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Major Histocompatibility Complex Class I Molecules Mediate Association of SV40 with Caveolae

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Simian virus 40 (SV40) has been shown to enter mammalian cells via uncoated plasma membrane invaginations. Viral particles subsequently appear within the endoplasmic reticulum. In the present study, we have examined the surface binding and internalization of SV40 by immunoelectron microscopy. We show that SV40 associates with surface pits which have the characteristics of caveolae and are labeled with antibodies to the caveolar marker protein, caveolin-1. SV40 is believed to use major histocompatibility complex (MHC) class I molecules as cell surface receptors. Using a number of MHC class I-specific monoclonal antibodies, we found that both viral infection and association of virus with caveolae were strongly reduced by preincubation with anti-MHC class I antibodies. Because binding of SV40 to MHC class I molecules may induce clustering, we investigated whether antibody cross-linked class I molecules also redistributed to caveolae. Clusters of MHC class I molecules were indeed shown to be specifically associated with caveolin-labeled surface pits. Taken together, the results suggest that SV40 may make use of MHC class I molecule clustering and the caveolae pathway to enter mammalian cells.

INTRODUCTION

Caveolae are small invaginations of the plasma membrane which are abundant in a number of specialized mammalian cell types (Severs, 1988; Anderson, 1993; Lisanti *et al.*, 1994). An important component of caveolae are the caveolins, integral membrane proteins present at high density within the caveolar membrane (for review, see Parton, 1996). Caveolin-1 was originally identified as a tyrosine substrate which is phosphorylated upon Rous sarcoma virus infection and cellular transformation and is the best studied caveolin family member (Glenney and Zokas, 1989; Glenney, 1992; Kurzchalia *et al.*, 1994). Recent investigations have shown the existence of additional caveolin family members which differ in tissue distribution and functional properties (Way and Parton, 1995; Scherer *et al.*, 1996; Tang *et al.*, 1996). For example, the caveolins differ in their specific interactions with trimeric G protein subunits in vitro (Li *et al.*, 1995; Scherer *et al.*, 1996). Caveolins are also thought to play a vital structural role in caveolae formation. Expression of caveolin-1 in lymphocytes, which have no morphologically identifiable caveolae, caused formation of typical caveolae at the plasma membrane (Fra *et al.*, 1995). Recent work has shown that caveolin is a cholesterol-binding protein (Murata *et al.*, 1995), and it has been suggested that the association of caveolin with cholesterol in glycosphingolipid-rich domains is important for caveolae formation and function (Parton and Simons, 1995).

Caveolae were first implicated in transcytosis in endothelial cells (Ghitescu *et al.*, 1986), but recently several other functions, such as signal transduction (Lisanti *et al.*, 1994) and calcium regulation (Fujimoto *et al.*, 1992; Fujimoto, 1993), have been proposed to occur in caveolae. Caveolae have also been suggested to perform potocytosis in which the narrow neck of the caveolae can close or open the connection to the extracellular media without the formation of intracellular vesicles (Anderson, 1993). The role of caveolae in conventional endocytosis is still debated (see van

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Deurs *et al.*, 1993) but at least under some conditions it appears that caveolae can be internalized (Tran *et al.*, 1987; Parton *et al.*, 1994).

Simian virus 40 (SV40), a non-enveloped virus belonging to the papovavirus family, has been shown to bind to the plasma membrane in small tight-fitting uncoated membrane invaginations and apparently endocytosed within small uncoated vesicles (Kartenbeck *et al.*, 1989). The virus is enclosed so tightly by the surface membrane that even fluid phase markers are excluded from the virus-containing vesicles. Following internalization, SV40 is then transferred to the endoplasmic reticulum (ER). Class I major histocompatibility proteins (MHC-I) have been proposed to be the cell surface receptors for SV40 (Atwood and Norkin, 1989; Breau *et al.*, 1992). MHC-I molecules, which present endogenous antigens to cytotoxic T-cells, are present at the plasma membrane of most nucleated mammalian cells. The MHC-1 complex, consisting of a heavy chain associated with β_2-microglobulin, binds endogenous antigenic peptides in the ER before being transported to the plasma membrane. Interestingly, earlier studies showed that cross-linking of MHC-I using antibodies to β_2-microglobulin induced internalization via small uncoated plasma membrane invaginations (Huet *et al.*, 1980).

The ultrastructural similarities between the SV40-containing invaginations (Kartenbeck *et al.*, 1989), the uncoated invaginations containing the possible SV40 receptor MHC-I (Huet *et al.*, 1980), and caveolae (Parton, 1996) prompted us to re-examine the SV40 entry pathway and, in particular, to investigate whether SV40 localizes to caveolae. We now show that SV40, as well as cross-linked MHC-I molecules, localizes to caveolin-1-positive uncoated invaginations of the plasma membrane, suggesting that SV40 uses the caveolar pathway to enter mammalian cells.

MATERIALS AND METHODS

Cells and Virus

CV1 cells were grown in DMEM containing 10% FCS. Preparation of viral stocks and infection were performed as described previously (Kartenbeck *et al.*, 1989). Briefly, stocks of SV40 were made by superinfection of CV1 cells. Cell supernatants were collected when cells were forming large intracellular vacuoles and starting to lyse 3–5 days after adding the virus. The cell supernatant was freeze-thawed several times before sonicating the supernatant. Before use, the supernatant was centrifuged to remove cellular debris. Stocks of virus were stored at −70°C.

For studies of viral entry, CV1 cells were incubated with SV40 in MEM/10 mM HEPES supplemented with 10% FCS or Hanks' salt solution containing 10 mM HEPES and 4 mM $NaHCO_3$ for 1 h on ice. After extensive washing in ice-cold PBS or medium, cells were either fixed immediately or incubated for varying times in normal growth medium at 37°C.

Antibodies

The mouse monoclonal antibodies MB40.5 and W6/32 were a generous gift from Guttorm Haraldsen (LIIPAT, Rikshospitalet, Oslo, Norway). MB40.5 recognizes a monomorphic determinant on the HLA heavy chain and W6/32 monomorphic determinants on the HLA-A, B, and C molecules (Atwood and Norkin, 1989). The rabbit anti-β_2-microglobulin antibody was a kind gift from Per Peterson (Scripps Clinic, La Jolla, CA).

Rabbit antiserum to SV40 was purchased from Lee Biomolecular Research (San Diego, CA) and mouse anti-SV40 T-antigen antibody (PAb 108) from PharMingen (San Diego, CA). Rabbit antibodies to the N-terminus of caveolin-1 (VIP21), mouse monoclonal antibodies to alkaline phosphatase, and secondary antibodies were from the sources described previously (Parton *et al.*, 1994).

Immunofluorescence Microscopy

For quantitation of the expression of T-antigen, CV1 cells grown on coverslips were incubated with SV40 as described above and incubated for 24 h in SV40-free medium. Cells were fixed with methanol and labeled for T-antigen followed by goat anti-mouse CY3 or FITC (Dianova, Hamburg, Germany).

To study the involvement of MHC-I as receptors for SV40 binding, CV1 cells grown on coverslips were preincubated with each of the various anti-MHC-I antibodies or combinations of these for 1 h on ice before incubation with SV40 for 1 h on ice in Hanks' salt solution containing the same anti-MHC-I antibodies as during preincubation. After washing in virus-free Hanks', the cells were incubated for 24 h in growth medium containing anti-MHC-I antibodies. After fixation, cells were labeled for T-antigens as described above. A minimum of 100 cells were examined for T-antigen expression. Control cells were incubated with SV40 only and similarly quantified for expression of T-antigen.

Electron Microscopy

For Epon embedding, cells grown in 3-cm culture dishes were processed as described previously (Parton *et al.*, 1994) using a primary fixative of 2.5% glutaraldehyde in 50 mM cacodylate, followed by postfixation in 2% OsO_4 and en block staining with 2% uranyl acetate. Ultrathin sections were cut perpendicular to the cell substratum, and the sections were stained using uranyl acetate and lead citrate.

For immunocytochemical labeling, cells grown in 6-cm culture dishes were detached using proteinase K (Sigma Chemical, St. Louis, MO) and fixed in Soerensen phosphate buffer containing 0.1% glutaraldehyde and 4% paraformaldehyde. Alternatively, cells were first fixed and then scraped from the culture dish without the use of the proteinase K. Identical results were obtained with the two methods. Following fixation, cell pellets were infused with polyvinylpyrrolidone (PVP)-sucrose, mounted, frozen, and stored in liquid nitrogen. Ultrathin cryosections were labeled as described by Griffiths (1993) using protein A-gold (purchased from G. Posthuma and J. Slot, Utrecht, Holland).

Quantitation of the number and localization of SV40 in cells were performed by counting the total number of viruses as well as the number of viruses localizing in invaginations (defined as pits less than 100 nm in diameter) per cell as seen in Epon sections cut perpendicular to the cell substratum.

Quantitation of the association of SV40 with caveolin-1-positive membranes was performed by counting the number of gold particles associated with SV40 surrounding membranes under different conditions.

Measurements of SV40 and Caveolae Size

Negatively stained SV40 were prepared by applying 5 μl of the sonicated virus suspension to formvar-coated grids for 1 min. Excess virus suspension was removed using filter paper, and after washing with distilled water 5 μl of 1% uranyl acetate were added for 1 min.

Measurements of the size of the caveolae were performed by negative staining of plasma membrane sheets. Formvar-coated grids were incubated with polylysine (1 mg/ml in water; Sigma Chemical) for 30 min at room temperature and then washed with water and dried. The coated grids were placed on the surface of coverslip-grown CV1 cells and then detached with part of the plasma membrane. The grids were fixed with 1% glutaraldehyde and labeled with antibodies to caveolin-1 and protein A-gold. After labeling, the grids were postfixed using 0.1% glutaraldehyde in 0.1 M cacodylate buffer followed by 1% OsO_4 and finally 1% tannic acid before staining with 0.25% uranyl acetate in methylcellulose.

Cell Surface Labeling with MHC-I Antibodies

CV1 or A431 cells were incubated with a mixture of the two monoclonal anti-MHC-I antibodies (MB40.5 and W6/32) at a concentration of 1 μg/ml in MEM/10 mM HEPES for 1 h on ice. After extensive washing, the cells were incubated sequentially with rabbit anti-mouse antibodies and 5 nm protein A-gold for 1 h on ice. The cells were then washed and incubated for 10 min at 37°C in prewarmed CV1 medium before preparation for either Epon embedding or frozen sectioning.

Incubation with MHC-I Antibodies

CV1 cells were preincubated with a mixture of the two mouse monoclonal anti-MHC-I antibodies (MB40.5 and W6/32) at two different concentrations (2 and 10 μg/ml) in MEM/10 mM HEPES containing 0.1% BSA for 1 h on ice. Cells were then further incubated with the same antibodies in the presence of the SV40 virus for 1 h on ice. After washing, the cells were incubated for 1 h at 37°C in MEM containing 0.1% BSA and MHC-I antibodies before fixation and embedding in Epon as described above. Control incubations with mouse monoclonal antibodies to alkaline phosphatase (Parton *et al.*, 1994) were performed in parallel under identical conditions with an antibody concentration of approximately 10 μg/ml.

RESULTS

SV40 Localizes in Caveolae-like, Caveolin-1-positive Invaginations

To study the mechanism by which cells internalize SV40, we incubated CV1 cells with virus at low temperature and then analyzed the viral entry pathway by electron microscopy after warming the cells to 37°C. Examination of ultrathin plastic sections of CV1 cells exposed to SV40 on ice for 1 h showed that the majority of virus particles localized to flattened areas of the plasma membrane as well as occasional invaginations with no visible cytoplasmic coat (our unpublished results). Incubation of the cells at 37°C caused an increasing fraction of the virus particles to localize to these invaginations (Figure 1). Virus particles were rarely observed in clathrin-coated pits or -vesicles at any time point (e.g., Figure 1). To quantitate the distribution of SV40 at the plasma membrane, CV1 cells were exposed to virus for 1 h on ice, washed, and then incubated at 37°C for varying times. On plastic sections cut perpendicular to the cell substratum, the number of cells with SV40 at the plasma membrane as well as the number and localization of the virus on each cell profile were quantitated. The localization of SV40 changed as a function of time at 37°C (Table 1). With increasing times at 37°C, less virus localized to the flat part of the membrane whereas the number of SV40 particles in invaginations increased.

The uncoated invaginations showed clear similarities to caveolae. Interestingly, however, the diameter of the invaginations consistently appeared slightly smaller than the normal caveolae found at the plasma membrane of the CV1 cells (Figure 1). This was most clearly illustrated by observations of SV40 in pits directly connected to normal sized caveolae (see Figure 1). Such a localization of SV40 was relatively common in CV1 cells at this time point. Measurements on negatively stained preparations of SV40 (Figure 2) showed that the virus has an apparent external diameter of approximately 46 nm as compared with an apparent inner diameter of 56 nm for caveolae stained using similar techniques (see MATERIALS AND METHODS).

To investigate the possibility that the SV40-containing invaginations correspond to caveolae, immunocytochemical labeling with anti-caveolin-1 antibodies (Dupree *et al.*, 1993) was performed on thawed cryosections of SV40-infected cells. Although the flattened plasma membrane with attached SV40 only rarely showed labeling for caveolin-1, SV40 particles within invaginations were often surrounded by a caveolin-1-positive membrane (Figure 3). Generally, the SV40-containing invaginations often localized to areas of the cell rich in caveolae and caveolin labeling. Quantitation of the amount of caveolin-1 labeling associated with invaginations or vesicular profiles containing SV40 showed that the number of SV40 particles surrounded by a caveolin-1-positive membrane increased as a function of time at 37°C as did the mean number of gold particles per virus (Table 2).

Antibodies to MHC-I Reduce the Number of Cells Expressing T-Antigens by Inhibiting Binding of SV40

To study the relationship between MHC-I and the binding of SV40, we preincubated cells with different well-characterized monoclonal antibodies to the MHC-I heavy chain (Atwood and Norkin, 1989) and polyclonal antibodies to β_2-microglobulin before exposing the cells to virus. The productive infection of cells with SV40 can be readily assayed by counting the proportion of T-antigen-expressing cells after a certain infection time. Control cells incubated with virus at 4°C and then incubated at 37°C showed nuclear T-antigen expression in approximately 80% of the cells after 24 h. Incubation with the MB40.5 antibody had a striking effect on the number of T-antigen-positive cells; after 24 h, the fraction of T-antigen-expressing cells was reduced by 66% (Figure 4 and Table 3). The other antibodies (see MATERIALS AND METHODS) showed smaller effects on T-antigen expression. The fact that these antibodies also specifically labeled sur-

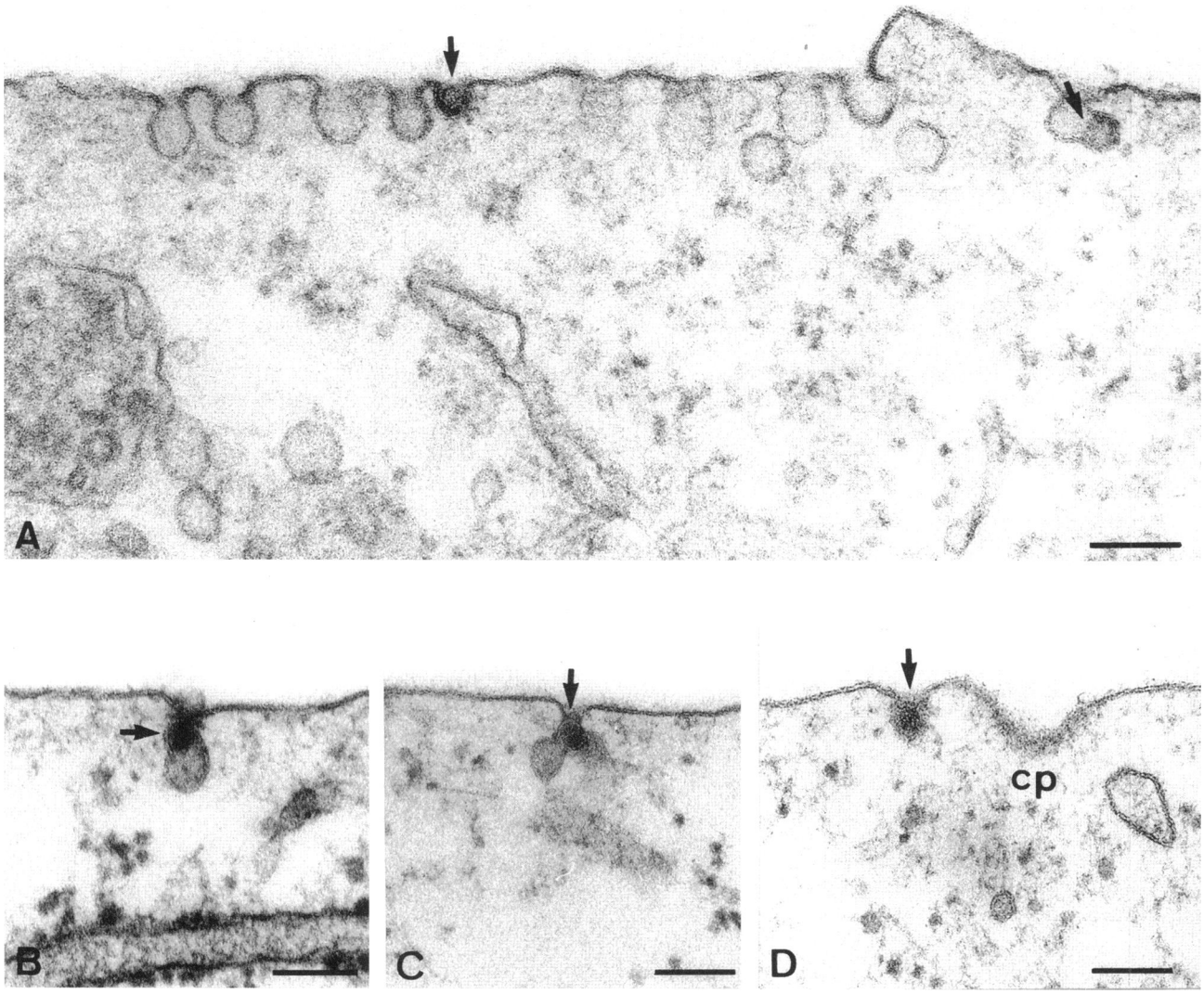

Figure 1. SV40 particles localize to small uncoated invaginations of the plasma membrane. CV1 cells were exposed to SV40 for 1 h on ice and then incubated at 37°C for 1 h. The SV40 particles (arrows) recognizable by their characteristic electron density and spherical shape localize in small tight-fitting uncoated invaginations of the plasma membrane. A shows two SV40 particles localized in an area of the plasma membrane rich in typical caveolae-like uncoated invaginations; note the slightly smaller size of the SV40-containing invaginations compared with the other caveolae-like structures. Note also how the invagination containing the SV40 particle on the right side of this figure is connected to a normal sized caveolae. Similar results are shown in B and C where virus particles can be seen in small outpocketings of the narrow caveolae neck. Essentially, no SV40 was found in coated pits or vesicles. An example is shown in D where an SV40-containing uncoated invagination and a virus-free clathrin-coated pit (cp) are shown to demonstrate the difference in size of the two invaginations. Bars, 100 nm.

face class I molecules under these conditions (our unpublished observations), although reactive with different domains of the MHC-I complex, shows the specificity of the effect. The greatest inhibition was achieved by preincubating the cells with a combination of the MB40.5 and W6/32 antibodies.

The inhibitory effect of anti-MHC-I antibodies on infection is consistent with the proposed role for MHC-I in virus binding (Atwood and Norkin, 1989; Breau *et al.*, 1992). To examine this directly, we investigated the effect of these antibodies on binding of SV40 by electron microscopy. We quantitated both the number of SV40-labeled cell profiles and the distribution of virus found at the plasma membrane with respect to localization in caveolae. As shown in Table 4, anti-MHC-I antibodies MB40.5 and W6/32 caused a considerable reduction in cell surface SV40. Of importance, this electron microscopic analysis showed that there was a considerable reduction in the number of virus particles associated with caveolae. As a control

Table 1. Incubation at 37°C causes increasing association of SV40 with surface invaginations

	Incubation time at 37°C		
	0	15 min	60 min
% SV40-labeled cell profiles	70	72	72
Surface virus particles/cell profile	3.1	3.5	2.9
% virus particles in surface pits	9	28	26

CV1 cells were incubated with SV40 for 1 h on ice and then incubated at 37°C for varying times. The number of cell profiles labeled with SV40 and the localization of the individual virus particles with respect to uncoated surface pits were quantitated on plastic sections cut parallel to the culture substratum.

we preincubated cells with monoclonal antibodies to alkaline phosphatase, which is also expressed on the cell surface. Although a small decrease in surface-associated SV40 was observed, there was no significant effect on the association with caveolae.

Antibody-clustered MHC-I Molecules Associate with Caveolae

The above results suggest that SV40 associates with caveolae and that this may be mediated by binding to MHC-I. Antibody-induced cross-linking of MHC-I with a β_2-microglobulin antibody has been shown to cause the MHC-I molecules to redistribute from the flattened part of the plasma membrane into uncoated invaginations (Huet *et al.*, 1980). We therefore examined whether antibody-clustered MHC-I molecules were concentrated within caveolae. Using the two anti-MHC-I antibodies (MB40.5 and W6/32) followed by rabbit anti-mouse antibodies and protein A-gold to cross-link surface-localized MHC-I, we found that the gold localized in large clusters at flat parts of the plasma membrane and in uncoated caveolae-like invaginations. Labeling cryosections of similarly treated cells with the caveolin-1 antibody confirmed that the uncoated invaginations containing cross-linked MHC-I are caveolin-1-positive caveolae (Figure 5). This appears to be a general feature of MHC-I molecules in fibroblasts since similar results were obtained with CV1 cells and with A431 cells.

DISCUSSION

The role of caveolae in different cell types is still a matter of considerable debate. Although caveolae were originally implicated in transcytosis in endothelial cells (Ghitescu *et al.*, 1986; Schnitzer *et al.*, 1994), several other functions, such as signal transduction (Lisanti *et al.*, 1994) and calcium regulation (Fujimoto *et al.*, 1992; Fujimoto, 1993), which do not necessarily depend on the formation of intracellular vesicles, have been proposed to localize to caveolae. Whether caveolae really internalize or remain connected to the plasma membrane as invaginations is still controversial (see van Deurs *et al.*, 1993 for a recent discussion). The results presented here raise the intriguing possibility that a virus, SV40, may use caveolae to enter cells. First, we have shown that SV40 is associated with caveolin-1-positive invaginations of the cell surface. Second, we have shown that the putative surface receptors for SV40, MHC-I molecules are concentrated within caveolae after clustering with antibodies. Finally, our results suggest that the association of SV40 with caveolae is relevant to the infectious entry pathway of SV40 because antibodies to MHC-I inhibit both the association of SV40 with caveolae and SV40 infection.

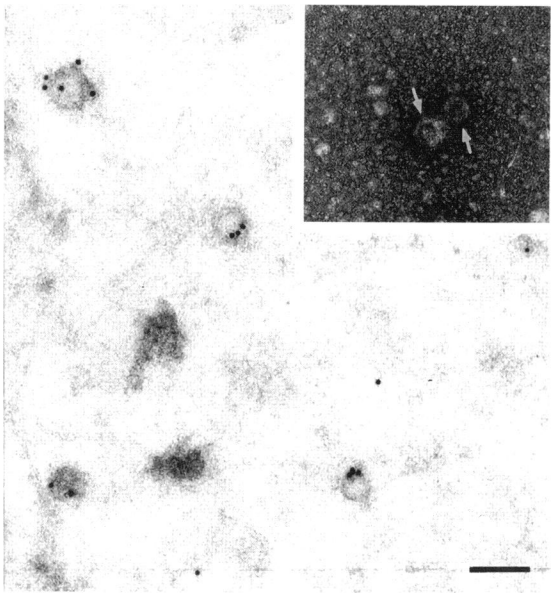

Figure 2. SV40 particles have a slightly smaller diameter than the average CV1 caveolae. To compare the size of caveolae in CV1 cells with the size of the SV40 particles, negatively stained caveolae and SV40 viruses were compared (see MATERIALS AND METHODS). This figure shows several caveolin-1-labeled caveolae (10 nm protein A-gold) localized at the cytoplasmic side of the plasma membrane in comparison to negatively stained SV40 particles (inset, arrows). Bars, 100 nm.

The initial event in SV40 entry is thought to involve binding of virus to MHC-I molecules on the cell surface (Atwood and Norkin, 1989; Breau *et al.*, 1992). In these studies, it was shown that preadsorption of SV40 to cells specifically inhibits binding of antibodies against MHC-I and that antibodies to MHC-I inhibited infection by SV40. Our assays for both viral association with caveolae and for SV40 infection are entirely consistent with these studies. In the present study, we showed that SV40 initially binds to flattened areas of the plasma membrane after incubation at 4°C. This presumably represents binding of virus to MHC-I

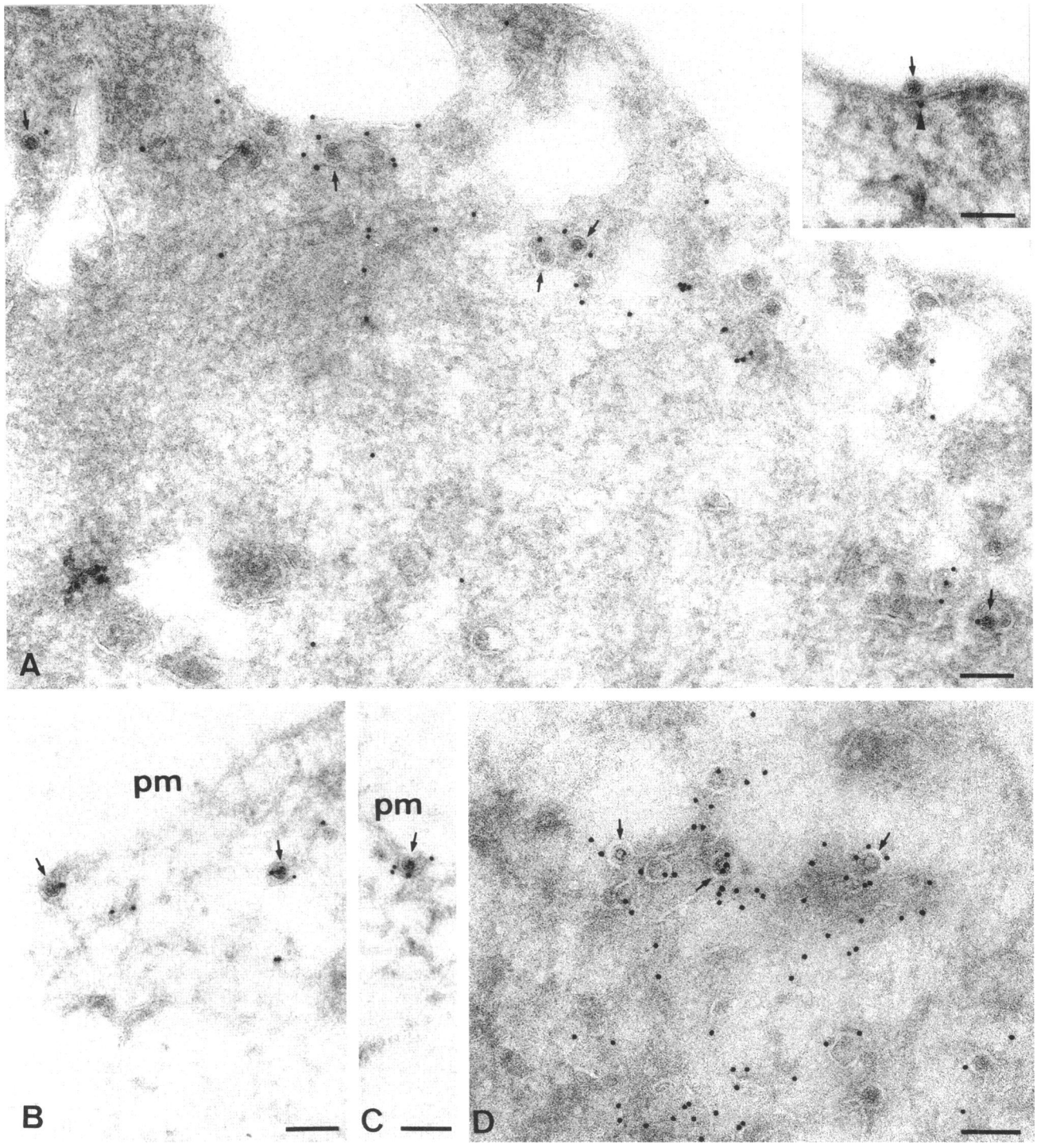

Figure 3. SV40 associates with caveolin-1-positive invaginations. To characterize the SV40 containing invaginations and vesicles, thawed cryosections of CV1 cells exposed to virus for 1 h on ice followed by various chase periods at 37°C were immunolabeled for caveolin-1. The membrane of the SV40-containing invaginations as well as possible intracellular SV40-containing vesicles contain caveolin-1 (SV40 surrounded by a caveolin-positive membrane is indicated by arrows). A–C are from specimens prepared after a 1-h incubation at 37°C. The inset in A, however, shows a surface SV40 particle found on the flat plasma membrane after binding on ice; note that even though the membrane is not invaginated, it is labeled for caveolin-1 (arrowhead). D is from a CV1 cell incubated for 2 h after incubation with SV40 and shows possible internalized virus in a caveolin-rich area of the cell. pm, plasma membrane. Bars, 100 nm.

Table 2. Incubation at 37°C increases the colocalization of SV40 and caveolin

	Incubation time at 37°C		
	0	15 min	60 min
% SV40 particles associated with caveolin-labeled membranes	13	20	40
Mean gold particles/SV40 particle	0.16	0.27	0.60

CV1 cells were incubated with SV40 for 1 h on ice and then incubated for varying times at 37°C. After immunocytochemical labeling of thawed cryosections, the number of SV40 particles associated with a caveolin-1-positive membrane and the mean number of gold particles per virus particle were quantitated.

molecules distributed more or less randomly over the cell surface. Subsequently, after warming the cells to 37°C, the virus becomes associated with flask-shaped uncoated invaginations with morphological similarities to caveolae. Immunoelectron microscopy confirmed that these structures are caveolin-1-positive invaginations. Combined with our results showing that MHC-I molecules clustered with antibodies localized to caveolae, these findings are consistent with a model in which the virus first binds to MHC-I molecule(s), which may be anywhere over the cell surface. As the surface-bound virus encounters and binds other MHC-I molecules, they would be clustered around the virus particle. The virus and bound receptors would then move into caveolae. Consistent with this model, antibody-induced clustering has been shown to redistribute a number of surface proteins to caveolae including glycosyl phosphatidylinositol-anchored proteins (Mayor *et al.*, 1994; Parton *et al.*, 1994), the β-adrenergic receptor (Dupree *et al.*, 1993), and VIP36 (Fiedler *et al.*, 1994). However, our observations suggest that the SV40-containing invaginations are slightly smaller than the normal caveolae found in CV1 cells; this was particularly evident when SV40-containing invaginations were observed in continuity with unoccupied caveolae (e.g., see Figure 1). This size difference could result from a very tight binding between viral capsid proteins and their receptors at the plasma membrane, which after moving the virus into caveolae may induce a slight change in the caveolar form. Another trivial explanation is that the virus-containing caveolae and the "empty" caveolae are affected differently by the fixation and processing procedures used for electron microscopy because caveolae morphology is known to be affected by these conditions (Severs, 1988). However, an alternative explanation for the difference in the size of the SV40-containing caveolin-1-positive pits and unoccupied caveolae is that SV40 does not move into caveolae but that caveolin is actually recruited around the surface-bound virus particle. Clustering of the viral receptors

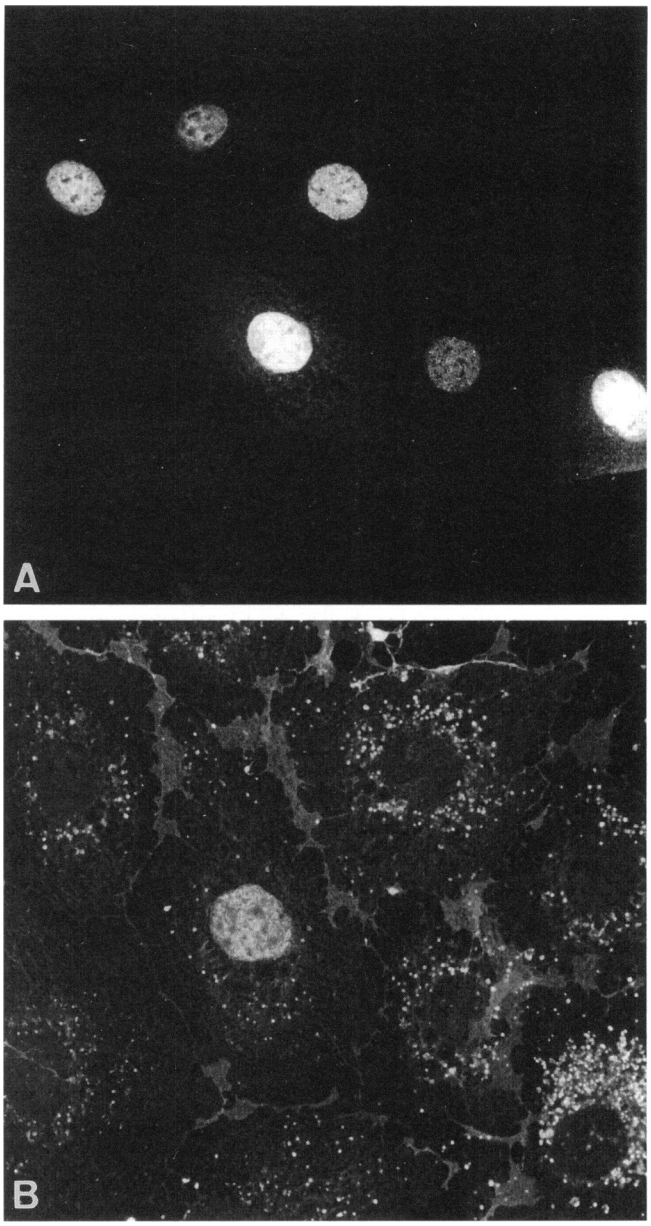

Figure 4. Antibodies to MHC-I inhibit infection by SV40. Immunofluorescent labeling with anti-T-antigen antibodies was used as an assay for infection with SV40. A shows control cells exposed to SV40 for 1 h on ice followed by a 24-h incubation in SV40-free medium. After labeling for T-antigen, the majority of the cell nuclei are positive. B shows cells which were incubated with antibodies to MHC-I before and during exposure to SV40 as well as during the 24-h chase period. The number of T-antigen-positive cells is clearly decreased. Cells were incubated with a mixture of the MB40.5 and W6/32 antibodies. Since both of these antibodies as well as the anti-SV40 T-antigen antibody are mouse monoclonal antibodies, the fluorescent marker (CY3-labeled sheep anti-mouse antibodies) shows the localization of both the T-antigens and MHC-I molecules. The single T-antigen-positive cell is, however, easy to distinguish from the surrounding cells, which all have T-antigen-negative nuclei. The labeling of small vesicular-like structures in the cells represents internalized anti-MHC-I antibodies.

Table 3. Preincubation with anti-MHC-1 antibodies inhibits SV40 infection

Antibody	% Inhibition of T-antigen expression
	0
MB40.5	66
W6/32	20
MB40.5 + W6/32	73
Anti-β_2-microglobulin	10

CV1 cells were preincubated with the indicated antibodies for 1 h on ice before incubation with SV40 for 24 h. Cells were then fixed and labeled for immunofluorescent detection of T-antigen. More than 100 cells were examined at random for nuclear T-antigen expression. The experiments were performed three times with identical results.

caused by binding of the virus may act as a nucleation center for the formation of caveolae. In this model, caveolin could associate with the SV40-containing pits after delivery to the cell surface from the trans-Golgi network (Dupree *et al.*, 1993) or could be recruited from preexisting caveolae. Although speculative, this model is supported by the consistent observation that the caveolin-1 density increased with incubation time at 37°C; at early times after the shift to 37°C, the majority of SV40-containing invaginations had only one gold particle. At later time points, however, more than five gold particles were found associated with some virus-containing invaginations. Since the labeling density for caveolin in caveolae is usually fairly constant, even after de novo formation of caveolae (Fra *et al.*, 1995), these results are more compatible with a nucleation model for caveolin association with SV40 than with movement of virus into caveolae. Rapid freeze, deep-etch microscopy has shown that the cytosolic side of the caveolae contain a striated coat, often with a spiral morphology, which is believed to consist of caveolin (Rothberg *et al.*, 1992). Caveolin has also been found to form large oligomers both in vivo and in vitro (Monier *et al.*, 1995). From the spiral morphology of the coat, it could be envisaged that one or a few caveolin molecules associated with the virus-containing membrane could act as a nucleation center for the oligomerization and formation of caveolae. Caveolin association with the membrane around the virus may be necessary for the budding of the virus-containing vesicle into the cell and for subsequent events leading to delivery to the ER.

The finding that MHC-I molecules cluster in caveolae may also be of interest in the context of MHC-I function. Once MHC-I molecules reach the cell surface, they are generally regarded as resident plasma membrane proteins which show negligible internalization (Neefjes *et al.*, 1990). In the absence of cross-linking, these proteins appear to show a near random distribution over the cell surface (Huet *et al.*, 1980; Neefjes *et al.*, 1990). However, a number of studies have documented MHC-I internalization after surface labeling with antibodies. In fibroblasts, cross-linking of MHC-I induced internalization via uncoated pits (Huet *et al.*, 1980; Machy *et al.*, 1987a); in T lymphocytes, however, a similar cross-linking procedure resulted in internalization of MHC-I via clathrin-coated pits (Machy *et al.*, 1987b). Dasgupta *et al.* (1988) also reported that in monocytes MHC-I molecules are internalized by coated pits, although the images provided also show the presence of MHC-I in uncoated invaginations. Although fibroblasts are rich in caveolae, lymphocytes do not express caveolin-1 and have no morphologically identifiable caveolae at the plasma membrane (Fra *et al.*, 1995). It is conceivable that this lack of caveolae might cause the MHC-I to use an alternative route of internalization in lymphoid cells. The ability to express caveolin in these cells and produce caveolae (Fra *et al.*, 1995) should prove interesting for future studies of both SV40 and MHC-I inter-

Table 4. Anti-MHC-1 antibodies prevent binding of SV40

	Antibody (concentration)			
	None	αC1 I (10 μg/ml)	αC1 I (2 μg/ml)	αALP (10 μg/ml)
% SV40-labeled cell profiles	72	38	41	57
Mean no. of virus particles/cell profile	2.9	0.9	1.3	2.7
Mean no. of virus particles in caveolae/cell profile	0.74	0.31	0.32	0.80

CV1 cells were preincubated with or without antibodies to MHC-I (αC1 I) or to alkaline phosphatase (αALP) for 1 h on ice before incubation with SV40. The cells were then warmed to 37°C for 1 h. The indicated antibodies were included in all incubation steps. The number of cells with SV40 at the plasma membrane and the localization of the individual virus particles were quantitated. Identical results were obtained in two separate experiments.

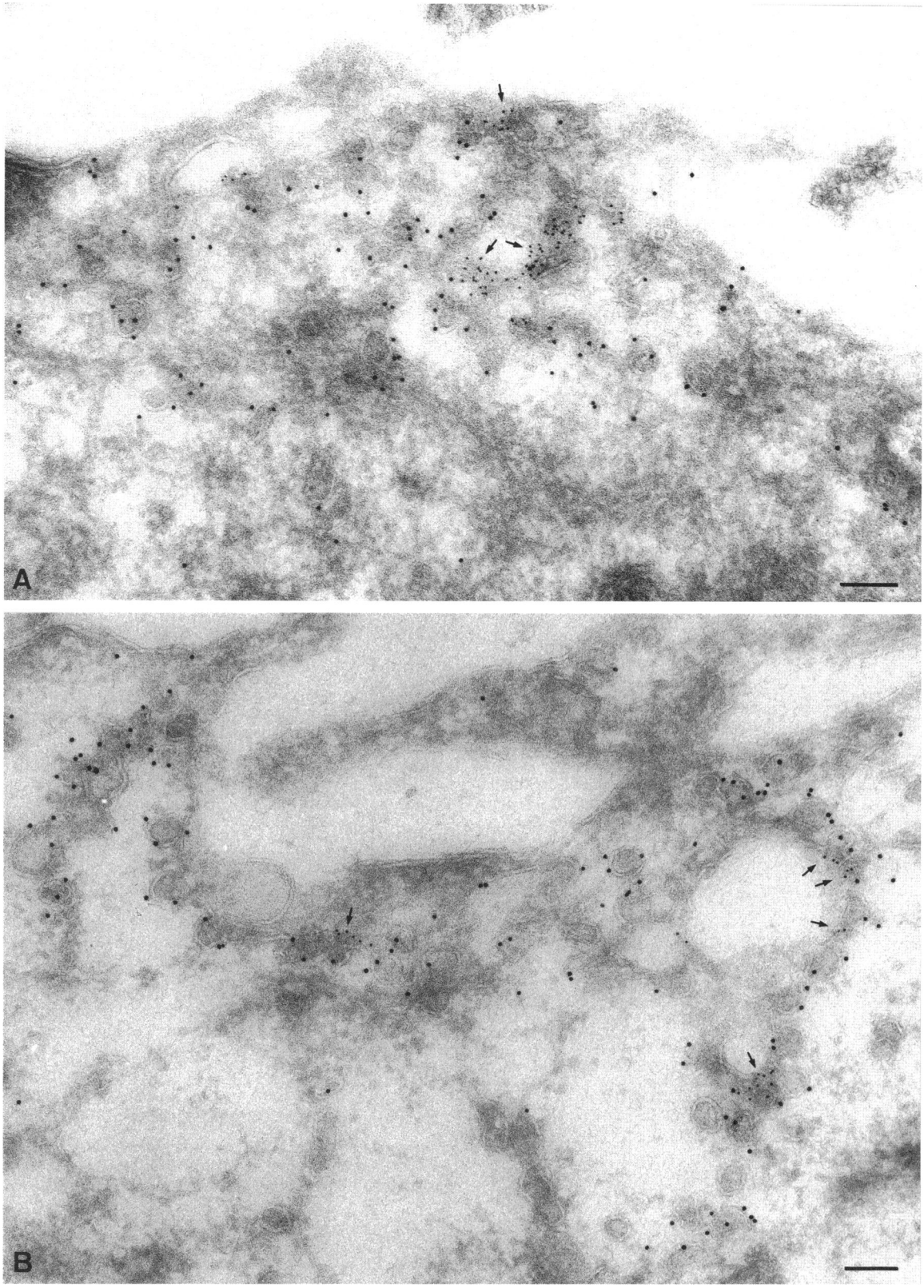

Figure 5. Cross-linked MHC-I molecules localize in caveolae. CV1 cells (A) or A431 cells (B) were surface labeled with mouse anti-MHC-I antibodies followed by rabbit anti-mouse antibodies and 5 nm of protein A gold. Gold particles (5 nm) are clustered in uncoated invaginations of the plasma membrane (arrows) which are labeled for caveolin-1 (10 nm of protein A-gold). Bars, 100 nm.

nalization. Additional work will also clearly be required to address the question of MHC-I internalization and the role of antibody-induced clustering of MHC-I. However, it is tempting to speculate that the return of unoccupied plasma membrane MHC-I molecules to the ER might be relevant to antigenic peptide loading. Perhaps some physiological condition or protein could cause a similar effect to SV40 to cluster and redistribute MHC-I molecules on the cell surface and hence trigger internalization.

These findings which suggest that a virus uses caveolae for cell entry are consistent with the view that caveolae are internalized at least under certain conditions. It remains to be seen whether SV40 is modifying the function of surface caveolae to allow its internalization and transport to the ER or whether the virus simply follows an existing pathway (Kartenbeck *et al.*, 1989). In this context the recent findings that caveolin cycles between the plasma membrane and the Golgi via the ER/Golgi intermediate compartment are of great interest (Smart *et al.*, 1994; Conrad *et al.*, 1995). The identification of a virus which has either modified or hijacked such a pathway should prove extremely valuable in future attempts to dissect this novel endocytic route.

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