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Bound Simian Virus 40 Translocates to Caveolin-enriched Membrane Domains, and Its Entry Is Inhibited by Drugs that Selectively Disrupt Caveolae

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Simian virus 40 (SV40) entry leading to infection occurred only after the virus was at the cell surface for 1.5 to 2 h. SV40 infectious entry was not sensitive to cytosol acidification, a treatment that blocks endocytosis via clathrin-coated vesicles. Instead, SV40 infectious entry was blocked by treating cells with the phorbol ester PMA or nystatin, which selectively disrupts caveolae. In control experiments, transferrin internalization was sensitive to cytosol acidification but was not sensitive to PMA or nystatin. Also, absorbed transferrin entered cells within minutes. Finally, bound SV40 translocated to caveolin-enriched membrane complexes isolated by a Triton X-100 insolubility protocol. Treatment with nystatin did not impair SV40 binding but did block the partitioning of virus into the caveolin-enriched complexes.

INTRODUCTION

Two general mechanisms of virus entry into cells have been described (Marsh and Helenius, 1989). Some enveloped viruses (e.g., HIV) enter cells by direct fusion of their envelopes with the plasma membrane. Other enveloped viruses (e.g., influenza), as well as nonenveloped viruses (e.g., simian virus 40 [SV40] and adenoviruses), enter cells by endocytosis. The most frequently described and best understood endocytic pathway for virus penetration is that which occurs constitutively through clathrin-coated vesicles. Endocytosis through uncoated vesicles has also been described (Mackay and Consigli, 1976; Maul *et al.*, 1978; Kartenbeck *et al.*, 1989; Basak and Turner, 1992) but is not well understood.

SV40 entry is unusual in several respects. First, the majority of SV40 particles are endocytosed into small tight-fitting uncoated vesicles (Maul *et al.*, 1978; Kartenbeck *et al.*, 1989). Second, these particles are targeted to the endoplasmic reticulum (ER; Kartenbeck *et al.*, 1989; Norkin and Anderson, 1996) rather than to the endosomal/lysosomal compartment, which is the usual target for endocytic traffic. Finally, SV40 infectious entry is dependent on an intracellular signal induced by SV40 from the cell surface (Dangoria *et al.*, 1996).

The small uncoated surface invaginations that seem to mediate SV40 entry have a morphology resembling that of caveolae (for a review of caveolae, see Anderson, 1993a). Caveolae are flask-shaped structures, ~70 to 100 mm in diameter, that are readily distinguished from clathrin-coated pits by their size and shape. Also, caveolae contain a characteristic marker protein called caveolin (Rothberg *et al.*, 1990). Ultrastructural and biochemical evidence shows that structures having the appearance of caveolae can be isolated in a low-density Triton X-100–insoluble fraction that is also highly enriched for caveolin (Rothberg *et al.*, 1992; Sargiacomo *et al.*, 1993; Lisanti *et al.*, 1994b).

The precise functions of caveolae are not clear, but they have been implicated in potocytosis, transcytosis, intracellular signaling, and endocytosis (Montesano *et al.*, 1982; Simionescu *et al.*, 1982; Anderson, 1993a; Lisanti *et al.*, 1994a,b; Parton *et al.*, 1994; Schnitzer *et al.*, 1994, 1995). To show that caveolae might mediate SV40 entry, we report that SV40 entry leading to infection was blocked by treatment of cells with either nystatin or phorbol ester, which selectively impairs

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caveolae function. In contrast, SV40 infectious entry was not blocked by acidification of the cytosol, which selectively impairs endocytosis through clathrin-coated pits. Furthermore, we show that preabsorbed SV40 translocated into the caveolin-enriched membrane domain isolated by the Triton X-100 insolubility protocol. Partitioning of SV40 into the Triton-insoluble fraction was blocked by nystatin. Finally, SV40 entry was notably slow, as compared with viruses that enter in clathrin-coated vesicles.

Despite much current interest in caveolae and non-clathrin-mediated endocytosis, it is not yet generally accepted that caveolae actually give rise to endocytic vesicles under physiologic conditions. The present findings suggest that SV40 may be an excellent marker to follow caveolae-mediated endocytosis under physiologically relevant conditions. A preliminary account of some of our results was reported (Norkin and Anderson, 1996).

MATERIALS AND METHODS

Chemicals

Nystatin, phorbol 12-myristate 13-acetate (PMA), and amiloride were purchased from Sigma (St. Louis, MO). Stock solutions of 5 mg/ml, 162 mM, and 10 mM, respectively, were made fresh in dimethyl sulfoxide (DMSO). Genistein was also purchased from Sigma and was reconstituted with 100% ethanol to a concentration of 20 mM and stored at −20°C until use.

Cells and Virus

CV-1 African green monkey kidney cells and HeLa cells were purchased from the American Type Tissue Culture Association (Rockville, MD) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin (Sigma). Cells were cultured at 37°C under a 5% CO_2 atmosphere. SV40 wild-type strain 776 was triple plaque purified. Virus was routinely grown by infecting 8×10^7 CV-1 cells at a multiplicity of infection (moi) of 1 plaque-forming unit (pfu) per cell. Cells were infected for 3–7 d. Virus was harvested from ~20 ml of cells by three freeze–thaw cycles. Cells debris was removed by centrifugation at 15,000 × *g*. Virus titers were determined by plaque assay, and titers of $\sim 10^8$ pfu/ml were routinely obtained. Virus stocks were stored at −20°C.

Preparation of SV40 Neutralizing Antiserum

SV40 virions were purified by centrifugation on a cesium chloride cushion at 24,000 × *g* for 24 h. The virus band was isolated with a syringe, and centrifugation on a cesium chloride cushion was repeated to insure viral purity (Khourg and Lai, 1979). Virions were injected intradermally in complete Freund's adjuvant into New Zealand white rabbits, and a booster immunization was given two weeks later. Four weeks after the first injection, rabbits were bled for antiserum. A 1:500 dilution of the anti-SV40 antiserum bound to CV-1 cells preadsorbed with SV40 but did not bind to uninfected CV-1 cells.

Flow Cytometry to Measure Bulk SV40 Entry

CV-1 cells grown on no. 1 glass coverslips were infected with SV40 (100 pfu/cell) for 30 min on ice in DMEM containing 10% FBS. Cells were washed twice and incubated at 37°C for various lengths of time to allow virus internalization. Cells were then detached from the coverslips with Versene (EDTA and phosphate-buffered saline [PBS]). Note that Versene did not remove SV40 from the cell surface. SV40 particles at the cell surface were detected by immunostaining on ice with rabbit anti-SV40 antiserum, followed by FITC-conjugated goat anti-rabbit IgG. Mean fluorescent intensity of 10,000 cells was measured by flow cytometry on a FACscan (Becton-Dickinson, San Jose, CA). Mean fluorescent intensity, converted to a linear scale for single cells only, was used to indicate relative amounts of surface SV40.

Postadsorption Neutralization Assay of SV40 Infectious Entry

Cells (CV-1, HeLa) grown on glass coverslips were infected with SV40 for 1 h on ice. Cells were then washed twice to remove unbound virions and incubated at 37°C for various periods to allow virus internalization. Cells were transferred to ice and treated with rabbit anti-SV40 neutralizing antiserum (1:500 dilution). Matched control cultures were mock treated with media rather than anti-SV40 antiserum. All samples were then incubated for 48 h at 37°C. Cells were fixed in acetone/methanol and stained by indirect immunofluorescence with SV40 T antigen-specific monoclonal antibody PAB101 (ATCC) and Texas Red-conjugated goat anti-mouse immunoglobulin (Ig)G (Jackson ImmunoResearch, West Grove, PA). T antigen-expressing cells were identified by fluorescence microscopy.

Transferrin Internalization

Transferrin entry into CV-1 cells was measured essentially as described by Karin and Mintz (1981) and Ciechanover *et al.* (1993). Apo-transferrin (10 mg; Sigma) was reconstituted in 1 ml of PBS containing ammonium iron III citrate (0.1 mg/ml) for 3 h at room temperature. Unbound iron was removed by dialysis in PBS. Transferrin (500 μg) was then labeled with ^{125}I (3 mCi) by the chloramine T method (Harlow and Lane, 1988). ^{125}I-Transferrin, in PBS containing $CaCl_2$ (0.1 mg/ml), $MgCl_2$ (0.1 mg/ml), and 1% BSA, was adsorbed to CV-1 cells for 30 min on ice. Cells were washed and incubated at 37°C for various lengths of time to allow ^{125}I-transferrin to internalize. Cultures were then washed twice with PBS, and surface transferrin was removed by treating cells with PBS containing Pronase (0.3%) for 1 h on ice. Cells were again washed twice, and the amount of Pronase-resistant (i.e., internalized) ^{125}I-transferrin was measured in a gamma counter. In control experiments in which cells were not cultured at 37°C after ^{12}I-transferrin binding, >95% of bound ^{125}I-transferrin was removed by Pronase treatment.

Cytosol Acidification to Inhibit Clathrin-dependent Endocytosis

Cytosol acidification was done essentially as described by Sandvig *et al.* (1987). CV-1 cells were incubated for 30 min in PBS, pH 7, containing NH_4Cl (50 mM), Ca (0.50 μg/ml), and Mg (50 μg/ml). Cells were then incubated in acidification buffer (KCl [140 mM], $CaCl_2$ [2 mM], $MgCl_2$ [1 mM], amiloride [1 mM], BSA [1%], and HEPES [20 mM], pH-adjusted to 7.0 with Tris) for various lengths of time.

Effects of PMA and Nystatin on SV40 Infectious Entry

CV-1 cells grown on glass coverslips were pretreated for 30 min at room temperature with either PMA (10 μM and 1 μM) or nystatin (25, 10, and 5 μg/ml) in DMEM. SV40 was adsorbed to cells for 1 h on ice in DMEM containing the inhibitors. Cells were washed twice, incubated for 4 h at 37°C in DMEM containing the various inhibi-

tors, washed five times with DMEM containing 10% FBS, and treated for 1 h on ice with media containing anti-SV40 neutralizing antisera. Cells were then incubated for 48 h at 37°C and stained by indirect immunofluorescence for SV40 T antigen. Parallel control cultures were not treated with the inhibitors.

Caveolae Isolation and Analysis

Caveolae were isolated essentially as described by Scherer *et al.* (1994) and Mastick *et al.* (1995). CV-1 cells from T175 flasks were washed and resuspended in 5 ml of ice-cold PBS, centrifuged, and resuspended in 1 ml of 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer (25 mM Mes, pH 6.5, and 150 mM NaCl) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml leupeptin and homogenized by pipetting up and down 10 times through a 1000-μl blue tip. The extract was adjusted to 40% sucrose, and a volume of 2 ml was transferred to an ultracentrifuge tube and overlaid with 1 ml of 35, 25, and 15% sucrose in MES buffer as above but minus the Triton X-100. The gradient was centrifuged for 17 h at 4°C in a Beckman SW50.1 rotor. Caveolin-enriched membranes were isolated as a band just below the 15–25% interface with an 18 G 1½ gauge needle and syringe (~1 ml). These fractions were diluted 1:2 in MES buffer containing Triton X-100, and the complexes were collected by centrifugation for 15 min in a microfuge at 14,000 rpm. Pellets were solubilized in 10 mM Tris, pH 8, containing 150 mM NaCl, 1 mM PMSF, and 1% Triton X-100. Protein in each sample was measured by Bio-Rad assay. The fractions were analyzed by 10 or 12% SDS-PAGE and Western blotting via standard procedures. Blots were initially probed for SV40 VP_1 and reprobed for caveolin. Caveolin was identified with rabbit anti-caveolin antisera (Santa Cruz Biotechnology, Tebu, France) and HRP-conjugated goat anti-rabbit IgG (Sigma). Bands were quantitated by densitometry with a Howtek model SM3 scanner and PDI Quantity One software (PDI, Huntington Station, NY).

RESULTS

Timing of SV40 Infectious Entry

Although most SV40 enters cells in uncoated vesicles, some particles do appear in clathrin-coated vesicles (Kartenbeck *et al.*, 1989). Because it is not clear which of these entry pathways actually leads to infection and because the timing of SV40 infectious entry is not yet known, we designed an assay to measure the kinetics of SV40 infectious entry exclusively, under the physiologically relevant condition of low-virus inputs. The assay was based on the following two features. First, we ascertained that SV40 at the cell surface is susceptible to neutralization by anti-SV40 antiserum. This enabled us to establish the time required for preadsorbed SV40 to internalize, because infection would then be protected from neutralization by antiserum. Second, to score entry events that result in infection, we immunostained cells for the SV40 T antigen, an early SV40 gene product. Our overall procedure was as follows. SV40 was adsorbed to cells at 4°C. Cultures were then washed, shifted to 37°C, and incubated for various periods of time before adding neutralizing anti-SV40 antiserum. Infected cells were identified by immunostaining for T antigen at 48 h. Our results show that, even at viral inputs of 100 pfu/cell, only 30% of the cells became infected by 2 h (Figure 1A). This slow rate of infection does not reflect a peculiarity

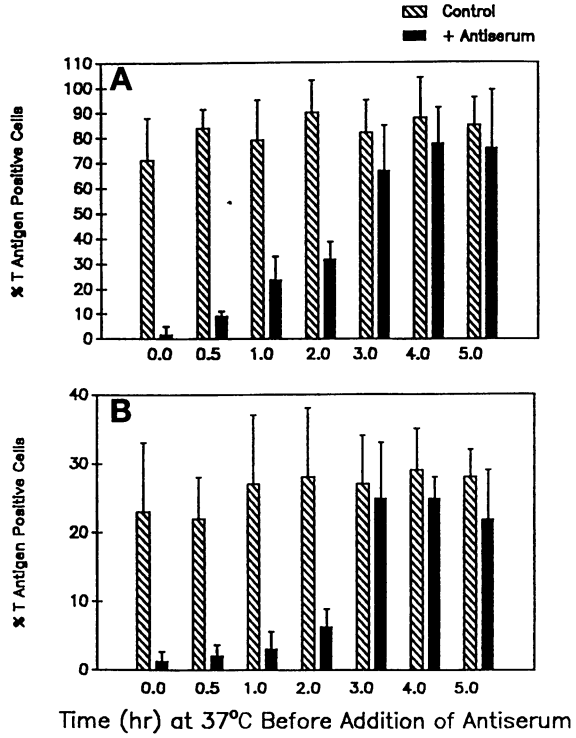

Figure 1. Timing of SV40 infectious entry. SV40 (100 pfu/cell) was adsorbed to CV-1 cells (A) or to HeLa cells (B) for 1 h at 4°C. Cultures were then incubated at 37°C for the indicated times before exposure to neutralizing anti-SV40 antiserum on ice for 1 h. Cultures were again incubated at 37°C. Infected cells were identified by indirect immunofluorescent staining for SV40 T antigen at 48 h. Matched control cells were mock treated with media rather than with anti-SV40.

of CV-1 cells, because very similar results were obtained with HeLa cells (Figure 1B). Corroborative results for the timing of infectious entry were obtained at viral inputs ranging from 0.1 to 100 pfu/cell. An assay based on postadsorption neutralization was previously used to analyze the infectious entry pathway of adenoviruses (Varga *et al.*, 1991).

To confirm that the slow rate of SV40 infectious entry seen here was not caused by the experimental protocol, we also used flow cytometry to follow bulk SV40 clearance from the cell surface. SV40 was adsorbed to cells at 4°C. The cultures were then shifted to 37°C, and the amount of virus at the cell surface at various times was measured by flow cytometry (Figure 2). This procedure showed that SV40 had a half-life at the cell surface of ~2.5 h. Also note that the greatest rate of clearance of SV40 from the cell surface occurred between 1.5 and 3 h postinfection, with virtually no virus leaving the cell surface during the first hour. Thus, SV40 entry was notably slow, regardless of whether infectious entry or bulk entry was measured. Also, in each case only a small amount of virus seemed to enter during the first hour.

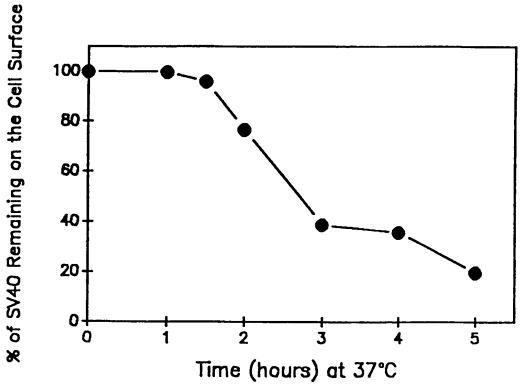

Figure 2. Timing of SV40 clearance from the cell surface. SV40 (100 pfu/cell) was adsorbed to CV-1 monolayer cells for 0.5 h at 4°C. Cells were then washed and incubated at 37°C. At the indicated times cells were detached with Versene, and SV40 virions at the cell surface were quantitated by indirect immunofluorescent staining with rabbit anti-SV40 antiserum and FITC-labeled goat anti-rabbit antiserum. Data are the mean fluorescent cell intensity for 10,000 cells per sample by flow cytometry.

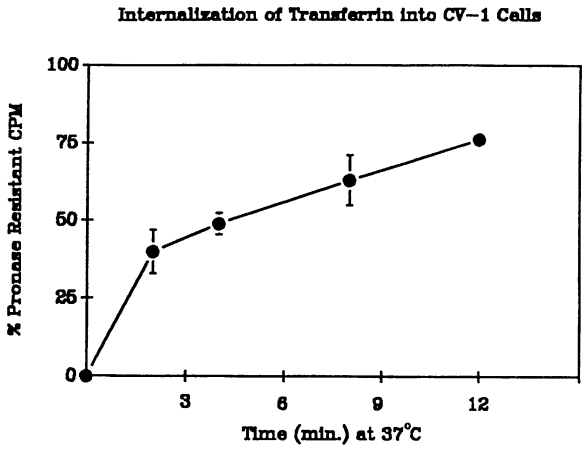

Figure 3. Timing of transferrin internalization into CV-1 cells. ^{125}I-labeled transferrin was adsorbed to CV-1 cells at 4°C for 0.5 h. Cultures were then incubated at 37°C. At the indicated times cell samples were treated with Pronase to remove transferrin still at the cell surface. Matched control cells were not treated with Pronase. Internalized ^{125}I-transferrin is expressed as the percentage of the total bound ^{125}I-transferrin that is resistant to Pronase digestion. Error bars are the SD of triplicate samples.

Transferrin is often used as a standard for a ligand that enters cells by receptor-mediated endocytosis in clathrin-coated vesicles. For the purpose of comparison to our findings with SV40, we measured the internalization of ^{125}I-labeled transferrin by following the time course over which preadsorbed transferrin became resistant to release from the cells by treatment with Pronase. In contrast to the slow rate of SV40 entry, we found that most ^{125}I-labeled transferrin was internalized within 4 min after shifting cells to 37°C (Figure 3).

SV40 Infectious Entry Is Not Dependent on Clathrin-coated Vesicles

As noted above, the majority of SV40 enters cells in uncoated vesicles (Kartenbeck *et al.*, 1989). Our next concern was whether this unusual entry pathway actually leads to productive infection. Thus, we asked whether infectious entry might occur under conditions that impaired endocytosis by coated vesicles. Cytosol acidification was used to block clathrin-mediated endocytosis. Under these conditions clathrin-coated pits are detected at the cell surface but are unable to pinch off from the plasma membrane. Consequently, ligands entering cells by receptor-mediated endocytosis become trapped in clathrin-coated pits (Sandvig *et al.*, 1987).

The effect of cytosol acidification on the ability of preadsorbed SV40 to penetrate and productively infect CV-1 cells was measured by the postadsorption neutralization assay. Cytosol acidification had no inhibitory effect on SV40 infectious entry. Indeed, it actually seemed to enhance infection. These effects of cytosol acidification were seen at SV40 inputs ranging from 1 to 100 pfu/cell (Figure 4).

To establish a positive control for the above experiment, we examined the effect of cytosol acidification on the internalization of transferrin. We measured the amount of transferrin internalized during 5 min after preincubation of cells under acidifying conditions for 0.5, 1, and 4 h. The longer preincubation times were used so that conditions might be comparable to those used in measuring the effects of cytosol acidification on SV40 penetration. Long exposures to acidifying conditions were necessary in the case of SV40 because of its slow rate of entry. As expected, cytosol acidification resulted in a significant decrease in transferrin internalization (Figure 5). Furthermore, the amount of transferrin internalized was similarly reduced after each time of exposure to acidifying conditions. Also, the longer exposures to acidifying conditions did not affect cell viability as judged by trypan blue dye exclusion. Greater than 90% of the cells remained viable in each instance.

SV40 Infectious Entry Is Inhibited by Agents that Selectively Disrupt Caveolae

Because SV40 infectious entry apparently occurs by a clathrin-independent process and because SV40 was seen in surface membrane invaginations that might now be called caveolae (Maul *et al.*, 1978), we were interested in the possibility that caveolae might mediate SV40 penetration leading to infection. To test this possibility, we performed our infectious entry assay under conditions that selectively disrupt caveolae.

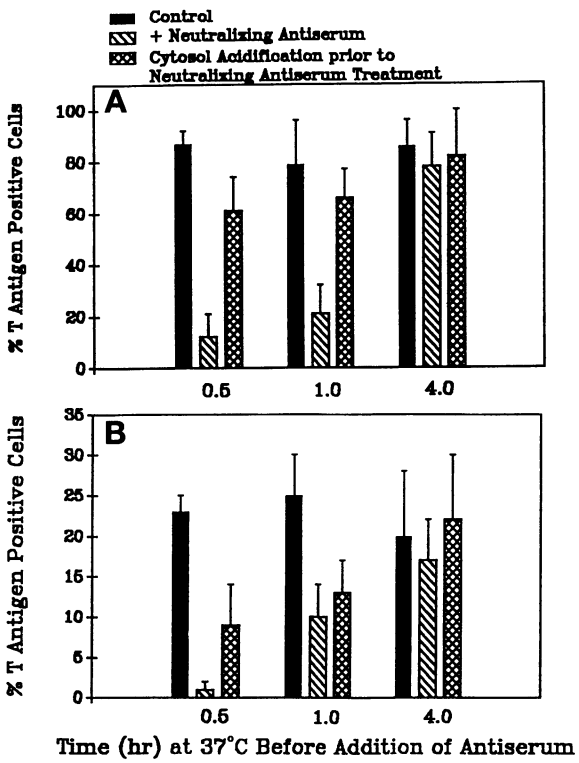

Figure 4. Cytosol acidification does not inhibit SV40 infectious entry. SV40 (100 pfu/cell; A) or (1 pfu/cell; B) was adsorbed to CV-1 cells for 1 h at 4°C. Cells were then incubated for various lengths of time at 37°C in either control buffer or acidification buffer. At the indicated times cultures were exposed to neutralizing anti-SV40 antisera or mock-treated, as indicated. All cultures were then incubated at 37°C and stained for SV40 T antigen at 48 h.

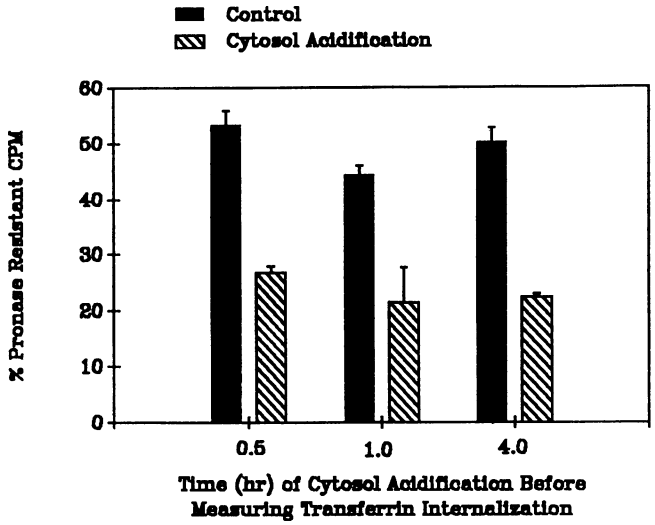

Figure 5. Cytosol acidification inhibits transferrin internalization. CV-1 cells were incubated for the indicated periods of time in either control buffer or acidification buffer. ^{125}I-labeled transferrin was adsorbed to cells at 4°C. The amount of transferrin internalized in 4 min after transfer to 37°C was measured in each instance. Error bars are the SD of triplicate samples.

Activators of protein kinase C, such as phorbol esters, disrupt caveolae and block their invaginations (Smart *et al.*, 1994). Exposure of CV-1 cells to PMA (1 and 10 μM) before, during, and for 4 h after SV40 preadsorption, followed by treatment with anti-SV40 neutralizing antiserum, resulted in a 70% reduction in the number of cells expressing T antigen, in comparison with cultures not treated with PMA (Figure 6). The effect of PMA is on a process that is necessary for penetration, because the effect is reversible if PMA is removed at 4 h and if cells are not treated with anti-SV40 antiserum at that time.

Caveolae are highly enriched for cholesterol (Lisanti *et al.*, 1993). Nystatin is a cholesterol-binding drug that can remove cholesterol from membranes and thereby selectively disrupt caveolae while not affecting clathrin-coated pits, actin cables, or other submembranous structures (Rothberg *et al.*, 1992; Lisanti *et al.*, 1993). Cells were exposed to nystatin before, during, and for 4 h after SV40 adsorption, followed by treatment with anti-SV40 antiserum. Nystatin at concentrations of 25 or 10 μg/ml, but not 5 μg/ml, inhibited SV40 infectious entry (Figure 7). As in the case of the PMA treatment above, the effect of the nystatin was reversible if cells were not treated with anti-SV40 antiserum at 4 h. This shows that the nystatin is blocking a process that is necessary for the virus to enter the cell.

Our finding that the effect of PMA and nystatin on infection was reversible, provided that bound virus at the cell surface was not neutralized with antisera before drug removal, suggests that caveolae are necessary for SV40 penetration, but not binding. This was confirmed by direct measurement of virus binding to

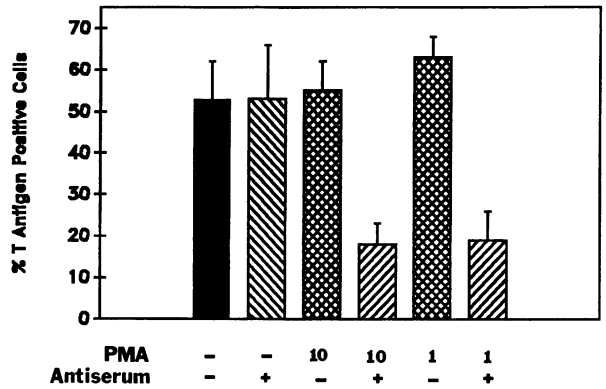

Figure 6. PMA inhibits SV40 infectious entry. CV-1 cells were treated with PMA (10 or 1 μM) or mock-treated, as indicated. Cells were infected with SV40 (10 pfu/cell) for 1 h on ice and then incubated for 4 h at 37°C. PMA was continuously present or not present, as indicated. Cultures were then treated with anti-SV40 antiserum, as indicated. Finally, all cultures were incubated in the absence of PMA and stained for SV40 T antigen at 48 h.

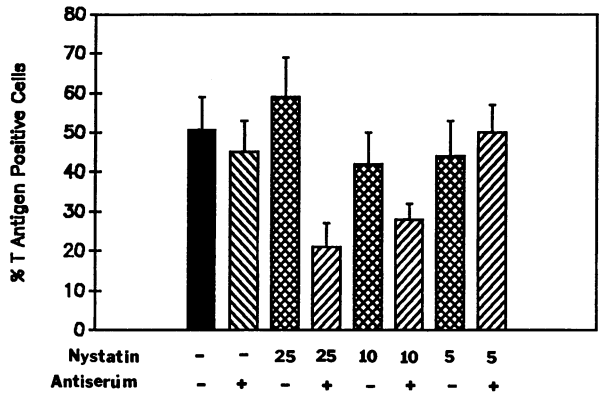

Figure 7. Nystatin inhibits SV40 infectious entry. CV-1 cells were treated with nystatin (25, 10, and 5 μg/ml) or mock-treated, as indicated. Cells were infected with SV40 (10 pfu/cell) for 1 h on ice and then incubated for 4 h at 37°C. Nystatin was continuously present or not present, as indicated. Cultures were then treated with anti-SV40 antiserum, as indicated. Finally, all cultures were incubated in the absence of nystatin and stained for SV40 T antigen at 48 h. Reprinted with permission from Norkin and Anderson, (1996).

drug-treated and control cells and by biochemical fractionation (see below).

To establish a control for possible effects of PMA and nystatin on clathrin-mediated endocytosis, we measured the effects of these drugs on the internalization of ^{125}I-labeled transferrin under the same conditions that blocked SV40 internalization. PMA and nystatin seemed to have no effect whatsoever on the internalization of transferrin (our unpublished observation). This argues that the effects of PMA and nystatin on SV40 penetration leading to infection were not on a clathrin-mediated endocytic pathway or on endocytosis in general.

Note that filipin, another cholesterol-binding drug, is also supposed to specifically disrupt caveolae (Rothberg *et al.*, 1992). We found that filipin also reversibly blocked SV40 penetration. However, under the conditions used (1 μg/ml), filipin also blocked the internalization of transferrin (our unpublished observation).

SV40 Partitions into a Caveolin-enriched Cell Fraction

To provide further evidence that caveolae might mediate SV40 entry, we asked whether preadsorbed SV40 would partition into a cellular fraction that is highly enriched for caveolae. For this purpose we isolated Triton X-100–insoluble complexes by flotation on sucrose density gradients. This is a well-established procedure for isolating a membrane fraction that is composed primarily of caveolae (Sargiacomo *et al.*, 1993; Chang *et al.*, 1994; Lisanti *et al.*, 1994a,b). Caveolin, a 22-kDa caveolar structural protein that is found at the cell surface exclusively in caveolae (Rothberg *et al.*, 1992), was used as a marker.

SV40 was preadsorbed to cells at 4°C, and samples were harvested initially and at 60 min after transfer to 37°C. Triton-insoluble complexes were isolated as a band just below the 25–15% sucrose interface. The Triton-insoluble complex, the 15% sucrose layer, the 40% sucrose layer (which contained the original lysate), and the whole-cell extract of each sample were analyzed by Western blotting for caveolin and for the major SV40 capsid protein VP_1. When equal amounts of protein (8 μg) were analyzed per sample, caveolin and VP_1 were seen only in the Triton-insoluble complexes (Figure 8A; the 40 and 15% sucrose layers are not shown). Caveolin and VP_1 could be seen in whole-cell extracts when a 15-fold concentrated sample was examined. By scanning densitometry of the Triton-insoluble complexes and the 15-fold concentrated whole-cell extracts, we determined that the Triton-insoluble complexes were enriched by ~70-fold for caveolin, in comparison with the unconcentrated whole-cell extracts. Similarly, at 0 min postadsorption there was a slight (1.5-fold) enrichment for VP_1 in the Triton-insoluble complex. However, the amount of VP_1 in the Triton-insoluble caveolin-enriched fraction increased ~14-fold between 0 min and 60 min postadsorption. This resulted in an enrichment for VP_1 in the caveolin-enriched fraction of ~20-fold at 60 min, relative to the whole-cell sample at 0 min.

Before asking whether nystatin treatment might effect the translocation of SV40 into the Triton-insoluble fraction, we first measured the effect of nystatin on SV40 binding. Treatment of cells with nystatin under conditions that blocked infection actually enhanced SV40 binding somewhat (Figure 8B). To corroborate this finding, we also measured the effect of nystatin on SV40 binding by using flow cytometry to quantify SV40 at the cell surface. This procedure also showed nystatin to cause a small increase in SV40 binding.

The effect of nystatin on the translocation of SV40 into the caveolin-enriched fraction was determined by comparing treated and control cells with respect to the ratio of the amount of VP_1 in the Triton-insoluble complex at 60 min to the amount in the corresponding

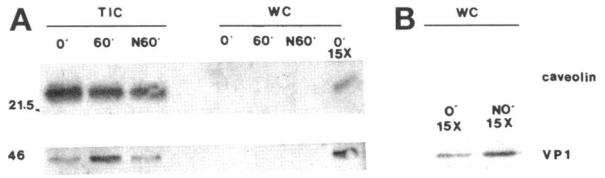

Figure 8. SV40 translocates into the Triton-insoluble caveolin-enriched fraction. Cells were infected with SV40 (100 pfu/cell) for 1 h on ice and then shifted to 37°C. (A) Western blots showing caveolin and SV40 VP_1 in Triton-insoluble complexes (TIC) and whole-cell extracts (WC) at 0 and 60 min postadsorption in samples from untreated and nystatin-treated (N) cells. (B) VP_1 in whole-cell samples from the same experiment as in A, but from a different gel and blot. Similar data were obtained in three independent experiments.

0-min whole-cell sample (15-fold concentrate). The ratios for the treated and control samples were 0.05 and 2.1, respectively. Thus, nystatin significantly prevented the partitioning of SV40 into the Triton-insoluble complex.

DISCUSSION

Viruses that enter cells by endocytosis are generally found to penetrate in clathrin-coated vesicles (Marsh and Helenius, 1989). Endocytosis through coated vesicles is constitutive. Furthermore, it is characterized by the rapid movement of bound ligands to clathrin-coated pits, followed by the quick pinching off of the coated vesicles from the plasma membrane.

Semlike Forest virus (SFV) is an example of a virus that enters cells via coated vesicles. Preadsorbed SFV has a half-life at the cell surface of ~10 min, and ~3000 SFV virions may enter a cell per minute in coated vesicles (Marsh and Helenius, 1980).

Ligands and receptors internalized in clathrin-coated vesicles are usually targeted to early endosomes from which sorting occurs (Trowbridge *et al.*, 1993). Targeting of many viruses to endosomes is important for their productive entry, because the low endosomal pH facilitates viral penetration into the cytosol (Marsh and Helenius, 1989).

SV40 entry was previously shown to differ in important ways from the process outlined above. First, whereas SV40 enters cells in both coated and uncoated vesicles, the majority of SV40 particles enter via the latter pathway (Kartenbeck *et al.*, 1989). Second, SV40 particles that enter in uncoated vesicles may be targeted to the ER (Kartenbeck *et al.*, 1989; Norkin and Anderson, 1996). This is particularly interesting because the ER is usually not considered to be a target for endocytic traffic (Kartenbeck *et al.*, 1989).

The minority of SV40 particles that enter cells in coated vesicles seems to accumulate in the endosomal/lysosomal compartment (Kartenbeck *et al.*, 1989). Although acidified vesicles have an active role in the entry pathway of a number of viruses, the activity of acidified vesicles does not seem necessary for infection by SV40, because lysosomotropic drugs do not impair SV40 infection (Norkin and Einck, 1978).

In contrast to viruses that are internalized rapidly in coated vesicles, we found that SV40 is internalized remarkably slowly. Using a postadsorption neutralization assay that we developed to specifically follow the timing of SV40 infectious entry, we found that SV40 infectious entry occurred only after the virus had been at the cell surface for 1.5–2 h. We also followed bulk SV40 entry by flow cytometry. In agreement with the results of our assay for infectious entry, flow cytometry showed that 80% of bound virions were still at the cell surface after 2 h. In a control experiment, we found that transferrin internalized within minutes.

Our postadsorption neutralization assay for infectious entry also enabled us to identify the SV40 entry pathway that leads to infection. SV40 infectious entry was not impaired by cytosol acidification, a treatment that blocks clathrin-mediated endocytosis (Sandvig *et al.*, 1987). Instead, SV40 entry was blocked by treating cells with PMA or the cholesterol-binding drug nystatin, which selectively disrupts caveolae (Rothberg *et al.*, 1992; Smart *et al.*, 1994). In control experiments we measured the effects of these treatments on the internalization of transferrin, a ligand known to be internalized in clathrin-coated vesicles. In contrast to SV40, transferrin internalization was sensitive to cytosol acidification but was insensitive to PMA and nystatin. Thus, the effects of PMA and nystatin on SV40 entry were specific for SV40 and were not due to an effect on clathrin-coated vesicle internalization or endocytosis in general. Note that SV40 entry leading to infection was actually enhanced by cytosol acidification. The basis for this effect is not clear. One possibility is that the low pH might stabilize the clustering of relevant caveolar components (Lisanti *et al.*, 1993).

In agreement with the above pharmacologic evidence that caveolae might mediate SV40 entry, SV40 was found to translocate into a caveolin-enriched fraction obtained with a standard Triton X-100 insolubility protocol (Sargiacomo *et al.*, 1993; Chang *et al.*, 1994; Lisanti *et al.*, 1994b). The following findings establish that this procedure yields a cellular fraction that is highly enriched for caveolae. First, immunogold labeling of caveolin in cells shows that it is localized at the cell surface exclusively in caveolae and also to a small extent near the trans-Golgi in tubulo-vesicular structures (Rothberg *et al.*, 1992; Dupree *et al.*, 1993). Second, the membrane structures in the low-density Triton-insoluble fraction resemble caveolae ultrastructurally, and nearly all could be decorated with anti-caveolin IgG (Lisanti *et al.*, 1994b). Also, when cells were slightly fixed in paraformaldehyde and extracted with Triton X-100 and then examined by electron microscopy, the insoluble membranes that remained were found to be caveolae (Moldovan *et al.*, 1995). A variety of biochemical techniques also show that the caveolin-enriched Triton-insoluble complexes are a highly purified caveolar fraction. In particular, many proteins associated with the plasma membrane and organelle-specific enzymes are excluded from the Triton-insoluble fraction (Sargiacomo *et al.*, 1993; Lisanti *et al.*, 1994b).

The low-density Triton-insoluble fractions that we obtained were enriched for caveolin by ~70-fold, as compared with the whole-cell homogenates. The amount of SV40 VP_1 in the caveolin-enriched complexes increased by ~14-fold during the first 60 min postadsorption. This implies that most SV40 initially bound to noncaveolar membrane domains, followed by translocation of the virus into caveolae. In agree-

ment with the above, nystatin treatment did not impair SV40 binding but did not block the partitioning of virus into the caveolin-enriched complexes. Also the effect of nystatin pretreatment on infection was reversible, provided that bound virus was not neutralized by antiserum before removal of the drug. Together, these results imply that caveolar integrity is necessary for SV40 infectious entry, but not binding.

Earlier, we found that MHC class I molecules are a necessary component of the SV40 receptor, being required for binding that leads to infection (Atwood and Norkin, 1989; Breau *et al.*, 1992). More recently, we found that MHC class I molecules do not internalize with SV40 but, instead, are shed into the extracellular fluid (Anderson and Norkin, unpublished results). Note that the Western blots in the present study were reprobed with antibodies against class I molecules. However, we were able to detect class I molecules only in the 15-fold concentrates of the whole-cell extracts (our unpublished results). More sensitive experiments that might show whether bound SV40 translocates to caveolae in association with class I molecules are in progress. Regardless, our results imply that SV40 uses MHC class I molecules to mediate initial binding and caveolae to mediate entry. Other viruses have also been found to use distinct cell surface factors to mediate the series of steps between initial binding and entry. These include nonenveloped viruses, such as adenoviruses (Wickham *et al.*, 1993), and enveloped viruses, such as herpesviruses (McClain and Fuller, 1994) and HIV (Golding *et al.*, 1995; reviewed, Norkin, 1995). Nevertheless, SV40 entry is different in important ways from entry of these other viruses. For example, adenoviruses enter cells rapidly, becoming resistant to neutralizing antisera within 15 min of binding to cells (Varga *et al.*, 1991). Furthermore, adenovirus penetration is sensitive to cytosol acidification (Varga *et al.*, 1991).

Our pharmacologic and biochemical results are the first to imply that caveolae might mediate virus entry. However, our findings might have been expected, because both SV40 and the related mouse polyomavirus were previously seen in small uncoated membrane indentations that were believed to pinch from the plasma membrane to form monopinocytotic vesicles (Maul *et al.*, 1978; Mackay and Consigli, 1976; Kartenbeck *et al.*, 1989). These vesicles can now be seen to closely resemble caveolae (compare Figure 1 of Maul *et al.* [1978] with Figure 1 of Rothberg *et al.* [1992]).

Caveolae were first identified as a possible endocytic compartment in endothelial cells, where they were thought to act in clathrin-independent endocytosis and in transcytosis of small and large molecules across the endothelia (Montesano *et al.*, 1982; Simionescu *et al.*, 1982; Milici *et al.*, 1987; Tran *et al.*, 1987). However, it is not yet generally accepted that caveolae can pinch off from the plasma membrane to form endocytic vesicles (Bundgaard *et al.*, 1983; Severs, 1988; Sandvig and Van Deurs, 1994). Caveolae have also been associated with a process called potocytosis, in which caveolae-bound ligands enter cells through carriers or channels in the membrane (Anderson, 1993b).

The controversy regarding the role of caveolae in endocytosis might be resolved if suitable marker ligands were identified. The present results show that SV40 might be an excellent marker to follow caveolae-mediated endocytosis under physiological conditions.

We recently reported that SV40 binding activates intracellular signal pathways that up-regulate early response genes and promote SV40 infectious entry (Dangoria *et al.*, 1996). The finding that SV40 transmits a signal from the cell surface is interesting with regard to the hypothesis that caveolae have a transmembrane signaling function (Lisanti *et al.*, 1994a). Note that a number of signal-transducing molecules (e.g., Src-family kinases, GTP-binding proteins, protein kinase C, and MAP kinase) and receptors that signal through these molecules have all been found enriched within caveolar complexes (Dupree *et al.*, 1993; Sargiacomo *et al.*, 1993; Chang *et al.*, 1994; Mastick *et al.*, 1995; Smart *et al.*, 1995; Mineo *et al.*, 1996). This suggests that multiple signaling pathways originate from this membrane domain (Mineo *et al.*, 1996). Our finding that SV40 entry is dependent on a transmembrane signal may be interesting with respect to the report that the internalization of caveolae (as induced by the phosphatase inhibitor okadaic acid) might be regulated by phosphorylation (Parton *et al.*, 1994). The precise role of the SV40-induced signal in entry is presently under investigation.

Because viruses that enter through clathrin-coated pits enter quickly and are targeted to the endosomal/lysosomal compartment, the slow rate of SV40 entry and the targeting of SV40 to the ER are likely related to the SV40 entry pathway through caveolae. It is not known how SV40 entry through caveolae might be related to SV40 targeting to the ER. As noted by Kartenbeck and colleagues (1989), the ER is an acceptor organelle for certain lipids, such as cholesterol, when they are applied extracellularly (Slotte and Bierman, 1987). Because caveolae are enriched for cholesterol, one interesting possibility is that SV40 penetration might exploit a caveolae-dependent pathway for lipid recycling.

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