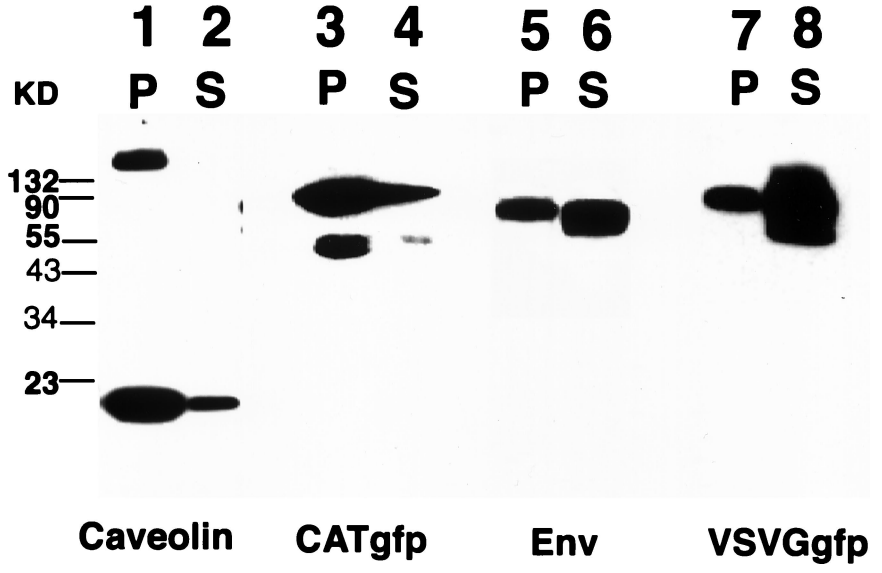

FIG. 2. Detergent solubility of caveolin, CATgfp, MLVenv, and VSV-G-GFP. Plain BHK cells (lanes 1 and 2) or BHK cells electroporated with RNA from Sindbis virus vectors encoding CATgfp (lanes 3 and 4), MLVenv (lanes 5 and 6), or VSV-G-GFP (lanes 7 and 8) were lysed with 1% Triton X-100 at 4°C and centrifuged to pellet membranes. Equal portions of pellets (P; lanes 1, 3, 5, and 7) and supernatants (S; lanes 2, 4, 6, and 8) were analyzed by Western blotting with antiserum to caveolin (lanes 1 and 2), GFP (lanes 3, 4, 7, and 8), or MLV Env (lanes 5 and 6).

of CAT1. To rule out the possibility that the inhibitory effect of cyclodextrin is due to reduced surface expression of CAT1, we biotinylated the surfaces of BHK cells electroporated with the Sindbis CATgfp vector, precipitated the biotinylated species with avidin agarose, and analyzed the precipitate by Western blotting with anti-GFP antibody. Cholesterol depletion had no effect on the amount of surface CATgfp (Fig. 3A), as has been observed with other membrane proteins in cells treated with cyclodextrin (15).

In the experiments described so far, the fusion-inhibiting effect of cyclodextrin was modest, perhaps because mammalian cells can be only partially depleted of cholesterol without significant toxicity. In contrast, insect cells can be extensively cholesterol depleted by growing them in medium containing delipidated serum (11, 30). To see if such treatment of insect cells would inhibit fusion more extensively, we repeated the experiments using C6/36 mosquito cells.

Extent of cholesterol depletion in insect cells. We first measured the amount of cholesterol in C6/36 cells passaged in delipidated serum. After three passages, total cell cholesterol fell more than 99%, to below the limit of detection of our assay. The cholesterol-depleted cells looked healthy by light microscopy, although their growth rate was lower than that of cells maintained in normal serum. In contrast, treatment of BHK cells with 10 mM cyclodextrin reduced their cholesterol by only about 35%, and raising the cyclodextrin dose caused the BHK cells to round up and detach from the tissue culture dish. These results are consistent with reports in the literature of BHK and other insect cell lines (11, 20, 37, 47).

Lack of effect of cholesterol depletion on expression of CAT1. We used puromycin to select C6/36 cells stably expressing CATgfp using the pSINrep19 stable Sindbis expression vector that works in insect as well as mammalian cells (1) and

TABLE 1. Effect of cyclodextrin on infectivity of MLVenv and CATgfp vesicles

Vesicle type	Cyclodextrin[a]	No. of syncytium-inducing units per[b]:			Titer (10^3)[b]
		10 μl	1 μl	0.1 μl	
MLVenv	−	71, 82	7, 10	0, 0	7.1, 8.2
	+	34, 35	3, 1	0, 0	3.4, 3.5
CATgfp	−	189, ND	20, 25	3, 4	20, 25
	+	13, 20	4, 2	0, 0	1.3, 2.0

[a] +, present; −, absent.
[b] Results of two experiments; ND, not determined.

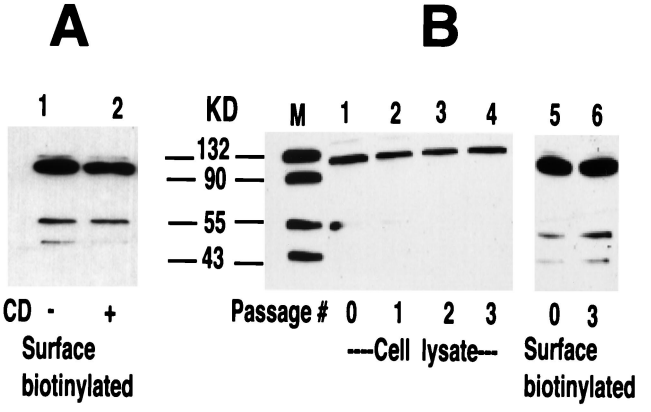

FIG. 3. Lack of effect of cholesterol depletion on expression of CATgfp; Western blot analysis with anti-GFP antibody. (A) Surface proteins labeled with biotin were purified with avidin agarose from BHK cells stably expressing CATgfp. Lane 1, mock-treated cells. Lane 2, cells treated (+) with cyclodextrin (CD). (B) Whole-cell lysates (lanes 1 to 4) or biotinylated surface proteins (lanes 5 to 6) from C6/36 cells stably expressing CATgfp grown in normal medium (lanes 1 and 5) or medium depleted of cholesterol for one (lane 2), two (lane 3), or three (lanes 4 and 6) passages.

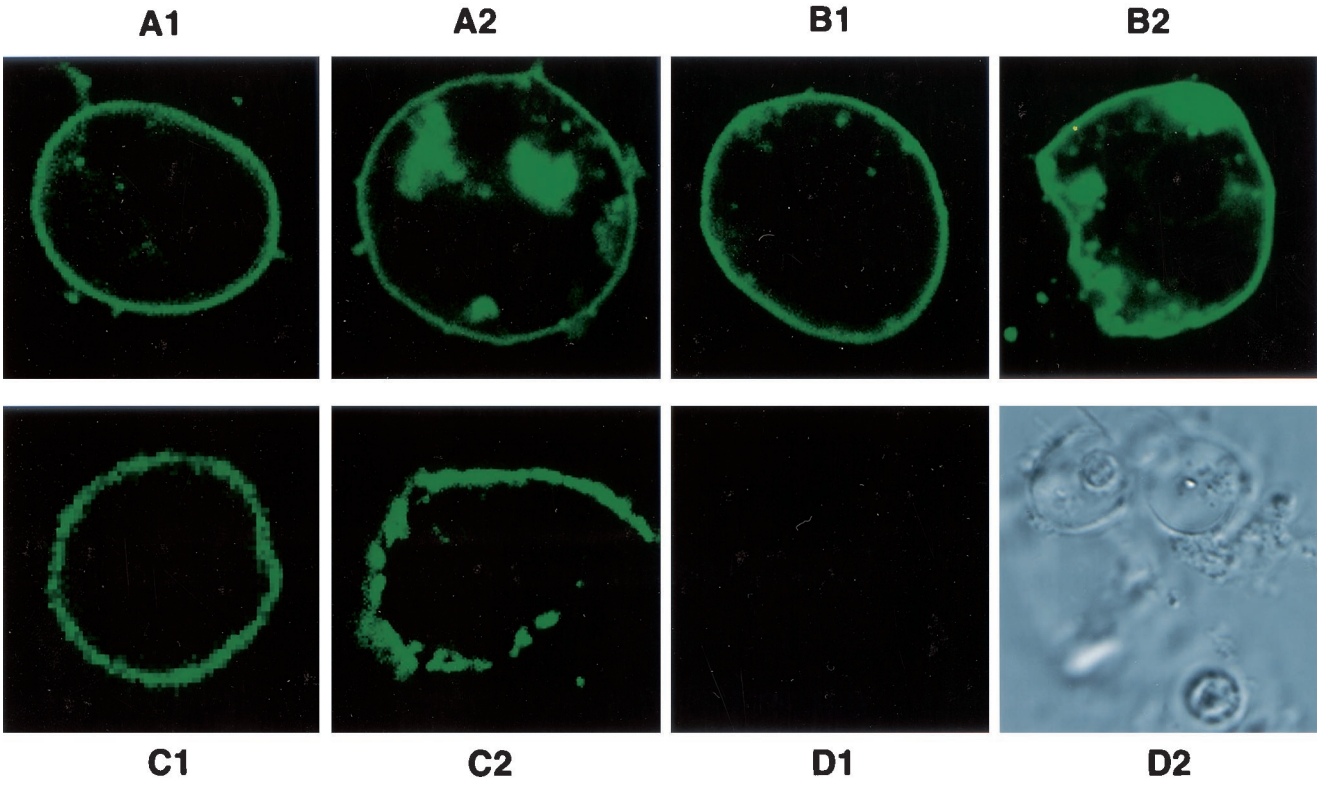

FIG. 4. Lack of effect of cholesterol depletion on surface expression of CATgfp in insect cells. Fluorescence images of C6/36 cells grown for three passages in normal medium (A and C) or cholesterol-depleted medium (B and D) and then electroporated with RNA from a Sindbis virus vector encoding CATgfp. Two cells of each type are shown in panels A1 and A2, B1 and B2, and C1 and C2 to illustrate the range of appearance of cells with respect to surface and intracellular CATgfp. The cells in panels C and D were treated with 0.5% Triton on ice for 10 min. The transmitted-light (D2) and fluorescence (D1) images of the same field show that CATgfp becomes Triton X-100 extractable in cholesterol-depleted cells. Image sizes, 15 by 15 μm (A, B, and C) and 30 by 30 μm (D).

also analyzed C6/36 cells acutely transfected with pSINrep5 vectors encoding CATgfp. Stable CATgfp expression was not altered by passage in delipidated serum, as determined by Western blot analysis of whole-cell lysate or cell surface biotin-labeled protein (Fig. 3B). The intensity of cell surface fluorescence from CATgfp following electroporation with transient expression vectors was not noticeably different in cells grown in normal versus delipidated serum (Fig. 4, A_1 and A_2 versus B_1 and B_2). In some cells, GFP was also located in intracellular aggregates; the extent of intracellular aggregation was not noticeably different for cells grown in the presence (A_2) versus the absence (B_2) of cholesterol. As expected for a raft-associated protein, the surface fluorescence was resistant to treatment with cold Triton X-100 in cells grown in normal serum (Fig. 4, C_1 and C_2) but was removed by Triton X-100 in cells grown in delipidated serum (Fig. 4, D_1 and D_2).

Inhibition of MLVenv-mediated fusion by cholesterol depletion of insect cells expressing CAT1. The insect cells stably expressing CATgfp formed syncytia when cocultivated with BHKenv cells (Fig. 5). Serial passage in delipidated serum inhibited syncytium formation by over 100-fold (Table 2). Passaging the cholesterol-depleted CAT1-expressing cells in medium containing normal serum restored their ability to form syncytia, showing that the cholesterol depletion did not have lasting effects.

Although the CAT1-expressing insect cells formed syncytia with cells expressing MLVenv, they were not infectible with MLV. This may be due to a block in expression of MLV in insect cells. To solve this problem, we used the infectious-vesicle assay, which relies on expression of the Sindbis virus vector to reveal fusion events. Vesicles made from BHK cells electroporated with RNA from pSINrep5MLVenv12 were able to infect and form syncytia in C6/36 cells stably expressing CAT-1 but not in plain C6/36 cells (Fig. 6). Serial passage in delipidated serum reduced the susceptibility of C6/36CATgfp cells to the infectious vesicles by 400- to 800-fold (Table 3).

Lack of inhibition of fusion by cholesterol depletion of insect cells expressing MLVenv. To see if cholesterol depletion would affect fusion in insect cells expressing MLVenv, we used a Sindbis virus vector encoding MLVenv plus GFP driven by a second subgenomic promoter; the GFP in this vector provided a marker to monitor the efficiency of electroporation. C6/36 cells grown in normal serum or serially passaged in delipidated serum were electroporated with the pSINrep5MLVenv-gfp vector. The efficiency of electroporation was determined by counting the number of GFP-positive cells 16 h later. One day after electroporation, XC cells were added to measure syncytium-forming ability. By comparing the number of syncytia 1 or 2 days after XC cells were added with the number of single green cells 1 day after electroporation, we determined the efficiency of syncytium formation. About 80 to 95% of C6/36 cells expressing *env*-GFP on day 1 went on to form syncytia

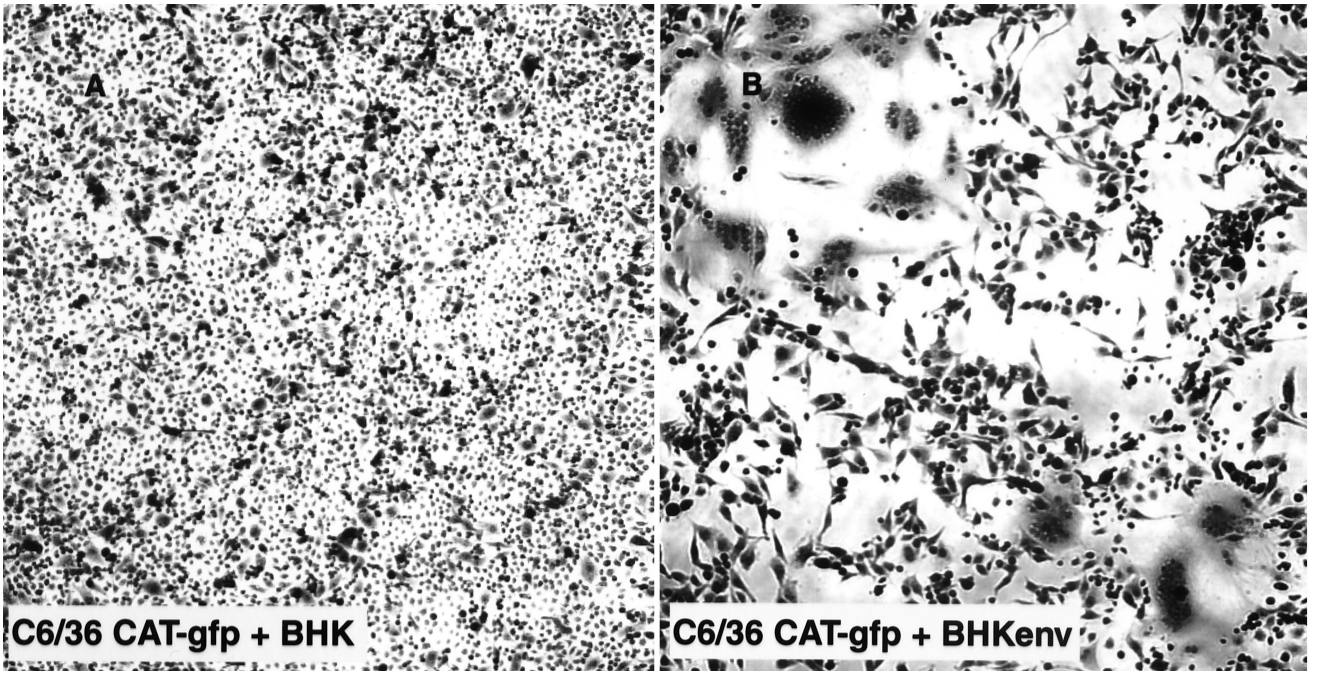

FIG. 5. Syncytium formation when C6/36 insect cells stably expressing CATgfp were cocultured with BHK cells (A) versus BHKenv cells (B).

with XC cells, independent of whether the C6/36 cells were grown in normal or delipidated serum (Table 4, top two lines, and Fig. 7A and B). In contrast, when C6/36 cells were electroporated with RNA from the pSINrep5CATgfp vector, the efficiency of syncytium formation with cells expressing MLVenv was reduced from 87 to 96% for cells grown in normal serum to about 1% for cells passed in delipidated serum (Table 4, bottom two lines, and Fig. 7C and D).

DISCUSSION

We found that insect cells provide a good model system for studying the cholesterol dependence of MLVenv-mediated fusion events because these cells can be extensively depleted of cholesterol without significant toxicity, and this makes them strongly resistant to fusion. Cholesterol-depleted, CAT-expressing insect cells were at least 10 times more resistant to MLVenv-mediated fusion than cholesterol-depleted mammalian cells. In assays using infectious vesicles, cholesterol depletion inhibited fusion ~1,000-fold (Table 3), while in cell-cell fusion assays, the inhibition was ~100-fold. The reduced inhibition in cell-cell fusion assays may be due to use of a low concentration of normal serum (we used 2%) to keep the indicator mammalian cells healthy; this amount of serum may have begun to replete the insect cells. It should be mentioned that growth in delipidated serum could deplete insect cells of other important factors besides cholesterol. However, the main effect is due to cholesterol, because adding back cholesterol to delipidated serum restored fusion to nearly control levels.

The possibility of artifacts due to overexpression of proteins is always a concern in experiments using potent expression vectors. Whenever possible in our experiments, we used the pSinRep19 vector (Fig. 5 and 6 and Tables 2 and 3) rather than the pSinRep5 vector, as the former encodes a mutant protease that results in ~20-fold less protein expression (1). The intracellular aggregates of CATgfp seen in some insect cells transfected with pSinRep5 vectors (Fig. 4, A_2 and B_2) could result from saturation of normal cell transport pathways. However, we feel it is unlikely that the cholesterol dependence of fusion is an artifact of overexpression because similar results were obtained with high and low expression vectors and cholesterol depletion inhibited fusion by over 99% in insect cells; if this were due to the use of an alternative, cholesterol-dependent transport process for CAT in overexpressing cells, that pathway would have to account for over 99% of the functional cell surface CAT.

Cholesterol-depleted insect cells should be a good system for studying the role of rafts in other cell functions. Despite slight differences in overall lipid composition compared to mammalian cells, insect cells are reported to have raftlike domains that are disrupted by cholesterol depletion (40). The survival of insect cells in the absence of cholesterol raises the possibility that rafts are nonessential or that insect cells have an alternative structure. A related study found that the insect

TABLE 2. Effect of serial passage in delipidated serum on ability of CATgfp-expressing C6/36 cells to form syncytia with BHKenv cells[a]

No. of passages in delipidated serum	No. of syncytia/dish	No. of syncytia after passage in normal serum
0	144, 161	143, 137
1	18, 23	131, 144
2	2, 2	99, 107
3	0, 0	90, 92

[a] Results of two experiments.

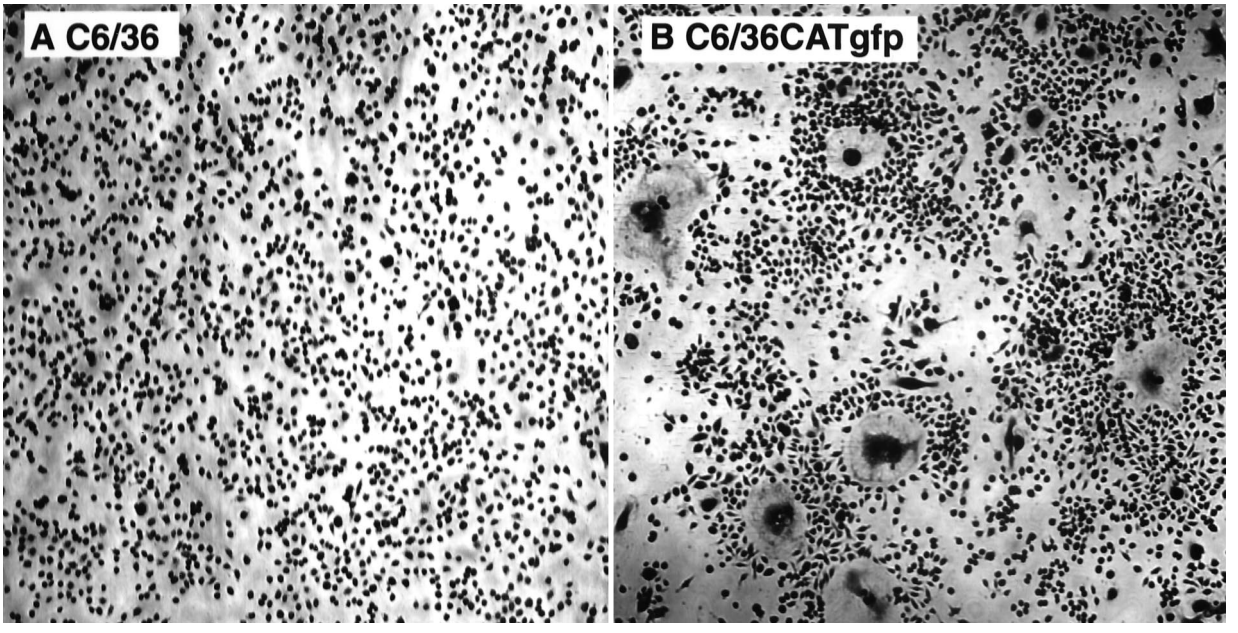

FIG. 6. Syncytium formation when C6/36 cells stably expressing CATgfp (B), but not plain C6/36 cells (A), were infected with vesicles from BHK cells electroporated with RNA from a Sindbis virus vector (pSINrep5MLVenv12) encoding MLVenv.

cell line High Five could be grown for at least 59 passages in delipidated serum without deleterious effects (11). These cholesterol-depleted cells were nearly fully susceptible to infection with VSV but impervious to the normally toxic effects of influenza virus ion channel M2, suggesting that cholesterol was necessary for ion channel activity.

It is useful to categorize ways in which cholesterol could affect fusion processes. (i) Cholesterol could interact directly with the virus receptor, altering its conformation so that it no longer binds virus or disrupts the membrane in a way that leads to fusion. As precedent, cholesterol is known to bind to some membrane proteins (34) and to alter the conformations of some membrane receptors so that they no longer bind their ligands (14). (ii) Cholesterol could affect more general membrane properties relevant to fusion, possible examples being fluidity, flexibility, spontaneous curvature (8), and the tendency to form an inverted hexagonal or other nonbilayer configuration related to fusion intermediates. (iii) The cholesterol requirement could be secondary to a need for phase-separated domains (rafts). Rafts could facilitate fusion by concentrating receptors; recruiting signaling molecules, such as lipid-modifying enzymes (42); limiting diffusion of modified lipids; or providing an edge between lipid domains more susceptible to membrane rupture. One approach to distinguishing among these possibilities is to take advantage of the variety of existing cholesterol analogues to see if any have differential effects on fusion and, for example, raft formation (14, 49). We think the possibility that fusion involves rafts through their role in signaling is less likely in view of our results with small receptor-bearing vesicles: for their infectiousness to involve signaling, they would have to have packaged all the elements of the signaling pathway, whereas in the intact cell, signaling presumably recruits factors distributed in the much larger cytosolic volume.

Our main finding is that cholesterol is needed in receptor-bearing but not envelope-bearing membranes. Similar results have been reported for alphaviruses (26, 47) and HIV (28). In the case of alphaviruses, the fact that point mutations in the envelope overcome the need for cholesterol in target membranes has been interpreted to indicate that the envelope interacts with cholesterol in the target membrane at some stage during fusion (26). The relative unimportance of cholesterol in

TABLE 3. Effect of serial passage in delipidated serum on susceptibility of CATgfp-expressing C6/36 cells to syncytia induction by vesicles from BHK cells electroporated with a Sindbis vector encoding MLVenv[a]

No. of passages in delipidated serum	No. of syncytium-inducing units per:				Titer (no./ml)
	100 μl	5 μl	1 μl	0.1 μl	
0	TMTC	79, 83	17, 16	2, 3	1.7, 1.6×10^4
2	14, 16	1, 2	0, 0	0, 0	140, 160
3	2, 4	0, 0	0, 0	0, 0	20, 40

[a] Results of two experiments. TMTC, too many to count.

TABLE 4. Effect of serial passage in delipidated serum on ability of C6/36 cells acutely electroporated with Sindbis vectors encoding MLVenv + GFP or CATgfp to form syncytia in indicator cells expressing CAT1 (XC cells) or MLVenv (BHK cells), respectively[a]

Gene(s) encoded by vector	No. of passages in delipidated serum	No. of GFP-positive cells	No. of syncytia	% GFP-positive cells that form syncytia
MLVenv + GFP	0	247, 892	199, 843	81, 95
MLVenv + GFP	3	123, 377	93, 340	76, 90
CATgfp	0	304, 850	292, 738	96, 87
CATgfp	3	187, 447	2, 3	1, 0.7

[a] Results of two experiments.

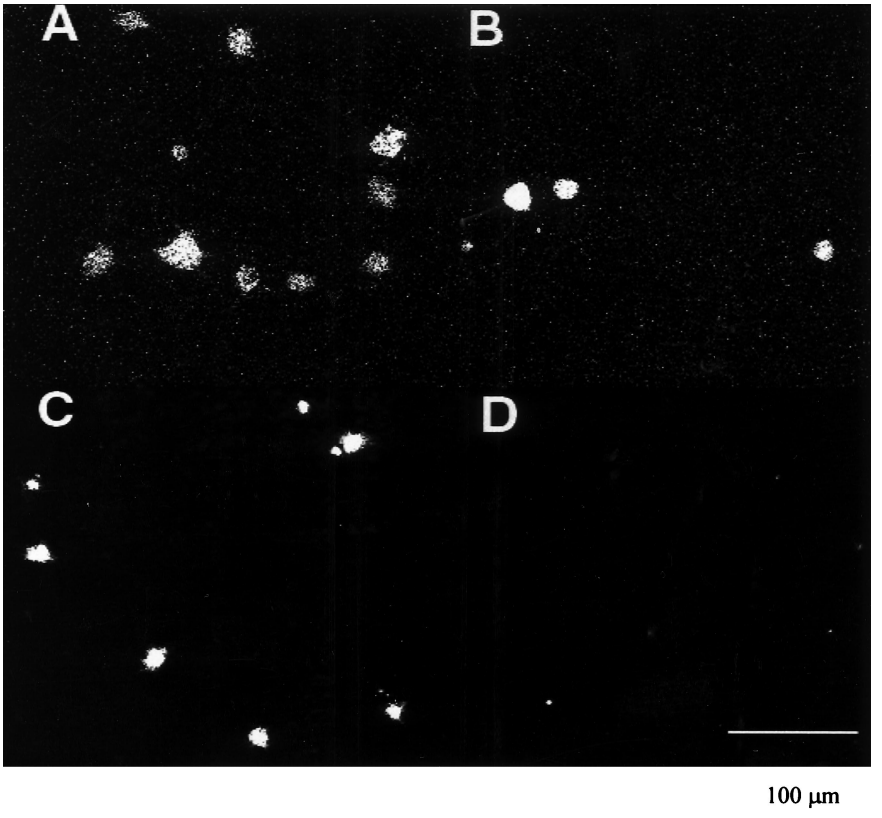

FIG. 7. Effect of cholesterol depletion on ability of insect cells transiently expressing MLVenv or CATgfp to fuse with indicator cells expressing CAT1 or MLVenv, respectively. Normal C6/36 cells (A and C) or C6/36 cells passaged three times in cholesterol-depleted medium (B and D) were electroporated with RNA from a Sindbis virus vector encoding MLVenv plus GFP and cocultured with XC cells (A and B) or with a Sindbis virus vector encoding CATgfp and cocultured with BHKenv cells (C and D). Fluorescence images 1 day after coculture are shown. In panels A, B, and C, the fluorescent patches come from syncytia, whereas in panel D, the small fluorescent spots come from single fluorescent cells that have not formed syncytia.

virus particles may seem at odds with the observation that viruses have a raftlike membrane lipid composition (3, 45). However, viral membranes may be raftlike because the viruses bud preferentially from rafts rather than because of a need for raft domains in virus particles during fusion (29, 36, 41, 51). The combined results argue against models in which cholesterol is needed in a symmetric fashion. For example, it is unlikely that the role of cholesterol is to reduce the energy of a hemifusion or fusion pore intermediate, as these structures are symmetric with respect to envelope and receptor membranes. Rather, inherently asymmetric steps in fusion are likely to be affected, such as insertion of fusion peptide into a target membrane or rupture of the inner leaflet of the target membrane (39). The relative size difference between virus and cell is not a relevant asymmetry because small vesicles bearing the envelope or receptor were equally infectious. Since the only obvious difference between the envelope and receptor infectious vesicles is which protein they contain, we favor a model in which the effect of cholesterol is mediated through its interaction with one of these proteins.

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