

Characterization of Early Steps in the Poliovirus Infection Process: Receptor-Decorated Liposomes Induce Conversion of the Virus to Membrane-Anchored Entry-Intermediate Particles

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The mechanism by which poliovirus infects the cell has been characterized by a combination of biochemical and structural studies, leading to a working model for cell entry. Upon receptor binding at physiological temperature, native virus (160S) undergoes a conformational change to a 135S particle from which VP4 and the N terminus of VP1 are externalized. These components interact with the membrane and are proposed to form a membrane pore. An additional conformational change in the particle is accompanied by release of the infectious viral RNA genome from the particle and its delivery, presumably through the membrane pore into the cytoplasm, leaving behind an empty 80S particle. In this report, we describe the generation of a receptor-decorated liposome system, comprising nickel-chelating nitrilotriacetic acid (NTA) liposomes and His-tagged poliovirus receptor, and its use in characterizing the early events in poliovirus infection. Receptor-decorated liposomes were able to capture virus and induce a temperature-dependent virus conversion to the 135S particle. Upon conversion, 135S particles became tethered to the liposome independently of receptor by a membrane interaction with the N terminus of VP1. Converted particles had lost VP4, which partitioned with the membrane. The development of a simple model membrane system provides a novel tool for studying poliovirus entry. The liposome system bridges the gap between previous studies using either soluble receptor or whole cells and offers a flexible template which can be extrapolated to electron microscopy experiments that analyze the structural biology of nonenveloped virus entry.

Despite the common requirement for efficient recognition by receptor molecules at the surface of the host cell, the entry mechanisms of the enveloped and nonenveloped viruses are profoundly different. The nucleocapsid of an enveloped virus is enclosed within a membrane, and cell entry can therefore be accomplished by fusing the viral membrane either with the external plasma membrane or with the membrane of an intracellular vesicle following endocytosis. Fusion events generally commence when a trigger initiates conformational changes in a viral glycoprotein, exposing hydrophobic sequences that insert into the host cell membrane. In many cases, best exemplified by influenza virus, the trigger is acidification as the virus passes through the endosomal pathway. In others, such as human immunodeficiency virus, the trigger is receptor binding.

In contrast, nonenveloped viruses require either the entire virion or the highly charged viral genome to pass across the hydrophobic barrier of the host cell membrane. Although details of the cell entry pathway for nonenveloped viruses are not well defined, a general model has been described for poliovirus and related viruses, in which receptor-mediated conformational changes and exposure of hydrophobic viral residues play crucial roles (17).

Poliovirus provides an excellent model system to study non-enveloped virus entry. The genetics and biochemistry of the

virus have been studied extensively, and preliminary structural information has been obtained for several stages of the virus life cycle (1, 2, 4). Poliovirus is a member of the enterovirus genus of the picornavirus family and is closely related to a number of other important pathogens including rhinoviruses, coxsackieviruses, and echoviruses. These viruses contain a positive-sense RNA genome (7,000 to 8,000 nucleotides in length) enclosed in an icosahedral protein shell consisting of four capsid proteins (VP1 to VP4) (27).

Infection is initiated when the virus attaches to a specific receptor, the poliovirus receptor (PVR or CD155), a cell adhesion molecule-like protein belonging to the nectin family (29). The receptor has an ectodomain composed of three immunoglobulin-like domains, a transmembrane region, and C-terminal cytoplasmic tail (24). The ectodomain can be transferred to a variety of anchors (28), including a glycosyl-phosphatidylinositol anchor (30), and remain fully functional for virus entry, demonstrating that neither the transmembrane nor the cytoplasmic domains play an essential role. The structure of the poliovirus-PVR complex has been solved by cryoelectron microscopy (cryo-EM) (2, 15, 16, 35) and shows that receptor molecules bind in a deep depression or “canyon” that surrounds the fivefold axis of the icosahedral virus particle.

At physiological temperatures, receptor binding initiates the cell entry process (8, 9, 22) by lowering the activation energy of a reaction, which causes an irreversible conformational change in the virus capsid (32). This conversion produces a particle with altered sedimentation properties (from 160S of the virion to 135S), antigenicity, and sensitivity to proteases. This particle is generally known as the 135S or A particle. The conforma-

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tional changes include the externalization of both the amino terminus of VP1 (12) and the small internal myristoylated protein, VP4 (6, 9, 14). At room temperature, both VP4 and the amino-terminal extension of VP1 are completely enclosed in the native virion protein shell (18) but are transiently and reversibly exposed at physiological temperatures (21). The exposed N terminus of VP1 allows the 135S particle to attach to lipid membranes in a receptor-independent manner (12); the externalized VP4 also partitions into membranes during infection (7). Both 135S particles and virus-receptor complexes have been shown to form channels in membranes (7, 31). Furthermore, the ability of viruses with mutations in VP4 to form channels correlates with their ability to infect cells (7).

After the conversion to 135S, the virus undergoes a second conformational change in which the viral RNA is ejected, resulting in the production of an empty particle which sediments at 80S. The 80S particle is antigenically distinct from both the 135S particle and native virus (12, 19). The trigger for RNA release is unknown.

Structures of the 135S and 80S particles have been solved by using cryo-EM (1, 4). To aid in their interpretation, pseudo-atomic models for these structures have been derived by docking atomic level-resolution models of the capsid proteins into cryo-EM reconstructions. Together, biochemical and structural information has led to a working model for the early steps of poliovirus infection in which the receptor captures the virus, orients it appropriately at the cell surface, and catalyzes conformational changes. These changes result in the insertion of viral protein sequences in a cell membrane to form a pore or hole in the membrane through which the viral RNA is translocated into the cytoplasm (1, 4, 17). Although this model is consistent with available data (including the link between the ability to make channels and the ability to release RNA), it is also possible that the inserted sequences disrupt membranes, resulting in the release of the 135S particle or externalized RNA into the cytoplasm.

Despite the wealth of structural and biochemical information, there are several key steps in the cell entry pathway that remain poorly understood. It is known that infection with poliovirus is not obligatorily dependent on acidification of endosomes or on dynamin-requiring processes such as classical clathrin-mediated endocytosis or internalization via caveoli (10). However, the site of RNA translocation (cell surface, intracellular vesicle), the trigger for RNA release, and the precise nature of the RNA translocation complex remain unknown.

Addressing these important questions will require a variety of approaches. One of the key obstacles to further understanding is the lack of an appropriate model system to bridge the considerable gap between studies using whole cells and studies using soluble particles. In this paper, we describe the development of a simple liposome-based model system for biochemical and structural characterization of the early steps in poliovirus entry. In this model, liposomes which incorporate lipids with nickel-charged nitrilotriacetic acid (NTA) head groups are used to capture recombinantly expressed PVR ectodomains containing a C-terminal (membrane-proximal) His tag. These receptor-decorated liposomes are used as simple surrogates for cells. The model circumvents the need to reconstitute full-length receptor into membranes and provides a convenient

way to control many of the parameters that may be involved in cell entry. We show that receptor-decorated liposomes are capable of (i) capturing virus, (ii) inducing the virion-to-135S particle transition, and (iii) facilitating the externalization of VP4 and the N terminus of VP1 and the formation of a receptor-independent 135S particle-membrane complex, mediated by the newly exposed N terminus of VP1.

MATERIALS AND METHODS

Virus growth, labeling, and purification. The type 1 Mahoney strain of poliovirus was propagated in HeLa cell suspension culture, using Joklik's minimal essential medium supplemented with 10% fetal calf serum, 0.5% pluronic acid, 100 μ M nonessential amino acids, 20 mM HEPES (pH 7.5), 12.5 U of penicillin, and 12.5 μ g of streptomycin per ml. Cells in log-phase growth were pelleted and resuspended at a density of 4×10^7 cells/ml in medium deficient in fetal calf serum and containing virus at a multiplicity of infection of 20. After incubation at room temperature for 1 h to allow virus attachment, cells were diluted 10 fold with prewarmed complete medium, and the suspension culture was incubated for 6.5 h at 37°C. The cells were then harvested by centrifugation, and virus was released by freeze-thawing the cell pellet twice. Cell debris was removed by low-speed centrifugation, and virus was purified with a CsCl density gradient as previously described (36).

Virus particles containing radiolabeled capsid proteins were produced using medium deficient in L-methionine and with the addition of [³⁵S]methionine to the culture 3.5 h postinfection, to yield purified virus with a specific activity on the order of 4×10^5 cpm/ μ g.

Preparation of liposomes. Phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, cholesterol, and phosphatidic acid were supplied in chloroform (Avanti Polar Lipids) and mixed in molar ratios of 1:1:1:1.5:0.3, respectively (12). Chloroform was evaporated with a stream of argon, leaving a thin lipid film, which was dried overnight under vacuum. Dried lipid was rehydrated at a concentration of 1 mg/ml in 50 mM HEPES (pH 7.3) and 50 mM NaCl, thoroughly mixed by vortexing, and extruded through a 0.1- μ m-pore-size membrane (Avanti Polar Lipids) to form a homogeneous population of unilamellar vesicles with a diameter approximately equivalent to the pore size. Liposomes were freshly prepared for each experiment.

Fluorescence-labeled liposomes were generated by the inclusion of phosphatidylethanolamine with lissamine rhodamine B-labeled head groups (Avanti Polar Lipids), at a final concentration of 0.5% (wt/wt), prior to evaporation of chloroform from the lipid mixture.

Nickel-charged liposomes, for binding of the His-tagged receptor, were similarly generated by the inclusion of the nickel salt of 1,2-dioleoyl-*sn*-glycero-3- $\{[N(5\text{-amino-1-carboxypentyl})\text{iminodiacetic acid}] \text{succinyl}\}$ (designated DOGS-NTA-Ni; Avanti Polar Lipids), at a final concentration of 20% (wt/wt) unless otherwise stated; they are referred to as NTA-liposomes.

Formation of receptor-decorated liposomes. Soluble PVR consisting of the ectodomain (amino acids 1 to 337) without the transmembrane and cytoplasmic domains and with a six-histidine tag at the C terminus (sPVRHis) was expressed in stably transformed mammalian cells and purified as previously described (23); it was a gift from V. R. Racaniello (Columbia University College of Physicians and Surgeons, New York, N.Y.).

Nickel-chelating liposomes with a total lipid concentration of approximately 1 mg/ml were incubated at room temperature (<25°C) in 50 mM HEPES (pH 7.3), 50 mM NaCl, with sPVRHis at a final concentration of 100 μ g/ml. After 10 min, a further 100 μ g of receptor/ml was added and incubated for an additional 10 min. Receptor-decorated liposomes were purified from unbound material by flotation through Ficoll gradients.

Virus binding to liposomes. Samples of purified receptor-decorated liposomes containing approximately 1-mg/ml lipid and 0.5-mg/ml bovine serum albumin (BSA) in 50 mM HEPES (pH 7.3)–50 mM NaCl were mixed at room temperature with radiolabeled poliovirus in a final volume, typically of 100 μ l. Virus was used at nonsaturating amounts (0.2 μ g) or in excess (20 μ g), depending on the experiment.

Ficoll gradient liposome flotation. The liposome sample was adjusted to contain Ficoll at a final concentration of 20% (wt/vol) and was overlaid with 10% (wt/vol) Ficoll, followed by buffer alone. After centrifugation at $100,000 \times g$ at 20°C for 30 min, gradients were fractionated from the top. Fractions were analyzed for the presence of liposomes by visualization of rhodamine, for the presence of receptor by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining, or for the presence of radiolabeled

virus by liquid scintillation counting (Packard Tri-Carb 1900TR). Alternatively, for preparative purposes, the visible floated liposome band was harvested from the gradient at the interface between the 10% Ficoll and buffer layers.

Temperature dependence of conversion. Receptor-decorated or nondecorated liposomes (10 μg of lipid) were incubated with 0.5 μg of radiolabeled poliovirus for 5 min at 37°C or room temperature in 50 mM HEPES (pH 7.3)–50 mM NaCl before analysis of particles by sedimentation through sucrose gradients. A broader temperature range was sampled by incubating the virus-receptor-liposome complexes for 32 min at room temperature, 31°C, 33°C, 35°C, 37°C, or 39°C. The longer incubation time was chosen to facilitate detection of potentially inefficient conversion at suboptimal temperatures.

Time course of conversion. Receptor-decorated liposomes (10 μg of lipid) were incubated with 0.2 μg of radiolabeled poliovirus (nonsaturating amounts) at room temperature in 50 mM HEPES (pH 7.3) and 50 mM NaCl. The use of nonsaturating levels of input virus ensured that the majority of virus was bound to liposomes at the start of the time course (data not shown). Samples were then diluted 1:20 into the same buffer (prewarmed) and incubated at 37°C for 0.5, 2, 8, and 32 min. The reactions were stopped at each time point by rapid freezing in liquid nitrogen.

Effect of receptor density on conversion. Receptor-decorated liposomes were prepared as described above, except that they contained DOGS-NTA-Ni in the range of 2 to 20% (wt/wt). Liposomes (100 μg of lipid) were mixed with 20 μg of radiolabeled poliovirus (saturating amounts). Virus-receptor-liposome complexes were separated by flotation through Ficoll gradients and incubated at 37°C for 40 min.

Release of virus from liposome complexes. Receptor-decorated liposomes (100 μg of lipid) were mixed with 20 μg of radiolabeled poliovirus (saturating amounts) at room temperature. Liposome-receptor-virus complexes were purified in a preparatory Ficoll flotation step. The complexes were then incubated at room temperature or 37°C for 40 min, cooled to room temperature, and adjusted to 1.5 M imidazole to disrupt receptor-virus interactions, or they were mock treated. Intact liposome-particle complexes were then purified by an additional flotation through Ficoll. Purified liposome fractions containing liposome-particle complexes were then subjected to digestion by *Staphylococcus aureus* V8 protease (Sigma) by incubation for 30 min at room temperature with protease at 200 $\mu\text{g}/\text{ml}$.

Sucrose gradient sedimentation of particles. Particles were extracted from liposome samples by 10-fold dilution and then solubilization of the lipid membranes with the addition of Triton X-100 to a concentration of 0.5%. Debris was pelleted in a microcentrifuge at 12,000 $\times g$ for 5 min, and the supernatant was layered onto 15 to 30% sucrose gradients and subjected to centrifugation for 35 min at 50,000 rpm and 4°C in a Sorvall AH650 rotor.

Analysis of particles released from liposomes was carried out by first separating particles from liposomes in Ficoll gradient flotations. The bottom two fractions of such flotation gradients, containing released particles, were then diluted 10 fold, layered onto 15 to 45% sucrose gradients, and subjected to centrifugation for 3.5 h at 25,000 rpm and 4°C in a Sorvall AH629 rotor.

All gradients were fractionated from the bottom, and the presence of radiolabeled virus particles was detected by liquid scintillation counting. Gradient profiles are displayed with values as percentages of the total counts per minute across the gradient, to simplify the comparison of samples with various signal strengths.

The nature and complexity of these experiments do not facilitate the direct comparison of data from separate experiments; however, all data shown are representative of the trends observed in experiments that have been repeated at least two or three times.

RESULTS

Formation of receptor-decorated liposomes. Liposomes were made from a mixture of lipids including variable amounts of lipids containing NTA-Ni-linked head groups. These nickel-charged head groups bound to the six-histidine tag on the C-terminal (membrane-proximal) end of the PVR ectodomain. Attachment of sPVRHis to liposomes was confirmed by the colocalization of the protein with the liposome fraction by lipid flotation assays. Rhodamine-labeled lipids were included so that liposomes could be identified visually as a pink band in flotation assays. The position of PVR in the same flotation assays was determined by SDS-PAGE analysis of aliquots of

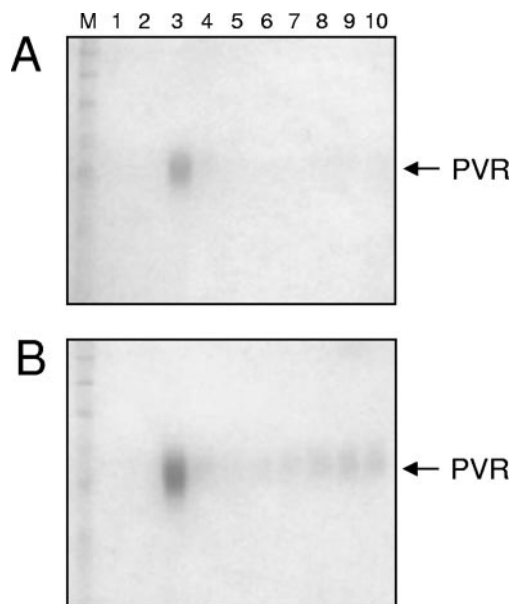


FIG. 1. Formation of receptor-decorated liposomes. Coomassie-stained SDS-PAGE of liposome flotation gradient fractions. Lanes 1 to 10 represent fractions, from top to bottom, of flotation gradients in which liposomes banded at fraction 3. M, molecular weight markers. (A) Flotation after binding of 4 μg of PVR onto 100 μg of liposomes. (B) Flotation after mixing 20 μg of PVR with 100 μg of liposomes: some unbound PVR does not float and remains in fractions 8 to 10, illustrating saturation of binding under these conditions. Images were captured with a gel documentation system (Bio-Rad).

the gradient fractions (Fig. 1A). When exposed to increasing quantities of sPVRHis, the binding capacity of NTA-liposomes became saturated (Fig. 1B), presumably when all available NTA-Ni sites were occupied. In all further experiments, receptor-decorated liposomes were prepared with excess receptor and were separated by flotation from unbound receptor prior to their use in virus binding experiments. Liposomes lacking NTA-Ni-linked head groups had no significant capacity for binding sPVRHis (data not shown).

Receptor-mediated binding of virus to liposomes. Radiolabeled poliovirus was mixed with liposomes for 5 min at room temperature, and virus binding was measured as the extent of colocalization of radioactivity and liposomes by lipid flotation assays. Receptor-decorated liposomes bound the majority of nonsaturating amounts of virus, while nondecorated liposomes supported only minimal binding (Fig. 2). Nondecorated liposomes made with or without NTA-Ni head groups displayed essentially the same low levels of virus binding (data not shown). Low levels of receptor-independent binding may be due to nonspecific membrane interactions or the process of virus breathing, in which hydrophobic residues capable of membrane binding are transiently exposed at the surface of the virus particle (21). Binding of unlabeled virus to receptor-decorated liposomes was also confirmed by the detection of viral capsid proteins in flotation gradient fractions by SDS-PAGE analysis and Coomassie blue staining (data not shown).

Receptor-decorated liposomes induce the temperature-dependent conversion of virus to cell entry-intermediate particles. To determine the functionality of receptor-decorated

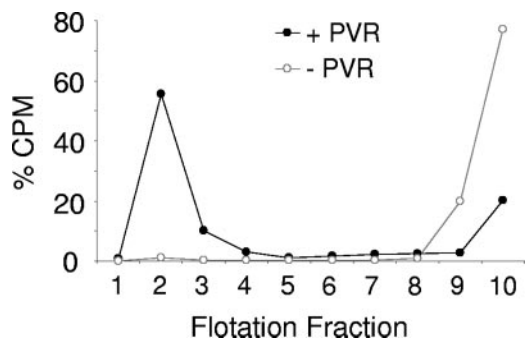


FIG. 2. Receptor-mediated binding of virus to liposomes. Location of radiolabeled virus in liposome flotation gradients, from top (fraction 1) to bottom (fraction 10), after incubation at room temperature for 5 min with either receptor-decorated (closed circles) or control (open circles) liposomes. Liposomes banded at fraction 2.

liposomes, we investigated their ability to induce conformational changes in virus particles analogous to those seen during infection of the cell. Liposomes were incubated with nonsaturating amounts of radiolabeled virus for 5 min. The resulting particles were extracted by solubilizing the lipid with detergent and separated by sedimentation through sucrose density gradients (Fig. 3). Exposure of native virus (160S) to receptor-decorated liposomes at 37°C resulted in conversion to type A particles (135S) with some 80S particles (Fig. 3B), whereas at room temperature the majority of virus remained as 160S (Fig. 3A). After exposure at either temperature to control liposomes lacking PVR, the majority of the virus was also unchanged from 160S.

The resolution of sucrose density gradients was reduced when separating particles recovered from liposomes by detergent extraction. Although some peaks appeared broader than normal, the positions of peaks on these gradients remained as expected. Furthermore, the exposure of 160S, 135S, or 80S particles in control experiments to nondecorated liposomes and subsequent extraction with detergent did not alter their sedimentation characteristics (data not shown).

The temperature dependence of virus conversion was further investigated by incubating liposomes for 32 min with nonsaturating amounts of radiolabeled virus at room temperature, 31°C, 33°C, 35°C, 37°C, or 39°C (Fig. 4). Efficient conversion to particles with altered sedimentation was seen only at 37°C and 39°C.

To determine the rate of virus conversion in this system, time course experiments were carried out in which virus (at nonsaturating concentrations) was bound to receptor-decorated liposomes and incubated at 37°C for 0.5, 2, 8, and 32 min (Fig. 5). By the first time point at 0.5 min, all 160S virus had converted to 135S particles. After 2 min, a proportion of these 135S particles had undergone further conversion to 80S particles. Beyond 2 min, no significant change was observed in this distribution of converted particles.

These experiments demonstrate that sPVRHis retains the functionality of soluble receptor when immobilized on liposomes and that these liposomes are capable of inducing the rapid transition of virus to converted particles in a receptor- and temperature-dependent manner.

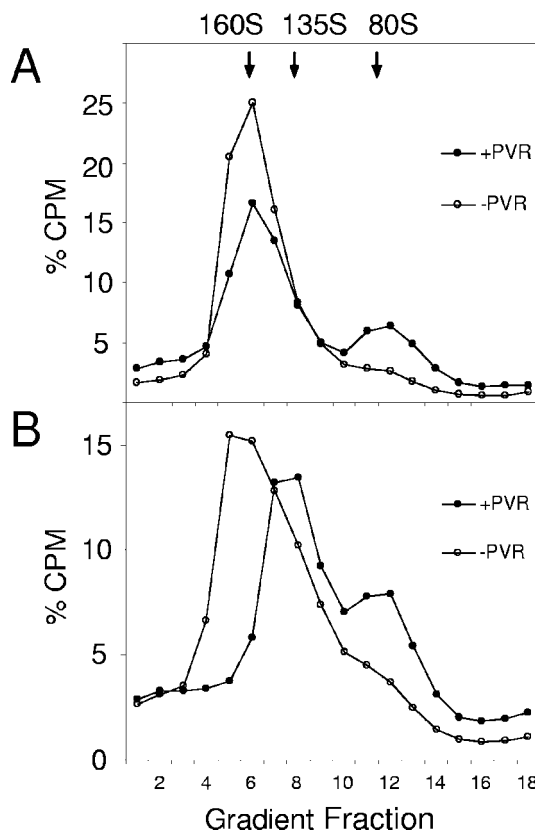


FIG. 3. Receptor-decorated liposomes induce conversion of virus to cell entry-intermediate particles at physiological temperature. Sucrose density gradient analysis of radiolabeled poliovirus after incubation with receptor-decorated (closed circles) or control (open circles) liposomes for 5 min at room temperature (A) or 37°C (B). Arrows indicate the positions of 160S, 135S, and 80S particles after sedimentation through parallel gradients.

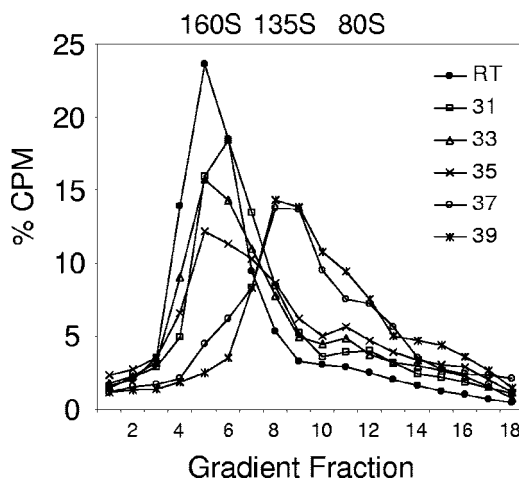


FIG. 4. Temperature dependence of virus conversion by receptor-decorated liposomes. Sucrose density gradient analysis of radiolabeled poliovirus after incubation for 32 min with receptor-decorated liposomes at room temperature (closed circles) and 31°C (open squares), 33°C (open triangles), 35°C (crosses), 37°C (open circles), and 39°C (barred crosses).

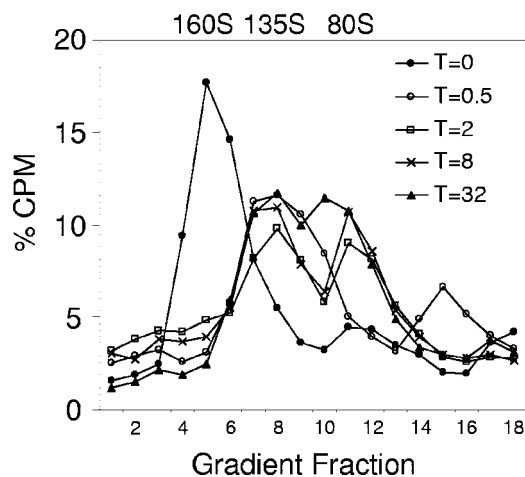


FIG. 5. Time course of conversion of virus bound to receptor-decorated liposomes. Sucrose density gradient analysis of radiolabeled virus particles before incubation ($T = 0$; closed circles) and after 0.5 (open circles), 2 (squares), 8 (crosses), and 32 (triangles) min of incubation at 37°C . Rapid temperature elevation was achieved by a sample dilution of 1:20 into prewarmed buffer; reactions were stopped by freezing in liquid nitrogen.

The density of receptors on the liposome surface dictates the efficiency of virus conversion. By changing the proportion of nickel-chelating lipids present in liposomes, the capacity for binding sPVRHis could be altered, enabling the formation of liposomes with a range of receptor densities. There was a linear relationship between the proportion of NTA-lipid (0 to 20%) in liposomes and their capacity for binding receptor (data not shown). Receptor-decorated liposomes, containing between 2 and 20% nickel-chelating lipid, were mixed at room temperature with excess virus. Liposome-receptor-virus complexes were separated from unbound virus in a preparatory flotation step. The purified liposome-receptor-virus complexes were then incubated at 37°C for 40 min, after which particles were extracted with detergent and analyzed by ultracentrifugation through sucrose density gradients. Radioactive counts in gradient fractions corresponding to native or converted particles were summated to display the relative proportion of these particles with increased clarity. Receptor-decorated liposomes containing between 10 and 20% NTA-lipid induced efficient conversion of virus at 37°C (Fig. 6). However, reducing the proportion of NTA-lipid in liposomes to $<10\%$ caused a dramatic reduction in the levels of particle conversion (Fig. 6), indicating that under these conditions sufficient receptor molecules were present on liposomes to allow virus binding but not efficient conversion.

Virus particles become membrane associated in a receptor-independent manner on conversion to 135S particles and are released from liposomes by V8 protease digestion. Previous work had demonstrated that 135S particles eluted from cells have the ability to bind to liposomes by membrane insertion of the externalized hydrophobic N terminus of VP1 (12). We hypothesized that receptor-mediated conformational changes induced in close proximity to the surface of receptor-decorated liposomes would result in the N terminus of VP1 becoming inserted directly into the membrane in a way that mimics the infection process at the cell membrane. To test this, we inves-

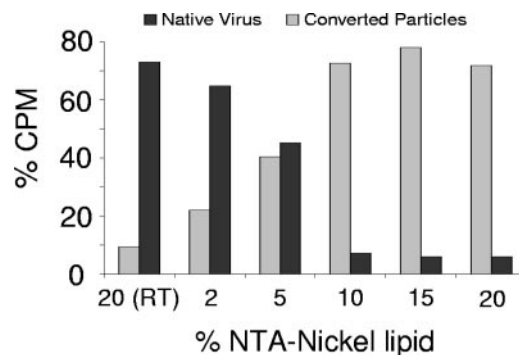


FIG. 6. Receptor density controls efficiency of virus conversion. The graph shows the relative proportions of native virus and converted particles after incubation with receptor-decorated liposomes for 40 min at 37°C . The density of receptor on liposomes was varied by altering the percentage of NTA-lipid during preparation of liposomes as indicated. Values represent the sum of counts assigned to peaks in sucrose density gradient profiles representing native or converted particles. RT, room temperature.

tigated the ability of converted particles to remain stably associated with the liposome membrane after the removal of receptor molecules from the liposome.

The interaction between sPVRHis and NTA-liposomes could be disrupted with imidazole, allowing liposomes to be separated from receptor by flotation in Ficoll gradients. The removal of receptor from liposomes by this method was confirmed by SDS-PAGE of flotation gradient fractions (data not shown). By this procedure, both receptor and receptor-bound virus could be removed from liposomes, facilitating analysis of the remaining proportion of virus particles that were interacting directly with the membrane. By carrying out this procedure before and after virus conversion, we were able to investigate the effect of particle conversion on virus-membrane interactions.

Purified virus-receptor-liposome complexes were incubated at 37°C for 40 min to induce conversion or maintained at room temperature. Liposome-receptor interactions were disrupted by adjusting samples to 1.5 M imidazole, and membrane-associated particles were then separated from unbound material by flotation assay. The concentration of imidazole used in these experiments was higher than is normally required to disrupt nickel-histidine affinity, due to the need to disrupt multiple tethers to release virions; however, previous studies have shown that binding of 135S particles to liposomes is not disrupted by high salt concentrations (12).

In samples maintained at room temperature, the attachment of virus to liposomes was almost entirely dependent on receptor binding; release of virus from liposomes was concomitant with removal of receptor by imidazole treatment (Fig. 7A, panels 1 and 2). After incubation at 37°C , a proportion of virus spontaneously released from liposomes (Fig. 7A, panel 3), presumably as a result of converted particles being released from the receptor without becoming membrane anchored. In samples treated sequentially with elevated temperature followed by imidazole, further release of virus was observed; however, a significant proportion of particles reproducibly remained liposome associated (Fig. 7A, panel 4), presumably

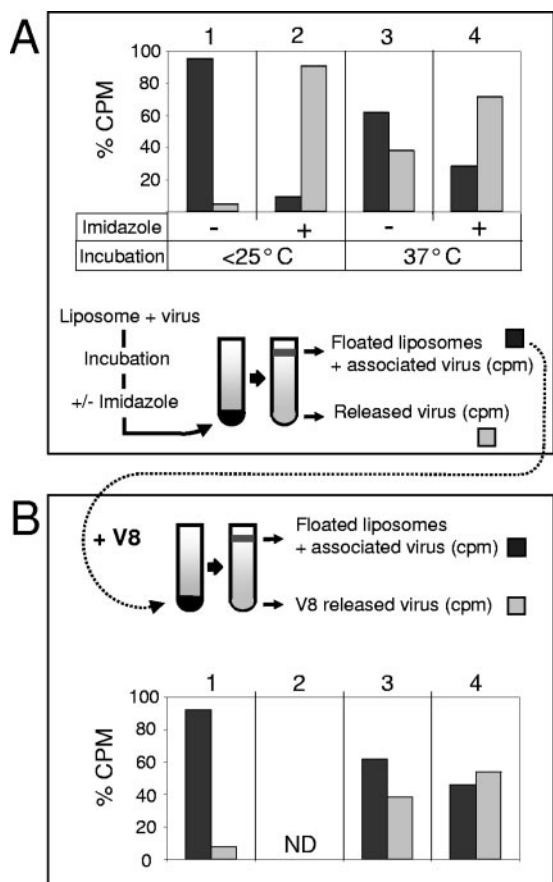


FIG. 7. (A) Converted virus particles remain membrane associated after removal of receptor. The proportion of radiolabeled virus particles (in counts per minute) remaining liposome associated (dark bars) or released (light bars) after temperature-dependent conversion of particles (37°C) and/or removal of receptor by imidazole is shown. Bars represent the sum of counts in top or bottom regions of flotation gradients. Liposome-associated counts (dark bars) in panel 4 represent converted particles anchored to the membrane in the absence of receptor. (B) Converted virus particles are released from liposomes by V8 protease digestion. The proportion of particles (in counts per minute) remaining liposome associated (dark bars) or released (light bars) from liposomes after V8 protease digestion of samples harvested from the flotations shown in panel A is shown. The particles released (light bars) in panels 3 and 4 have been freed from liposomes by protease cleavage of the VP1 membrane anchor. ND, not done, due to the low level of liposome-associated counts in sample 2, as shown in panel A.

due to a membrane interaction mediated by the externalized N terminus of VP1.

To investigate the involvement of the N terminus of VP1 in membrane anchoring, liposomes harvested from the flotation assays described above were exposed to V8 protease, which specifically cleaves VP1 to release the N-terminal 30 residues responsible for tethering the 135S particle to membranes (12). Particles released by the action of V8 protease were separated from those remaining liposome bound by additional flotation assays (Fig. 7B). Few particles were released from liposomes by V8 in samples previously maintained at ambient temperatures (Fig. 7B, panel 1), consistent with native virus particles being attached to liposomes via receptor binding. In samples

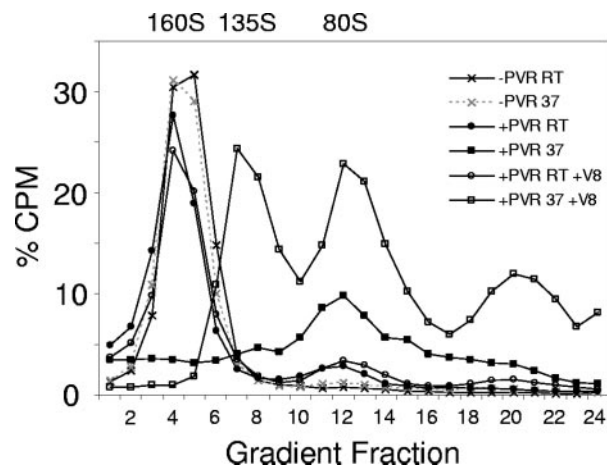


FIG. 8. Virus particles released from liposomes by V8 protease are 135S. Sucrose density gradient profiles showing sedimentation of radiolabeled particles spontaneously released from liposomes at 37°C (closed squares) or released by a combination of incubation at 37°C, followed by V8 protease digestion (open squares), are shown. 160S material in control reactions (remaining profiles, as indicated) represents excess virus not bound to liposomes.

previously incubated at 37°C, a large proportion of the particles that had remained associated with liposomes were now released by protease cleavage (Fig. 7B, panel 3), confirming involvement of the N terminus of VP1 in membrane anchoring of converted particles in this system. Furthermore, in samples that had previously been both incubated at 37°C and treated to remove receptor such that only membrane-anchored particles should remain, the proportion of particles released by V8 was indeed increased (Fig. 7B, panel 4).

To confirm the identity of particles released from liposomes by V8 protease, their sedimentation was measured by sucrose density gradient analysis (Fig. 8). Liposomes were mixed with excess amounts of radiolabeled virus so that multiple rounds of receptor-driven conversion could occur. After incubation with receptor-decorated liposomes at 37°C (or room temperature), samples were cooled to room temperature and incubated either with or without V8 protease. Unbound particles were separated from liposomes in flotation gradients before centrifugation through sucrose gradients. In samples incubated at 37°C and mock digested, only 80S particles were found not associated with liposomes, presumably being particles eluted from the liposome-receptor complex during conversion. However, after both temperature-induced conversion and V8 digestion, both 80S and 135S particles were now observed. This indicated that 135S particles formed during virus conversion become attached to the liposome by a VP1-membrane interaction and are released by V8 digestion. In control reactions at ambient temperature or with undecorated liposomes, only residual excess 160S particles were detected.

VP4 is released from particles and becomes membrane associated after conversion of virus by receptor-decorated liposomes. Previous studies have documented the loss of VP4 from the virus during conversion to the 135S particle (6, 9) and the insertion of VP4 into the membrane during infection (7). We therefore investigated the fate of VP4 after conversion of radiolabeled virus in the liposome model by comparing lipo-

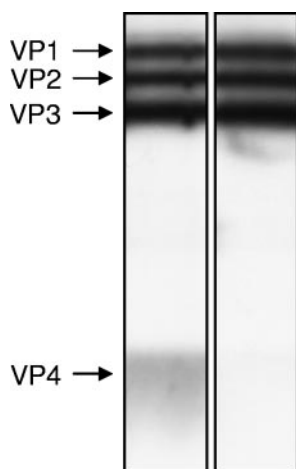


FIG. 9. VP4 is membrane associated after conversion of virus by receptor-decorated liposomes. SDS-PAGE autoradiograph of the liposome fraction (left) and nonfloating fraction (right) of a flotation gradient following incubation of receptor-decorated liposomes with radiolabeled virus at 37°C for 1 h. Autoradiographs were scanned (Epson 4870).

some-bound material with released particles. Liposomes were mixed with excess amounts of radiolabeled virus and incubated at 37°C for 1 h such that multiple rounds of receptor-driven conversion could occur. In SDS-PAGE autoradiographs of flotation gradient fractions, VP4 was clearly visible in the membrane-containing fractions but was not detected in the fractions containing released particles (Fig. 9). As the loading of the remaining capsid proteins VP1, VP2, and VP3 was approximately equal in both fractions, this liposome enrichment confirmed the association of VP4 with the membrane.

DISCUSSION

The development of a simple model membrane system provides an important tool for studying many aspects of nonenveloped virus entry. Here, we have established the methodology for the assembly and purification of a receptor-decorated liposome composed of soluble His-tagged PVR and nickel-chelated NTA-liposomes. The liposome complex was able to bind virus and at physiological temperatures to induce conversion of virus to the 135S entry intermediate, anchored to the membrane via an interaction with the hydrophobic N terminus of VP1. During this conversion, VP4 was also externalized and became membrane associated. These findings are consistent with previous studies of poliovirus entry and with an existing working model of the entry mechanism.

In this system, the level of radiolabeled virus binding to liposomes at ambient temperature was measured by flotation assays in which the majority of nonsaturating levels of virus were shown to comigrate with floated receptor-decorated liposomes. Virus binding was shown to be mediated specifically by the presence of the PVR ectodomain on the surface of liposomes. At temperatures close to physiological conditions, receptor-decorated liposomes induced the conversion of virus to 135S and 80S entry-intermediate particles. Particles were identified by sedimentation through sucrose density gradients after the liposomes were solubilized with detergent.

The minimum temperature required for efficient conversion was close to physiological conditions (between 35°C and 37°C), similar to those reported previously for conversion of virus with soluble receptor (13). Receptor-decorated liposomes induced a rapid rate of virus conversion at 37°C, with efficient conversion to 135S particles observed after 30 s, followed by further conversion of a proportion of these particles to 80S after 2 min. This population of particles appeared relatively stable, longer incubations or elevated temperature (39°C) caused no further conversion to 80S. Both the rate of conversion to 135S and the percentage of 135S particles that remains associated with the membrane are greater with the receptor-decorated liposomes than with receptor-expressing cells. The rate of conversion using the liposome model system also supersedes that for conversion with excess soluble receptor (13). This may indicate that the levels of receptor are superoptimal in the liposome system. Alternatively, it may be attributed to the increased accessibility of the membrane surface on the liposomes, which lack an extracellular matrix.

Although the trigger for conversion to the 80S particle remains unclear, the 135S particles appeared to be stabilized in this system by the presence of serum or purified BSA. In the absence of these components, virus conversion was able to proceed directly to the 80S particle, independent of both temperature and receptor (data not shown), perhaps triggered by a membrane interaction resulting from transient externalization of VP1. Previous studies have documented the ability of BSA (or fatty acid components present in preparations of BSA) or myristate to stabilize picornavirus particles (11, 33, 34). In the current study, BSA may also have stabilized the virus, leading to reduced virus breathing and therefore reduced nonreceptor-mediated membrane interaction.

The working model for poliovirus infection predicts that the fivefold axis of virus particles is the site of membrane interaction, allowing for multiple copies of membrane-anchored PVR to attach to receptor binding sites around the fivefold axis. We investigated the potential requirement for multiple receptor interactions by reducing the proportion of nickel-chelating lipids in the total lipid composition, thus limiting the number of receptor molecules per liposome. At high receptor densities, conversion of virus was efficient, suggesting that the required number of receptor binding sites on the virus had been saturated. When the receptor density was lowered, virus remained able to bind to liposomes, but conversion now appeared to be much less efficient, suggesting that multiple receptor interactions may indeed be required *in vivo*.

Receptor molecules could be removed from liposomes by disrupting the nickel-histidine interaction with imidazole and floating liposomes away from released receptor molecules. At ambient temperatures, liposome-bound virus was also released during this process, indicating that virus-liposome interactions were receptor mediated. After conversion at physiological temperature, a proportion of particles remained membrane associated, independent of the receptor. A proportion of these converted particles were released by the action of V8 protease, which specifically cleaves the externalized N terminus of VP1 (12), thus confirming the VP1-mediated membrane interaction of converted particles. Incomplete release of particles by V8 may indicate that VP1 was partially protected from proteolytic cleavage by virtue of being inserted in the membrane. Al-

though approximately 50% of converted particles could be released from liposomes by protease, the level of VP1 cleavage of remaining liposome-associated particles was much higher than this when analyzed by SDS-PAGE (data not shown), suggesting that VP1 was externalized and available for cleavage at each fivefold axis of the virus, except for those partially membrane-protected molecules of VP1 at the membrane-proximal axis.

After receptor-driven conversion, VP4 was shown to be absent from released particles and to accumulate in the liposome membrane, in agreement with previous studies showing loss of VP4 from the virus (6, 9) and the insertion of VP4 into the membrane during infection of the cell (7).

The externalization of VP4 and the N terminus of VP1 is triggered by receptor binding. It is clear that these components interact with the membrane and that VP4 is also involved in both the formation of channels with electrical conductance (30, 31) and delivery of RNA into the cytoplasm (7). The formation of size-selective membrane pores by a minor group rhinovirus has been elegantly demonstrated in experiments using endosomes isolated from virus-infected cells (3, 26). A recent study also provided the first visualization of the exit of RNA from a minor group human rhinovirus, in which release of RNA is triggered by exposure to low pH (20). Although this work clearly indicates that RNA exits the particle from a single point, presumably a fivefold axis, little is known about the mechanism which allows RNA to exit in such a coordinated manner. For viruses such as poliovirus, which can infect cells independently of endosomal acidification (25), the trigger for genome release remains unknown.

The NTA-liposome system developed in this study provides a controlled environment in which the biochemical characteristics of entry can be explored, with the flexibility to study the effects of further factors such as lipid composition, membrane curvature, membrane potential, and the inclusion of cellular components. The topology of the receptor-decorated liposome membrane is similar to that encountered by virus particles at the cell surface. With simple modifications, the system could be adapted to trap the virus-receptor complex on the inside surface of the liposome, mimicking the situation where the virus has been taken up in an endocytic vesicle. This strategy would also greatly facilitate exploration of conditions that could result in the release of the viral RNA and its translocation across the membrane.

In addition to biochemical assays, this model membrane system can be used for structural studies to explore the mechanism of nonenveloped virus cell entry. Recently, it has been used to produce the first visualization of the native poliovirus-receptor-membrane complex by cryo-EM (5). In this structure, the binding of five receptors orients the viral fivefold axis towards the membrane and causes a deformation of the lipid bilayer. Further analysis using cryo-EM of entry intermediates imbedded within the membrane could lead to visualization of a pore, with the ultimate goal of catching the virus in the process of RNA translocation.

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