MHC class I molecules are enriched in caveolae but do not enter with simian virus 40

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Simian virus 40 (SV40) binds to MHC class I molecules anywhere on the cell surface and then enters through caveolae. The fate of class I molecules after SV40 binding is not known. Sensitivity of 125I-surface-labelled class I molecules to papain cleavage was used to distinguish internalized class I molecules from class I molecules remaining at the cell surface. Whereas the caveolae-enriched membrane microdomain was found to also be enriched for class I molecules, no internalized papain-resistant 125I-surface-labelled class I molecules could be detected at any time in either control cells or in cells preadsorbed with saturating amounts of SV40. Instead, 125I-surface-labelled class I molecules, as well as preadsorbed 125I-labelled anti-class I antibodies, accumulated in the medium, coincident with the turnover of class I molecules at the cell surface. The class I heavy chains that accumulated in the medium were truncated and their release was specifically prevented by the metalloprotease inhibitor 1,10-phenanthroline. Thus, whereas class I molecules mediate SV40 binding, they do not appear to mediate SV40 entry.

Introduction

Simian virus 40 (SV40) initiates infection by binding to class I molecules encoded by the major histocompatibility complex (MHC) [human lymphocyte antigens (HLA) in humans] (Atwood & Norkin, 1989; Breau et al., 1992). MHC class I molecules are best known for their role in presenting endogenously synthesized antigenic peptide fragments for recognition at the cell surface by cytotoxic T lymphocytes. These molecules are heterodimers, composed of a 44 kDa transmembrane heavy chain and a noncovalently associated 12 kDa β2-microglobulin light chain.

Viruses that enter cells by endocytosis are generally considered to enter through clathrin-coated pits (Marsh & Helenius, 1989). In contrast, SV40 enters cells through caveolae (Anderson et al., 1996; Stang et al., 1997), which are flask-shaped plasma membrane invaginations, approximately 70 to 100 nm wide. Caveolae are distinguished from clathrin-coated pits in electron microscopy images by their size and shape. Also, caveolae contain a characteristic marker protein called caveolin (Rothberg et al., 1992). The regular functions of caveolae are not yet entirely clear, but they have been implicated in endocytosis, transcytosis, potocytosis and intracellular signalling (Montesano et al., 1982; Simionescu et al., 1982; Anderson, 1993a, b; Lisanti et al., 1994a, b; Schnitzer et al., 1995a, 1996).

In general, little is known about the fate of virus receptors following virus binding. We are interested here in the fate of MHC class I molecules following SV40 binding for several reasons. First, an earlier study reported that cross-linking class I molecules with antibodies against β2-microglobulin induced the internalization of class I molecules through small noncoated plasma membrane invaginations that would now be recognized as caveolae (Huet et al., 1980). Second, the caveolae-mediated SV40 entry pathway delivers the virus to the endoplasmic reticulum (ER), rather than to the endosomal/lysosomal compartment, which is the usual target for endocytic traffic (Kartenbeck et al., 1989; Norkin & Anderson, 1996). Since MHC class I molecules acquire antigenic peptides in the ER (Cox et al., 1990), and since it is not clear whether class I molecules at the cell surface might recycle to the ER, we were interested in the possibility that SV40 might target the ER through its association with class I molecules.

Several studies provided evidence that class I molecules are

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international by T cells (Capps et al., 1989; Dasgupta et al., 1988; Machy et al., 1987; Tse & Pernis, 1984; Vega & Strominger, 1989), and perhaps other cells as well (Huet et al., 1980; Reid & Watts, 1990). However, internalization was not observed in other studies (Eichholtz et al., 1992; Neefjes et al., 1990, 1992). These discrepancies might be due to cell type-specific differences and perhaps to differences in experimental approach. Regardless, although we found that the caveolin-containing membrane microdomain is enriched for MHC class I molecules, we could not detect any spontaneous, or antibody-induced, or SV40-induced internalization of class I molecules on CV-1 monkey kidney fibroblasts, which are standard permissive host cells for SV40. Instead, class I molecules appeared to be spontaneously released from the cell surface by the action of a metalloprotease. These results imply the existence of a putative coreceptor for SV40 that might be necessary for SV40 to enter and infect cells.

**Methods**

- **Chemicals.** The following chemicals were purchased from Sigma: 1,10-phenanthroline, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, soybean trypsin inhibitor, papain and lactoperoxidase. A 1:0 M stock of 1,10-phenanthroline was prepared before use in 95% ethanol. The protease inhibitors PMSF, aprotinin, leupeptin and soybean trypsin inhibitor were prepared, and stored, as described by Harlow & Lane (1986). Papain was reconstituted to a concentration of 7.5 mg/ml in PBS (pH 7.2), and frozen at −20 °C until use. Lactoperoxidase was reconstituted with PBS to 268 units/ml (2.5 mg/ml), and stored at −20 °C until use.

- **Cells and virus.** CV-1 African green monkey kidney fibroblasts were purchased from the ATCC and maintained in DMEM containing 10% foetal bovine serum (Atlanta Biologicals). SV40 wild-type strain 776 was triple plaque-purified and grown by infecting CV-1 cells at an m.o.i. of 1 p.f.u. per cell. Virus lysates, harvested at 3 days, were frozen and thawed three times, and cell debris was removed by centrifugation at 15 000 × g. Virus titres were determined by plaque assay on CV-1 cells.

- **Antibodies.** W6/32 hybridoma (Parham et al., 1979) was obtained from the American Type Tissue Culture Association, and maintained in RPMI containing 10% FBS until cells reached maximum density. Hybridoma cells were removed by centrifugation at 1000 g, and W6/32 tissue culture supernatant was frozen until use. W6/32 hybridoma was also injected into mice for ascites production (Harlow & Lane, 1988). Rabbit antisera specific for a peptide sequence on the cytoplasmic tail of human MHC class I heavy chain (Rougon et al., 1984), and denatured HLA-B27 (Neefjes et al., 1986) were generous gifts from H. L. Ploegh.

- **Cell surface iodination.** CV-1 cells were grown to confluency and washed twice with PBS. Radiolabelling was performed as described by Philips & Morrison (1970). Lactoperoxidase (90 µg/ml, 10 units/ml) in 1 ml of PBS was added to each plate. NaI (0.25 mM; ICN) was then added to each plate, followed by addition of 10 µl of H2O2 (7 µM final concentration), every minute, for 5 min. Cells were incubated for 5 min, and the iodination reaction was stopped by addition of 2 ml of stop buffer (PBS, 1 mg/ml tyrosine). Cells were incubated for an additional 5 min, and washed twice with PBS. After iodination, cell viability was greater than 90%, as judged by trypan blue dye exclusion. Cells did not display any altered morphology.

- **Immunoprecipitations and SDS–PAGE.** CV-1 cells were solubilized with 500 µl of lysis buffer (50 mM Tris–HCl, 1 mM MgCl2, 1% NP40, 1 mM PMSF, 15 µg/ml aprotinin, 2 µg/ml leupeptin, 5 mM 1,10-phenanthroline, 10 µg/ml soybean trypsin inhibitor (pH 7.4) for 5 min. Protein concentrations were measured using the Bio-Rad protein assay (Bradford, 1976). Extracts were preclariﬁed with 5 µl of normal rabbit serum, for 1 h on ice, followed by addition of 100 µl of formalin-fixed *Staphylococcus aureus* for 1 h on ice. *S. aureus* was pelleted by centrifugation at 15 000 × g for 5 min. Extracts were usually preclariﬁed three times to remove proteins that bound nonspeciﬁcally to immunoprecipitation complexes. MHC class I proteins were immunoprecipitated with 100 µl of W6/32 tissue culture supernatant fluid or with 5 µl of rabbit antiserum specific for the MHC class I cytoplasmic tail. Antibodies were added to cell extracts for 1 h, followed by the addition of 100 µl of *S. aureus*, for 1 h. To immunoprecipitate class I proteins using antiserum specific for denatured class I proteins, samples were first boiled for 10 min. Samples were cooled on ice, preclariﬁed, and class I proteins were immunoprecipitated with 5 µl of antiserum against denatured HLA-B27, for 1–3 h, followed by the addition of 100 µl of *S. aureus*. All immunoprecipitates were washed three times with 500 µl of lysis buffer, resuspended in 50 µl of sample buffer (63 mM Tris, 10% glycerol, 2% SDS, 40 mM DTT, 0.01% bromphenol blue), and boiled for 10 min. Samples were then resolved by SDS–PAGE. Gels were fixed with 25% methanol and 5% acetic acid, dried, and visualized by autoradiography.

- **Papain treatment to remove surface proteins.** Cell surface proteins were labelled with 125I. Cells were then washed and incubated for up to 3 h at 37 °C in DMEM containing 10% FBS. At the indicated times cells were detached by gently scraping monolayers with a rubber policeman and then treated with papain (1 mg/ml) in 500 µl of PBS containing 1 mM EDTA for 1 h on ice in glass sealed vials that were pulsed for 10 s with N2 gas. Viability of cells after this procedure was routinely about 80% as judged by trypan blue dye exclusion. Cells were washed three times with PBS to remove papain, and solubilized with lysis buffer.

Papain was also used to remove 125I-labelled W6/32 and 131I-labelled transferrin prebound to cells. W6/32 was purified from mouse ascites using a protein A affinity column (Harlow & Lane, 1988). Purified W6/32 and transferrin were labelled with 125I by the chloramine T method (Parham et al., 1979). 131I-labelled W6/32 or 125I-labelled transferrin were bound to cells on ice in PBS for 1 h, and cells were washed three times. Cells were cultured for various lengths of time at 37 °C in DMEM containing 10% FBS. Cells were then treated with papain as described above, washed, and the amount of radioactivity associated with cells after papain treatment was determined with a gamma counter.

- **Isolation of caveolin-enriched membrane.** Caveolin-enriched membrane was isolated by the Triton X-100-based procedure as previously described (Anderson et al., 1996). Caveolin-enriched membrane was isolated by a detergent-free procedure, essentially as described by Song et al. (1996). In brief, CV-1 cells were washed in ice-cold PBS and resuspended in 1 ml of lysis buffer containing 500 mM Na2CO3 (pH 11) and homogenized by pipetting up and down 14 times through a 1000 µl blue tip, followed by three cycles of sonication (10 s each). The lysate was then adjusted to 45% sucrose by adding 1 ml of 90% sucrose in MES buffer (25 mM MES, pH 6.5, and 150 mM NaCl). The lysate was then transferred to an ultracentrifuge tube and overlaid sequentially with 2 ml of 35% sucrose and 1 ml of 5% sucrose, each in MES buffer containing 250 mM Na2CO3. The tube was centrifuged at 39000 r.p.m. for 14 h at 37 °C in a SW50.1 rotor. Caveolin-enriched membrane was isolated as a band at the 3/35% sucrose interface. It was then diluted 1:3 in buffer containing 1:1 500 mM Na2CO3 (pH 11) and MES buffer (pH 6.5) with 1 mM PMSF and pelleted by centrifugation at 14000 r.p.m. in a microcentrifuge. The proteins in the pellet were solubilized in a buffer
containing 10 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 60 mM n-octyl β-D-glucopyranoside, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 mM sodium vanadate, and 10 μg/ml leupeptin. Protein concentrations were determined using the Bio-Rad protein assay (Bradford, 1976).

Purification of whole plasma membrane. Plasma membrane was isolated using the colloidal silica technique, essentially as described by Stolz & Jacobson (1991). In brief, CV-1 cells were washed with MES-buffered saline (MBS) containing 20 mM MES, pH 5.5, 135 mM NaCl, 1 mM MgCl₂ and 0.5 mM CaCl₂ with protease and phosphatase inhibitors. Cells were then coated with 1% colloidal silica (kindly provided by B. S. Jacobson) in MBS for 2–5 min. Cells were next washed with MBS and then overcoated with 1 mg/ml polyacrylic acid (mol. mass 50000) in MBS to balance the positive charges on the silica beads. Cells were washed again in MBS and lysed by osmotic pressure in the lysis buffer containing 2.5 mM imidazole, 1 mM MgCl₂ and 0.5 mM CaCl₂ with all inhibitors. Cells were then scraped off the flask and vortexed briefly. The exposed surface of the cell membrane was pelleted at 900 g for 5 min in a clinical centrifuge. The membrane pellet was cleaned by washing gently in the lysis buffer and by pelleting through a 70% metrizamide cushion for 20 min at 20000 r.p.m. in an SW50.1 rotor. The membrane pellet was then solubilized in a strong detergent and sonicated three times (5 s each). The proteins were precipitated in ice-cold acetone for 20 min (1 part solubilized protein to 4 parts of acetone). The precipitated proteins were then solubilized as described for the caveolin-enriched fraction, and concentrations determined using the Bio-Rad protein assay (Bradford, 1976).

Results

Caveolin-enriched membrane regions are enriched for MHC class I molecules

SV40 initially binds to MHC class I molecules anywhere on the cell surface and then translocates to the caveolin-enriched membrane microdomain (Anderson et al., 1996). We ask here whether class I molecules might mediate the translocation of bound SV40 to the caveolin-containing membrane microdomain.

A standard and stringent procedure for the isolation of a membrane fraction highly enriched for caveolin is based on the insolubility of caveolae in Triton X-100 and on the low density of caveolae in sucrose density gradients (Rothberg et al., 1992; Lisanti et al., 1994a, b). Both of these properties of caveolae are attributed to their high content of glycosphingolipids (Brown & Rose, 1992). We began, as before (Anderson et al., 1996), by using a detergent-based procedure to isolate caveolin-enriched membrane fractions. However, we did not detect any class I molecules in the detergent-insoluble low density fraction from either control or SV40-infected cultures (data not shown). Since some caveolae-associated proteins do not cofractionate with caveolin following extraction with Triton X-100, we next isolated caveolin-enriched membrane using a detergent-free method (Song et al., 1996). In this procedure Triton X-100 was replaced with sodium carbonate buffer and cells were homogenized by pipeting and then finely disrupted by sonication. A low-density fraction was then obtained by sucrose density gradient centrifugation.

Class I molecules were readily observed in the caveolin-enriched fractions isolated from control and infected cells using the detergent-free method (Fig. 1). To ask whether the low-density membrane fraction might actually be enriched for class I molecules relative to the whole plasma membrane, we isolated highly purified whole plasma membrane using a silica coating-based procedure (Stolz & Jacobson, 1991). Membrane samples were then normalized to total protein in order to

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**Fig. 1.** MHC class I molecules in caveolin-enriched low-density membrane fractions. (a) Low-density caveolin-enriched membrane (L) was isolated by the detergent-free procedure and whole plasma membrane (M) was isolated using the cationic colloidal silica technique. Equal amounts of protein were added per lane and class I heavy chains were identified by SDS–PAGE and Western blotting. (b) SV40 (100 p.f.u. per cell) was preadsorbed to CV-1 cells for 1 h on ice and cultures were incubated at 37 °C as indicated. Equal amounts of protein from low-density caveolin-enriched fractions and whole cell extracts were added per lane and analysed by SDS–PAGE and Western blotting.
Fig. 2. ^{125}I surface-labelled MHC class I molecules do not internalize but accumulate in the culture fluid. (a) Surface-labelled cultures were incubated at 37 °C. At the indicated times cell extracts were prepared and immunoprecipitated with W6/32. MHC class I heavy chains were resolved by SDS–PAGE and autoradiography. (b) Cells were mock infected or exposed to SV40 on ice and then incubated at 37 °C for the indicated times prior to treatment with papain. Class I proteins were immunoprecipitated from detergent extracts with anti-CT and analysed by SDS–PAGE and autoradiography. Detergent extracts were also prepared from samples not treated with papain, as indicated. (c) Media from the control cell samples shown above were immunoprecipitated with antiserum against a denaturation-insensitive epitope on the class I heavy chain. Immunoprecipitates were resolved by SDS–PAGE and autoradiography.

compare roughly equal amounts of low-density membrane (L) and whole plasma membrane (M). When equal amounts of protein from the low-density membrane fraction and whole plasma membrane were examined by immunoblotting, the low-density membrane was seen to indeed be enriched for class I molecules (Fig. 1a).

The above finding that the caveolin-enriched membrane fraction is also enriched for class I molecules supports the premise that class I molecules might mediate the translocation of bound SV40 to the caveolin-enriched membrane microdomain. However, exposing cells to saturating amounts of SV40 (100 p.f.u. per cell; note that the ratio of SV40 particles to p.f.u. is greater than 100) did not cause a detectable increase in the amount of class I molecules in the caveolin-enriched membrane fraction (Fig. 1b). The slight increase seen here between 0 and 1 h was not representative of multiple repetitions of this experiment. Note that the caveolin-enriched fraction is seen to also be enriched for class I molecules when compared to whole cell extracts containing equal amounts of protein. Also, SV40 translocated to the caveolin-enriched fraction by 1 h, as seen before using the detergent-based procedure (Anderson et al., 1996).

The above data do not eliminate the possibility that class I molecules might mediate the translocation of bound SV40 to the caveolin-enriched membrane microdomain, since any SV40-induced increase in the already high basal levels of class I molecules in that membrane fraction might be below the level of sensitivity of our procedures (see Discussion).

SV40 does not cause entry of surface MHC class I molecules

We next asked whether class I molecules might enter cells with SV40. The rate for the spontaneous turnover of surface-labelled class I molecules on uninfected CV-1 cells is shown in Fig. 2(a). Cell surface proteins were directly labelled with ^{125}I using the lactoperoxidase technique. At various times whole cells were detergent-solubilized and class I molecules were immunoprecipitated using anti-MHC-I monoclonal antibody
(MAb) W6/32. This MAb recognizes an epitope that is present only on intact native class I molecules, which contain the 44 kDa transmembrane heavy chain and the non-covalently associated 12 kDa β2 microglobulin. Analysis of the autoradiograph by densitometry indicated a half-life for valently associated 12 kDa β2 microglobulin. Thus, internalized class I molecules would be resistant to cleavage and release by papain. Cells were then solubilized and immunoprecipitated with anti-CT, an antiserum specific for a peptide sequence on the cytoplasmic tail of the MHC class I heavy chain (see below). No papain-resistant, 125I-labelled class I molecules could be detected in control whole cell extracts at any time up to 3 h (Fig. 2b). Identical results were obtained when W6/32 was used to immunoprecipitate the cell extracts (data not shown).

Whereas we could not detect papain-resistant surface-labelled class I molecules in whole cell extracts, we readily detected the accumulation of surface-labelled class I molecules in the media using an antiserum against a denaturation-insensitive epitope on the class I heavy chain (Fig. 2c). Thus, the spontaneous turnover of surface class I molecules seen in control cells appeared to result primarily from shedding. Note that the class I molecules in the media were truncated to about 37 kDa (Fig. 2c) and could not be detected using W6/32 or anti-CT (see below).

To ask whether entry of class I molecules might be induced by SV40, cells were exposed to saturating inputs of the virus (400 p.f.u. per cell). Nevertheless, we still did not detect any papain-resistant 125I-labelled class I molecules in cells exposed to high inputs of SV40, even as late as 3 h (Fig. 2b). Indeed, in multiple repetitions of this experiment we never detected internalized class I molecules, even on grossly overexposed gels. Note that the SV40-infected and control samples seen in Fig. 2 were generated concurrently. Anti-CT was used to immunoprecipitate the samples shown in Fig. 2(c) since, unlike W6/32, it does not compete with SV40 for binding to class I molecules.

To establish additional controls for the above experiments, and since some investigators reported that class I molecules internalize when indirectly labelled or cross-linked with antibodies (e.g. Huet et al., 1980; Machy et al., 1987), we followed the fate of surface class I molecules by indirect labelling with 125I-labelled W6/32. The labelled antibody was preadsorbed to CV-1 cells at 4 °C and cultures were then incubated for various periods of time at 37 °C. Internalized antibody would be resistant to release from cells by papain treatment. No papain-resistant 125I-labelled W6/32 could be detected when CV-1 cells were incubated for up to 3 h at 37 °C. Instead, the labelled antibody was found to accumulate in the media, coincident with its loss from cells (Fig. 3a).

The data are from an experiment in which W6/32 was not cross-linked with secondary antibody. Identical results were obtained when W6/32 was cross-linked with rabbit anti-mouse IgG. Identical results were also obtained when the direct surface-labelling procedure was used to label class I molecules. Thus, class I molecules do not appear to internalize into CV-1 cells either following SV40 binding or cross-linking with antibodies.

To establish a positive control for the above experiments, we asked whether bound 125I-labelled transferrin might become resistant to release by protease treatment. All of the bound
transferrin became resistant to papain within 5 min of transferring cells to 37 °C (Fig. 3 b). Note that analysis of CV-1 cells by flow cytometry indicated that these cells have 100-fold more MHC class I molecules that bind W6/32 than transferrin receptors that bind antibodies against the transferrin receptor (data not shown). Thus, we should have detected the internalization of even a small percentage of surface class I molecules, if it was occurring.

Results from some earlier studies imply that when peptide dissociates at the cell surface from native class I molecules, the β2-microglobulin moiety dissociates and the heavy chain is cleaved and released by the action of a protease that is sensitive to the metalloprotease-specific inhibitor 1,10-phenanthroline (Demaria et al., 1992; Ljunggren et al., 1990; Neefjes et al., 1992; Powers & Harpur, 1986). In agreement with those reports, we found that the turnover of anti-HC-reactive (but not W6/32-reactive) surface-labelled class I molecules could be prevented by treatment with 1,10-phenanthroline (Fig. 4). Note that the drug also prevented the accumulation of heavy chains in the media. Also, aprotinin, a serine protease inhibitor, and leupeptin, an inhibitor of serine and thiol proteases, were without effect on class I shedding (data not shown). These findings explain why we could not detect surface-labelled class I molecules in the media using W6/32 or anti-CT as noted above, and why the class I heavy chains in the media were truncated (Fig. 2 c).

Since the low-density caveolin-enriched membrane is also enriched for class I molecules, and since class I molecules are shed from the membrane by a metalloprotease, we asked whether we might observe an SV40-induced increase in the level of class I molecules in the caveolin-enriched membrane if 1,10-phenanthroline was present throughout the experiment. However, the inhibitor had no effect on the levels of class I molecules in the caveolin-enriched fractions from either infected or control cultures (Fig. 5).

**Discussion**

MHC class I molecules are necessary for SV40 binding that leads to infection (Atwood & Norkin, 1989; Breau et al., 1992). The present study was prompted by our interest in the fate of class I molecules during subsequent stages of SV40 entry. While little is known in general about the fate of virus receptors during later stages of virus entry, the fate of the SV40 receptor is particularly interesting for several related reasons. First, SV40 enters cells through caveolae (Anderson et al., 1996; Stang et al., 1997), and this poorly understood entry pathway targets SV40 to the ER (Kartenbeck et al., 1989; Norkin & Anderson, 1996). Second, class I molecules acquire antigenic peptides in the ER for presentation at the cell surface (Cox et al., 1990), and there is no consensus concerning whether class I molecules at the cell surface might recycle to the ER (see below). Thus, if class I molecules were to mediate SV40 entry, they might then account for the unusual targeting of SV40.

Evidence that MHC class I molecules might internalize was initially provided by following the fate of molecules labelled at the cell surface with anti-class I antibodies (for review see Yewdell & Bennink, 1992). Since the internalization of a variety of cell surface receptors is induced by their ligands or by cross-linking with antibody (Goldstein et al., 1985), this approach left unanswered whether class I internalization is spontaneous or needs to be induced. This distinction is important here since we needed to determine the baseline for spontaneous class I internalization in order to determine any effect of SV40 on class I entry. It is also significant concerning any possible physiological relevance of class I internalization.
In subsequent studies, approaches to measuring class I internalization were developed that were independent of cross-linking with antibodies. For example, it was possible to follow the fate of class I molecules that were radiolabelled at the cell surface (Reid & Watts, 1990; Vega & Strominger, 1989). Internalization of class I molecules has also been followed by measuring the uptake of fluorescent β2 micro-globulin (Hochman et al., 1991). Results from studies in which several variations of these approaches were used have not provided a consensus regarding whether class I molecules are internalized constitutively, inducibly, or even at all (Abdel Motal et al., 1993; Aragnol et al., 1986; Capps et al., 1989; Eicholtz et al., 1992; Hochman et al., 1991; Huet et al., 1980; Machy et al., 1987; Neefjes et al., 1992; Reid & Watts, 1990; Tse & Pernis, 1984; Vega & Strominger, 1989).

Since these studies involved a variety of cell types, cell-type-specific effects might be responsible for some of the dissimilar findings. Differences in experimental methodology might account for others. For example, it remains to be shown that traffic of labelled antibody reagents reflects traffic of class I molecules. Indeed, most of the studies reporting the internalization of class I molecules used reagents (e.g. antibodies) labelled with isotopes or fluorescent dyes to track class I molecules. It was assumed that these reagents remained conjugated to the class I molecules and reflected their movements. In contrast, the few reports which did not find evidence for internalization (Eicholtz et al., 1992; Neefjes et al., 1990, 1992) used a more direct surface-labelling biochemical approach. Nevertheless, other studies in which a direct surface-labelling approach was used did report evidence for internalization of class I molecules (Reid & Watts, 1990; Vega & Strominger, 1989).

Since different experimental methodologies might lead to different conclusions, we followed the fate of surface class I molecules on CV-1 cells using labelled antibodies as well as by direct surface-labelling. Thus, it is noteworthy that we did not see any evidence that surface class I molecules might internalize using either of these standard and sensitive procedures.

Our direct surface-labelling procedure depended on isolating 125I-surface-labelled class I molecules from cell extracts by immunoprecipitation. Thus, some internalized class I molecules might have gone undetected if they were degraded soon after entry. However, this seems unlikely in the case of class I molecules that might have entered in association with SV40, since entering SV40 is largely targeted to the ER, rather than to the endosomal/lysosomal compartment (Kartenbeck et al., 1989; Norkin & Anderson, 1996). Furthermore, degradation is not an issue in experiments where surface class I molecules were indirectly labelled with 125I-labelled W6/32.

To establish a positive control for the above experiments, we followed the internalization of 125I-labelled transferrin. In contrast to our findings using labelled W6/32, preadsorbed transferrin completely internalized within 5 min of preadsorption. Furthermore, analysis by flow cytometry showed that CV-1 cells have 100-fold more class I molecules that bind W6/32 than transferrin receptors that bind antibodies against the transferrin receptor. Together, these results suggest that we should have detected internalized surface-labelled class I molecules, even if only a small percentage of the surface-labelled molecules had internalized.

Since we found no evidence for the internalization of class I molecules coincident with SV40 entry, despite using saturating amounts of SV40 and the highly sensitive direct surface-labelling procedure to detect internalized class I molecules, we conclude that class I molecules do not deliver SV40 into the cell, nor can they account for the targeting of SV40 to the ER. Instead, our results imply the existence of a putative coreceptor for SV40 that might mediate its entry. In this regard, other viruses have also been found to use distinct cell surface factors to mediate the series of steps between initial virus binding and entry. Examples include nonenveloped viruses such as adenoviruses (Wickham et al., 1993), and enveloped viruses such as herpesviruses (McClain & Fuller, 1994) and human immunodeficiency virus (Deng et al., 1996; Dragic et al., 1996; reviewed Norkin, 1995).

It is interesting to consider where in the SV40 entry pathway the virus might dissociate from class I molecules and associate with the putative coreceptor. In this regard, SV40 initially binds to class I molecules that are on flat regions of the plasma membrane. The virus later becomes associated with caveolae. This was indicated by both cell fractionation (Anderson et al., 1996) and ultrastructure (Stang et al., 1997). The virus then enters in tight-fitting vesicles (Kartenbeck et al., 1989) that may derive from caveolae.

The present findings do not eliminate the possibility that class I molecules might mediate the translocation of bound SV40 to caveolae. Consistent with this possibility, we found that the caveolin-enriched membrane fraction is also enriched for class I molecules relative to whole plasma membrane. To the best of our knowledge this finding is presently unique. Furthermore, Stang et al. (1997) recently reported that cross-linking class I molecules with W6/32 causes them to cluster in caveolae, as shown by immunoelectron microscopy. However, we could not detect any SV40- or W6/32-induced increase in the level of class I molecules in the caveolin-enriched membrane fraction. A possible explanation is that any SV40- or antibody-induced increase in the level of class I molecules in the caveolin-enriched fraction might have been below the level of detection by our procedure, since that fraction is already highly enriched for class I molecules.

We recently reported that SV40 entry is promoted by an intracellular signal that SV40 induces from the cell surface (Dangoria et al., 1996). More recently, we found that signal transmission by SV40 requires the functional integrity of the low-density membrane microdomain that surrounds the caveolae (Y. Chen & L. C. Norkin, unpublished results). Furthermore, electron microscopy of detergent-insoluble low-density complexes showed that SV40 particles accumulated at
the ‘mouths’ of the caveolae when signalling was blocked, whereas the virus was found within the caveolae when signalling was unobstructed (Y. Chen & L.C. Norkin, unpublished results). Note that the annular regions about the caveolae, as well as the caveolae per se, are enriched for a variety of signal transducing molecules (Schnitzer et al., 1995b).

It may be of interest that whereas W6/32 did not induce internalization of class I molecules, this MAbs (as well as other anti-class I MAbs) did induce a transmembrane signal in CV-1 cells. However, the antibody-induced signal led to the activation of mitogen-activated protein kinase (MAPK), whereas the SV40-induced signal did not (Dangoria et al., 1996). This finding suggests that the SV40 signal might be transmitted through the putative coreceptor, rather than through class I molecules.

The following model is consistent with the above findings. First, SV40 binds to class I molecules anywhere on the cell surface. Then, in association with class I molecules SV40 translocates to the annuli surrounding the caveolae. Within that microdomain SV40 dissociates from class I molecules and associates with the putative coreceptor. The SV40-induced signal is then transmitted, promoting virus entry into the caveolae, followed by later stages of entry into the cell.

Although we detected no internalization of either directly or indirectly labelled class I molecules into CV-1 cells, we readily detected the accumulation of truncated directly labelled class I heavy chains and 125I-labelled W6/32 in the media. Our findings are entirely consistent with earlier reports (Demaria et al., 1992, 1994; Ljunggren et al., 1990; Neefjes et al., 1992), which implied that surface class I molecules are cleaved by a surface metalloprotease activity after the spontaneous release of peptide and the β2 microglobulin moiety. This instability of empty class I heterodimers and their release from the cell surface would prevent the rebinding of peptide that might sensitize bystander cells to lysis by cytotoxic T cells (e.g. Demaria et al., 1994; Neefjes et al., 1992; Ortiz-Navarret & Hammerling, 1991; Rock et al., 1990).

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