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Morphological Changes in the T=3 Capsid of Flock House Virus during Cell Entry†

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We report the identification and characterization of a viral intermediate formed during infection of *Drosophila* cells with the nodavirus Flock House virus (FHV). We observed that even at a very low multiplicity of infection, only 70% of the input virus stayed attached to or entered the cells, while the remaining 30% of the virus eluted from cells after initial binding. The eluted FHV particles did not rebind to *Drosophila* cells and, thus, could no longer initiate infection by the receptor-mediated entry pathway. FHV virus-like particles with the same capsid composition as native FHV but containing cellular RNA also exhibited formation of eluted particles when incubated with the cells. A maturation cleavage-defective mutant of FHV, however, did not. Compared to naïve FHV particles, i.e., particles that had never been incubated with cells, eluted particles showed an acid-sensitive phenotype and morphological alterations. Furthermore, eluted particles had lost a fraction of the internally located capsid protein gamma. Based on these results, we hypothesize that FHV eluted particles represent an infection intermediate analogous to eluted particles observed for members of the family *Picornaviridae*.

Nonenveloped viruses are stable protein complexes designed to protect and transport the viral genome from cell to cell. During assembly and disassembly, these complexes undergo transitions through meta-stable intermediates. Metastable assembly intermediates known as provirions have previously been identified and characterized for several viruses (9, 15, 19), but less is known about the intermediates formed during viral cell entry. Members of the family *Picornaviridae*, including poliovirus, human rhinovirus, and coxsackievirus B3, are known to form "eluted particle" intermediates during cell entry (3, 8, 11, 14, 22, 27–29). Eluted particles are virions that, after initial binding to their cognate receptor, have dissociated from the receptor in an altered form. They have lost the internal capsid protein VP4 and sediment at a decreased rate on sucrose gradients. They also display altered antigenic properties, show increased protease susceptibility, and, most importantly, are no longer able to reattach to their receptor (11, 13, 14, 17, 27).

Here we report that Flock House virus (FHV), a member of the family *Nodaviridae*, also forms eluted particles during the initial stages of viral infection. FHV is a nonenveloped icosahedral insect virus with a bipartite positive-strand RNA genome (for a review, see references 2 and 37). Its life cycle is confined to the cytoplasm of infected cells. FHV has a well-characterized T=3 capsid that is initially assembled from multiple subunits of the single structural precursor protein alpha (16, 19). Following assembly, alpha protein undergoes a maturation cleavage, which gives rise to the major coat protein beta and a small peptide, gamma, which remains associated with mature virions and is located inside the virus particle near the packaged RNA (16, 19). Previous results have shown that the C-terminal portion of gamma peptide is involved in specific packaging of the FHV genome during assembly of virus particles (36). The N-terminal portion, on the other hand, was shown to disrupt artificial membranes in in vitro experiments (4, 5, 21). Based on these results, it has been suggested that gamma peptide functions as a membrane disrupting agent during viral entry, allowing release of the viral RNA into the cytoplasm. This latter aspect is analogous to the function associated with the VP4 peptide of picornaviruses (17, 38, 39).

In this study we have investigated early cell entry events associated with FHV infection of *Drosophila* cells. We observed that nearly all input virus bound to cells at a low temperature, whereas a significant portion could be recovered in the medium after the cells were warmed to physiological temperature. The particles recovered from the medium had undergone morphological changes and were no longer able to rebind to the cells. Furthermore, they had acquired sensitivity to low pH and had lost a portion of the gamma peptide. These results indicate that nodavirus particles form infection intermediates whose properties are analogous in many aspects to eluted particles, an infection intermediate observed for members of the family *Picornaviridae*. Our observations are particularly intriguing, given that nodavirus capsids contain only one type of coat protein subunit whereas picornavirus capsids are formed by three different types of subunits.

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MATERIALS AND METHODS

Cells. *Drosophila melanogaster* cells (Schneider's line 1) were propagated in Schneider's insect medium supplemented with 15% heat-inactivated fetal bovine serum and antibiotics as described previously (18). *Spodoptera frugiperda* cells

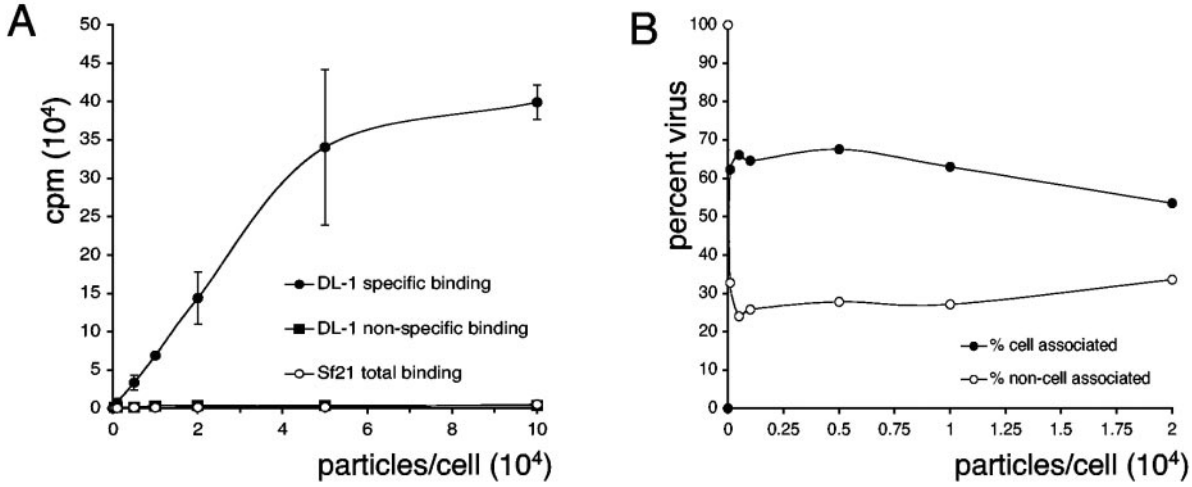

FIG. 1. FHV binding assay. (A) Increasing amounts of gradient-purified [^{35}S]methionine-labeled FHV particles were added to a suspension of *Drosophila* cells and incubated for 1 h at room temperature. Cells were pelleted and washed, and cell-associated radioactivity was measured by liquid scintillation counting. Radioactivity due to nonspecific binding was subtracted after the assay was repeated in the presence of a 100-fold excess of unlabeled FHV. Nonspecific binding was on the same order as binding to the nonpermissive Sf21 cell line. Each data point represents an average of 15 independent experiments. Error bars represent standard deviations from the means. (B) Cell-associated radioactivity and non-cell-associated radioactivity for each particle-to-cell ratio were plotted as a percentage of total radioactivity added to the sample. Even at low particle-to-cell ratios, a constant fraction of radioactivity was recovered in the medium after a 1-h incubation with *Drosophila* cells at room temperature.

(line IPLB-Sf21) were propagated in TC100 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics as described previously (35).

In vitro transcription of RNA2. Plasmid p2BS(+)-wt was linearized with XbaI and used as a template for in vitro transcription of capped RNAs as described previously (36).

Source of FHV RNA1. In vitro synthesized, capped transcripts of RNA1 were first amplified in *Drosophila* cells using liposome-mediated transfection. Total RNA was purified from the cells, and an aliquot containing approximately 100 ng of RNA1 was used as a source for the generation of FHV particles. Details of this procedure have been described previously (36).

Transfection of *Drosophila* cells. *Drosophila* cells were transfected with FHV RNA1 and RNA2 using Cytofectene (Bio-Rad) as a transfection reagent. Details of this procedure are described elsewhere (30).

Purification of virus particles. FHV was purified as previously described (30). Briefly, infected or transfected cells were lysed with NP-40, and cell debris was removed by low-speed centrifugation. Particles were then pelleted through a 30% (wt/wt) sucrose cushion, resuspended in buffer, and sedimented through a 10 to 40% (wt/wt) sucrose gradient. Virus particles were either collected from the gradient tube by needle insertion and aspiration into a syringe or by fractionation on an ISCO gradient fractionator.

Preparation of [^{35}S]methionine- and [^{3}H]uridine-labeled FHV. *Drosophila* cells were infected with gradient-purified FHV at a multiplicity of infection of 30 PFU per cell and incubated at 27°C as described previously (25). At 8 h postinfection, medium was removed from the cells, and the cell monolayer was rinsed once with methionine-free Shields and Sang medium (Sigma). Cells in each 100-mm culture dish were covered with 5 ml of methionine-free medium, followed by the addition of 1 mCi of Easytag (Perkin-Elmer). After incubation for 4 h at 27°C, radiolabeled virus was purified as described above. [^{3}H]uridine-labeled FHV was generated and purified as previously described (30).

Preparation of [^{35}S]methionine-labeled FHV VLPs. Monolayers consisting of 8×10^6 Sf21 cells in 100-mm dishes were infected at a multiplicity of infection of either 5 or 10 PFU per cell by adding recombinant baculovirus AcR2. AcR2 contains the cDNA of FHV RNA2 under control of the polyhedrin promoter and was described previously (40). Following addition of the virus, cells were incubated at room temperature with rocking for 1 h. Unattached virus was then removed and replaced with 7 ml of complete TC100 growth medium. Incubation was continued at 27°C for 24 h without agitation. For ^{35}S-labeled virus-like particles (VLPs), cells were rinsed with 2 ml of methionine-cysteine-deficient Sf-900 II SFM medium (Gibco). Each 100-mm dish was covered with 3 ml of fresh methionine-cysteine-deficient medium and rocked gently for 1 h at room temperature. Medium was then replaced with 2 ml of fresh medium containing 200 μCi of ^{35}S-EasyTag Express protein labeling mix (Perkin-Elmer) per ml. Plates were rocked for an additional 3 h before the addition of 3.5 ml of complete TC100 medium. The plates were incubated at 27°C until 4 days postinfection, when VLPs were isolated as described above.

Virus binding assay. *Drosophila* cells were resuspended in Shields and Sang medium (Sigma) at a density of 10^8 cells/ml. In contrast to Schneider's insect medium, Shields and Sang medium does not contain a pH indicator and therefore did not cause color quenching during liquid scintillation counting of samples generated in the course of this assay. Different amounts of gradient-purified [^{35}S]methionine-labeled FHV were added to the cells, and the suspension was incubated with mild agitation at various temperatures for 1 h. Cells were then pelleted at $3{,}000 \times g$ for 3 min; the supernatant was then transferred to a fresh tube and saved. The cell pellet was resuspended in 0.5 ml of phosphate-buffered saline (PBS), lightly vortexed, and pelleted as indicated above, and the supernatant was saved as before. The wash step was repeated once more, and the final cell pellet was resuspended in TEN lysis buffer (10 mM Tris-HCl, pH 8, 1 mM NaCl, 1 mM EDTA, 1% NP-40), vortexed, and put on ice for 10 min to promote complete cell lysis. The level of radioactivity in the cell lysate, PBS washes, and cell culture medium was then determined by liquid scintillation counting. To test for the ability of eluted particles to rebind to cells, radiolabeled virus recovered in the cell culture medium, i.e., the initial supernatant, was subjected to the same binding assay one more time.

Electron microscopy. A drop of gradient-purified virus, naïve or eluted, was applied to a glow-discharged collodion-covered copper grid (400 mesh) and allowed to adsorb for 2 min. Excess solution was removed with filter paper, and the grids were washed and blotted with filter paper three times by floating them on droplets of 50 mM HEPES, pH 7.0. Each grid was then treated three times with a drop of 2% (wt/vol) uranyl oxalate (Ted Pella, Tustin, CA) filtered through a 2-μm-pore-size filter; the third drop was left on for 2 min before the grid was blotted and air dried. The samples were viewed in a Phillips CM 100 transmission electron microscope at 100 keV.

Electrophoretic analysis of eluted particles. Electrophoresis was performed on discontinuous 16.5% sodium dodecyl sulfate–Tris-Tricine gels (Bio-Rad) according to the procedure of Laemmli. Samples were mixed with an equal volume of 2× electrophoresis buffer (125 mM Tris-HCl [pH 6.8], 4.6% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 20% glycerol) and heated in a boiling water bath for 5 min. Polypeptides were electrophoresed at 200 V for 1 h. Gels were fixed and stained with Coomassie brilliant blue using standard procedures. Band intensities were determined by densitometry using an Alphaimager 2200 densitometer equipped with a documentation and analysis program (Alpha Innotech Corporation).

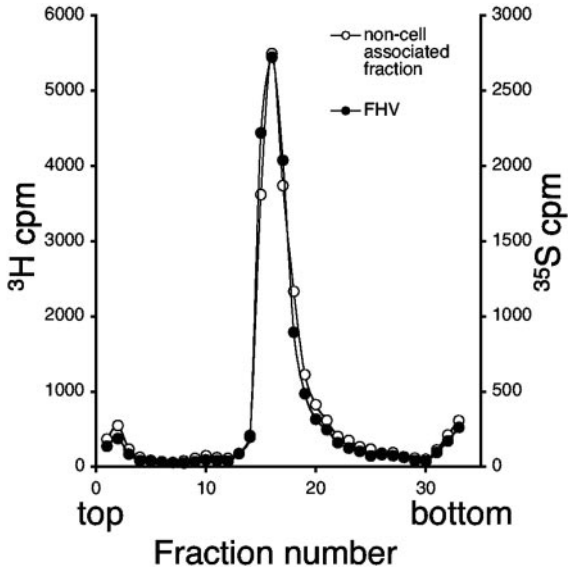

FIG. 2. Sucrose gradient sedimentation profile of naïve and eluted FHV particles. [^{35}S]methionine-labeled non-cell-associated radioactivity and gradient-purified [^{3}H]uridine-labeled FHV, which had not been exposed to cells previously, were combined and cosedimented through a 10 to 40% (wt/wt) sucrose gradient at pH 7. The gradient was fractionated, and radioactivity in each fraction was determined by liquid scintillation.

RESULTS

Binding of FHV to *Drosophila* cells is saturable. Cultured *D. melanogaster* cells (Schneider's line 1, DL-1) are used for routine propagation of FHV in our laboratory. These cells are thought to carry a surface protein that serves as a receptor for FHV entry. To characterize binding of FHV to these cells, increasing numbers of virus particles labeled to high specific activity with [^{35}S]methionine were added to a suspension of DL-1 cells at room temperature (note that DL-1 cells are cultured at 27°C and that infection is typically carried out at room temperature). After a 1-h incubation period, the cells were pelleted, washed, and lysed with NP-40. Radioactivity recovered in the cell lysate, i.e., cell-associated FHV, was determined by liquid scintillation counting. To establish how much of this radioactivity was due to nonspecific binding of virus to cells, the assay was repeated in the presence of a 100-fold excess of nonlabeled FHV. Specific binding was then calculated by subtracting the cell-associated radioactivity observed in the presence of cold virus from that observed in its absence. As shown in Fig. 1A, the amount of cell-associated FHV initially increased linearly with input virus but reached a plateau at approximately 50,000 particles per cell. Beyond this point, cell-associated radioactivity increased only slightly. The saturation of virus binding at this level was indicative of a specific molecule serving as the receptor for FHV. Binding of FHV to nonpermissive *S. frugiperda* cells (line IPLB-Sf21) was also tested. FHV does not infect Sf21 cells (unpublished data), but its genome can replicate in these cells when launched from baculovirus vectors (25). This suggests that the block to infection is during viral entry. As expected, the binding profile of FHV to Sf21 cells was essentially identical to that representing nonspecific binding to DL-1 cells (Fig. 1A).

Only a fraction of input FHV becomes cell associated in the binding assay. Closer inspection of the data obtained in the cell binding assay revealed that radioactivity associated with the cell lysate represented only a fraction of the input value (Fig. 1B). Specifically, on average only 67% of input radioactivity was recovered in the cell lysate even at levels well below saturation of virus binding. The remaining 33% was present primarily in the medium and to a lesser extent in the PBS washes. Incubating cells with virus for an additional hour did not change these results (data not shown). To determine whether radioactivity recovered in the medium represented intact virus or degraded particles, it was sedimented through a 10 to 40% (wt/wt) sucrose gradient. As an internal control, [^{3}H]uridine-labeled FHV was added to the sample. The gradient was fractionated, and radioactivity in each fraction was determined by liquid scintillation counting. As shown in Fig. 2, both the [^{3}H]-labeled FHV marker and the [^{35}S]-labeled material cosedimented through the gradient, indicating that the radioactivity recovered in the medium represented intact virions.

FHV particles recovered in the medium do not bind to fresh DL-1 cells. In order to further investigate why a significant portion of FHV was recovered in the non-cell-associated supernatant, we investigated whether these particles could bind to fresh DL-1 cells. Accordingly, the particles were isolated from the supernatant and subjected to a second binding assay as described above. Interestingly, only 14% of the radioactivity was recovered in the cell-associated fraction, whereas 86% of the total radioactivity was recoverable in the supernatant (Table 1). Thus, only a small fraction of the virus that was present in the medium after the first binding assay was able to bind to cells in a second attempt. The inability of the majority of the particles to bind to cells implied that they were no longer infectious using the normal cell entry pathway.

FHV forms eluted particles. A simple explanation for the results obtained so far was that a given population of FHV particles contains a constant fraction of defective particles that are unable to bind to and enter DL-1 cells. On the other hand, the results were reminiscent of observations made previously for picornaviruses. In particular, studies with poliovirus revealed that most virions bind efficiently to susceptible cells at 4°C but that a certain fraction elutes from the receptor when the sample is brought to physiological temperature. These so-called eluted particles have lost capsid protein VP4 and no

TABLE 1. Effect of temperature on FHV cell binding

Temperature (°C)	% Radioactivity ± SD[a]		
	Cell-associated I	Supernatant I	Cell-associated II[b]
21	67 ± 16	33 ± 16	14 ± 4.5
4	98 ± 2	2 ± 2	NA
4 → 21[c]	64 ± 11	37 ± 11	19 ± 6
4 → 21 → 4[d]	71 ± 7	29 ± 7	11 ± 5

[a] Roman numerals refer to the first (I) and second (II) binding assays.
[b] This column represents the fraction of radioactivity in supernatant I that was cell-associated in a second binding assay. The second binding assay was performed at 21°C for 1 h. NA, not applicable.
[c] Temperature was kept at 4°C for 1 h and then moved to 21°C for 1 h.
[d] Temperature was kept at 4°C for 1 h, moved to 21°C for 1 h, and then returned to 4°C for 1 h.

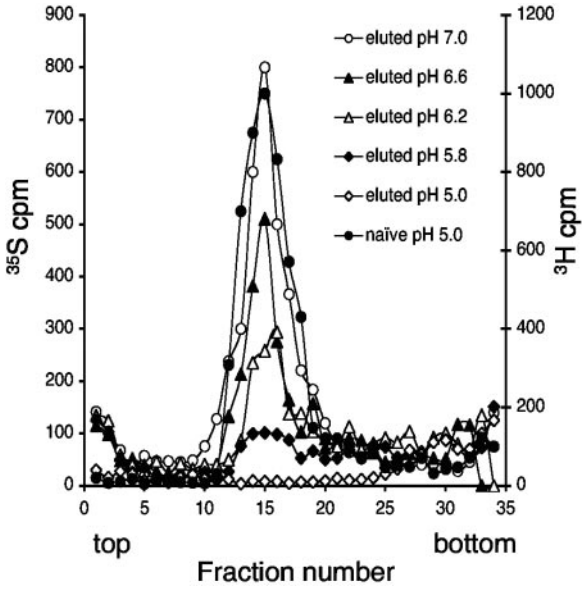

FIG. 3. Sucrose gradient sedimentation profile of naïve and eluted FHV particles after incubation at acidic pH. [^{35}S]methionine-labeled eluted FHV was mixed with [^{3}H]uridine-labeled naïve FHV, and the mixture was incubated for 1 h in buffer at the indicated pH. The mixture was then sedimented through a 10 to 40% (wt/wt) sucrose gradient in the same buffer, the gradient was fractionated, and radioactivity in each fraction was determined by liquid scintillation counting. Concurrent with decreasing pH, an increasing portion of eluted ^{35}S-labeled FHV particles formed aggregates that pelleted to the bottom of the tube during centrifugation.

longer bind the poliovirus receptor (17). To determine whether such behavior could explain the results obtained for FHV, the cell binding assay with radiolabeled particles was repeated at 4°C. As shown in Table 1, at this temperature 98% of the radioactivity was found associated with DL-1 cells after 1 h. In contrast, when an aliquot of the sample was transferred from 4°C to room temperature and incubated for an additional hour, only 67% of the radioactivity remained cell associated. Interestingly, decreasing the temperature back to 4°C did not cause the particles to reassociate with the cells (Table 1). Taken together, these results demonstrated that initially almost all of the particles in a population of purified FHV are competent for binding to DL-1 cells but that a certain fraction disassociates from the cells at room temperature. Since most particles in this fraction were unable to reassociate with the receptor, they likely had undergone a physical alteration. We refer to these particles as eluted particles, whereas FHV particles that have never been incubated with cells will be referred to as naïve FHV particles.

Eluted FHV particles are sensitive to low pH. Compared to naïve poliovirions, eluted poliovirus particles have increased hydrophobicity due to surface exposure of the N terminus of capsid protein VP1, which is normally located in the interior of the particle (17). To determine whether eluted FHV particles had properties that distinguished them from naïve FHV particles, both types of particles were subjected to a range of pH conditions and then sedimented through a sucrose gradient. Specifically, [^{3}H]uridine-labeled naïve FHV and [^{35}S]methionine-labeled eluted FHV particles were mixed and incubated in buffers between pH 7 and pH 5 for 1 h. They were subsequently centrifuged through a 10 to 40% sucrose gradient prepared in the same buffers, the gradient was fractionated, and radioactivity in each fraction was determined by liquid scintillation counting. Naïve FHV particles were stable at all pH values tested, sedimenting to the same position in each case. Eluted FHV particles, on the other hand, cosedimented with naïve FHV particles at pH 7, but radioactivity associated with the peak fractions progressively decreased as the pH value dropped (Fig. 3). Below pH 6.2, eluted particles no longer formed a distinct peak; rather, the radioactivity was recovered as a pellet at the bottom of the centrifuge tube (Table 2). Thus, eluted virions behaved as a homogeneous population of particles that were acid sensitive and aggregated under low pH conditions. This phenotype was evidently a result of prior interaction with DL-1 cells and, more specifically, probably with the cell surface receptor.

Electron microscopy reveals morphological changes in eluted particles. We next investigated whether eluted particles showed morphological differences under the electron microscope compared to naïve particles. Given that eluted particles aggregated under acidic conditions, we chose uranyl oxalate as a negative stain because it has a neutral pH, in contrast to uranyl acetate, which has an acidic pH. Naïve particles were stained and viewed under identical conditions. As shown in Fig. 4A, naïve FHV particles appeared as a homogeneous collection of virions impenetrable to stain. In contrast, eluted particles exhibited a distinct stain-permeable phenotype, but the particles were intact overall and had the same diameter (35 nm) as naïve virions. Interestingly, the stain did not uniformly fill the interior of the virus particle. Rather, it appeared somewhat angular and off center (Fig. 4B). The ratio of absorbance at 260 nm and 280 nm of eluted FHV particles and naïve FHV particles was indistinguishable (1.65), indicating that eluted particles retained their genome. This was also suggested by the fact that both types of particles cosedimented on a sucrose gradient.

Formation of eluted particles requires mature FHV coat protein but not the viral RNA genome. In order to explore which components of the FHV virion were required for formation of eluted particles, we took advantage of FHV variants that either differed in the nature of the coat protein or in the composition of the encapsidated RNA. Specifically, one type of particle was a mutant of FHV, D75N, in which the maturation cleavage of precursor protein alpha to beta and gamma is inhibited (41). When [^{35}S]methionine-labeled D75N particles

TABLE 2. Phenotype of native and variant FHV particles in cell-binding assay and acid sensitivity test

Particle	Percentage of particles in cell supernatant after incubation at:		Reattachment of particles in supernatant	Percentage of particles in supernatant aggregating at low pH	Eluted particle phenotype
	4°C	21°C			
FHV	2	33	No	95	Yes
VLP	2	33	No	98	Yes
D75N	50	50	Yes	15	No

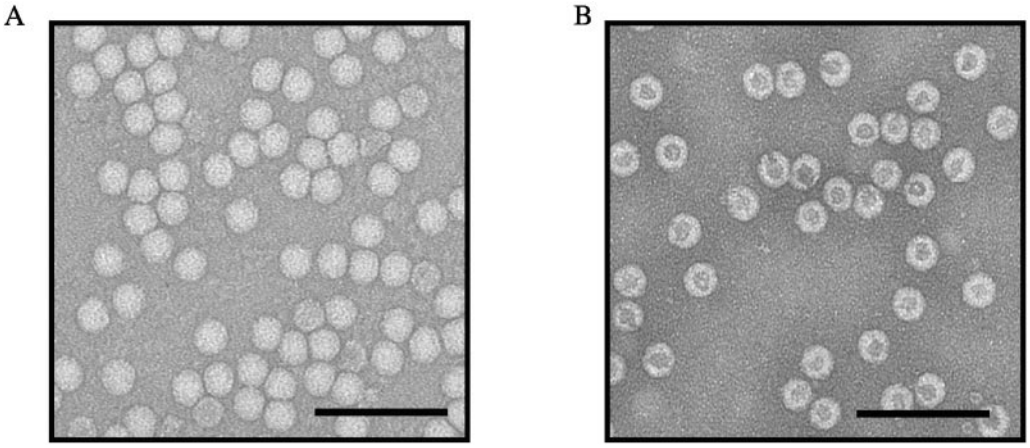

FIG. 4. Electron micrographs of naïve FHV particles (A) and eluted FHV particles (B). Scale bar = 150 nm.

were incubated with DL-1 cells for 1 h at 4°C, only half of the particles were recovered in association with the cells (Table 2). This fraction did not change when incubation was continued for an additional hour at room temperature. Interestingly, the unattached virus was not acid sensitive (data not shown) and reattached to fresh DL-1 cells with the same 50% efficiency. These results suggested that formation of eluted particles requires the presence of mature coat proteins beta and gamma.

We also evaluated the role of the packaged RNA genome in formation of eluted particles. To this end, we used VLPs of FHV, which are generated in a baculovirus expression system (35). Expression of FHV coat protein alpha in Sf21 cells from a recombinant baculovirus vector results in spontaneous assembly of particles, followed by normal maturation cleavage. The structure of the particles is indistinguishable from that of native virions as shown by X-ray crystallography (unpublished data). However, in contrast to native FHV particles, which contain one copy of viral RNA1 and RNA2, VLPs contain a random mixture of cellular RNAs (35). When DL-1 cells were incubated for 1 h with [^{35}S]methionine-labeled FHV VLPs at 4°C and then pelleted, only 2% of the radioactivity was recoverable in the supernatant (Table 2). These results indicated that FHV VLPs bound as efficiently as native virions to DL-1 cells. When the cell-associated VLPs were moved to room temperature and incubated for an additional hour, one-third of the VLPs eluted and were recovered in the supernatant (Table 2). VLPs in the supernatant were then dialyzed to pH 5.0 and centrifuged through a 10 to 40% sucrose gradient. The resulting sedimentation profile showed that the particles exhibited the same pH sensitivity as eluted particles generated from native FHV (data not shown). These results indicated that the FHV genomic RNA does not play a specific role in the transition from naïve particles to eluted particles.

Eluted particles have lost a fraction of gamma protein. The phenotypic changes observed for eluted particles, such as inability to rebind to cells, permeability to negative stain, and sensitivity to acidic pH, were evidently a consequence of changes in the protein component of FHV. In picornaviruses, eluted particles were shown to have lost the internal VP4 peptide, which, like the FHV gamma peptide, is the result of a maturation cleavage of precursor protein VP0 to VP2 and VP4 (17). Based on this analogy, we examined whether the capsid composition of eluted FHV particles differed from that of naïve particles. To this end, equal amounts of the two types of particles were electrophoresed through a Tris-Tricine polyacrylamide gel, followed by staining with Coomassie brilliant blue. Casual examination of the stained gel revealed the presence of capsid proteins beta and gamma in both samples, but the amount of gamma protein in eluted particles appeared to be reduced (Fig. 5). To confirm this, the gel was subjected to densitometry analysis, which verified that there was substantially less gamma protein present in eluted FHV than in naïve FHV. Specifically, we found that the ratio of beta to gamma was significantly higher in eluted particles than in naïve particles. The beta-to-gamma ratio was used to correct for possible gel loading differences between the two samples. From this ratio, it was calculated that approximately 25% ± 1% of the gamma protein had been lost from the eluted FHV particles. The homogeneous behavior of the particles when subjected to pH 6.2 and sucrose gradient sedimentation (Fig. 3) suggested that all particles in the population had lost 25% of the gamma peptides and not that the particles formed a mixture in which 25% had lost all gamma peptides and 75% had lost none.

DISCUSSION

FHV cell binding. Viruses generally initiate infection of susceptible cells by first binding to a cell surface receptor. *Drosophila* cells are thought to carry such a receptor for FHV, but a specific attachment protein has yet to be identified. By analyzing FHV binding as a function of the number of virus particles added per cell, we were able to show that binding of FHV to *Drosophila* cells can be saturated. This indicates that FHV binds to a discrete site or sites on the cell surface. The lack of ideality in saturation, i.e., slowed, but continued increase of cell-associated radioactivity after the addition of more than 50,000 particles per cell (Fig. 1A), could have been the result of one or more factors: (i) receptor recycling during the time required for the binding assay (31), (ii) the existence of a subpopulation of receptors or cells that show decreased affinity toward virions (27), (iii) the existence of a subpopulation of virions with altered receptor affinity (26), or (iv) some other,

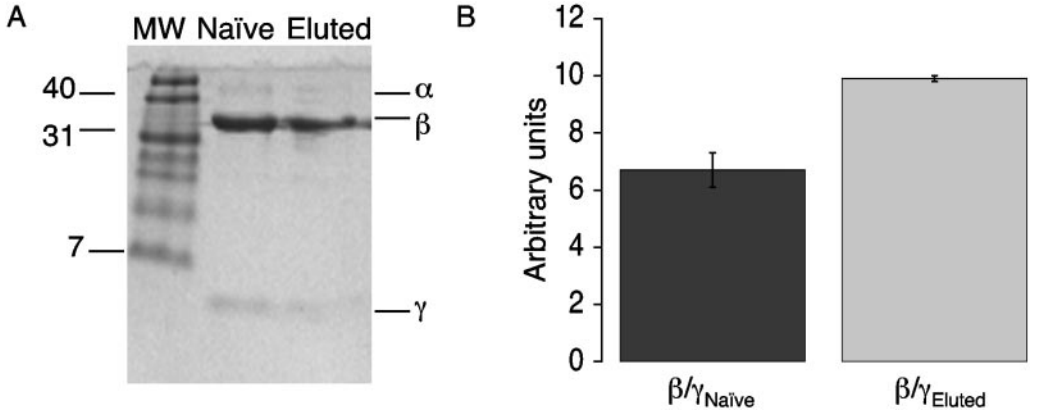

FIG. 5. (A) Electrophoretic analysis of naïve and eluted FHV particles on a 16.5% Tris-Tricine polyacrylamide gel. (B) Bands representing capsid proteins beta and gamma shown in panel A were quantified by densitometry. By computing the beta-to-gamma ratio, differences in sample loading were taken into account. Values shown represent an average of six independent experiments.

nonspecific mechanism for virus uptake such as pinocytosis in addition to the specific receptor binding (10). Despite the lack of ideal saturation, analysis of FHV binding to a nonpermissive cell line, Sf21, was virtually indistinguishable from FHV nonspecific binding to *Drosophila* cells, indicating that the binding profile obtained for specific binding to *Drosophila* cells was in fact the result of attachment to a high-affinity FHV receptor on the cell surface. Binding analysis showed that there were approximately 5×10^4 virus binding sites per cell. This prediction agrees well with the number of receptor binding sites reported for other viruses, for example 10^4 sites for adenovirus (33) and human rhinovirus type 2 (HRV2) on HeLa cells (27). Typically there are 10^3 to 10^5 virus binding sites per cell (23).

FHV eluted particles. Binding assays showed that essentially all naïve FHV particles were able to bind to *Drosophila* cells at 4°C but that only about 70% of these particles remained bound or entered the cells at room temperature. The remaining 30% were detected in the cell supernatant, mostly unable to reattach to cells and therefore no longer infectious through a receptor-mediated pathway. We refer to these particles as eluted particles. Abortive elution, or sloughing, was first discovered for poliovirus (14, 22) and later for HRV14, HRV2, and coxsackie B3 (1, 27–29). For poliovirus type 1, 27% of the virions that attach to cells in the cold elute upon subsequent incubation at physiological temperature for 1 h (14, 22). For rhinovirus, up to 40% of cell-attached virus was reported to elute from HeLa cells within 1 h (27, 32). As observed for FHV, eluted picornavirus particles do not rebind to their cognate receptor and are no longer competent to infect susceptible cells by this pathway (11, 14, 22, 24, 27–29). However, low infectivity for poliovirus eluted particles has been detected and ascribed to direct interaction of the particles with the cellular membrane (12). We did not detect receptor-independent infectivity of FHV eluted particles in *Drosophila* cells.

Eluted picornavirus particles are known to display altered antigenic properties and increased susceptibility to proteases (11, 13, 14, 17, 27). We have not yet performed a systematic analysis of the stability and antigenic properties of eluted FHV particles. Instead, our investigations focused on the behavior of eluted particles at low pH. This was based on unpublished results from our laboratory that FHV requires low pH for cell entry and uncoating. Indeed, we found that eluted particles aggregated at low pH, which is in line with the notion that this condition contributes to uncoating of FHV in vivo.

Using FHV variants that differed in protein or RNA content, we also showed that the formation of eluted particles is a function of the capsid protein, specifically, its ability to undergo the postassembly maturation cleavage. This is based on the observations that under equivalent conditions, the cleavage-defective D75N mutant did not form eluted particles. The possibility that alternate, nonphysiological conditions, e.g., increased temperature, allow this transition to occur remains to be tested.

The nature of the packaged RNA, on the other hand, did not affect the ability of FHV to form eluted particles. This suggests that conformational transitions are achieved independently of specific nucleic acid sequences. However, the packaged RNA per se may play a vital role in this process, given that it represents an important structural component of the virion (16). Unfortunately, FHV does not form empty particles, which would be the ideal reagent to address this issue.

Loss of structural protein from eluted particles. Closer inspection revealed that eluted FHV particles had lost a fraction of gamma protein. Since this was not observed upon incubation of FHV with the nonsusceptible Sf21 cell line, we conclude that formation of eluted particles and loss of gamma peptide are a result of interaction with the FHV receptor. The fraction of gamma lost from the particles represented 25% ± 1%. Based on the T=3 symmetry of FHV, one would expect this number to be closer to 33% or multiples thereof. It is possible that the population of eluted particles was contaminated with naïve virions, which would have resulted in a reduced apparent value for the amount of gamma released. On the other hand, all virions in a given preparation of eluted particles were sensitive to low pH, suggesting that the degree of contamination must have been small. Clearly, this issue requires further investigation.

Loss of a capsid polypeptide was also observed for eluted picornavirus particles. For example, poliovirus eluted particles, also known as 135S particles, lack capsid protein VP4 (6, 17, 22). Similar to FHV gamma peptide, VP4 is generated during maturation cleavage of capsid precursor protein VP0 to VP2

and VP4. However, VP4 is derived from the N terminus of VP0, whereas gamma is derived from the C terminus of precursor protein alpha. Moreover, VP4 is myristylated at its N terminus, whereas gamma peptide is not.

Compared to FHV eluted particles, poliovirus 135S particles also have a different sedimentation coefficient relative to naïve poliovirus particles (160S) (13, 17). For rhinovirus, eluted particles have a sedimentation rate that is 10% lower than that of the naïve virions (27, 29), and a similar observation was made for coxsackievirus B3 (11). In FHV, loss of one-fourth of the gamma peptides from the capsid equates to a loss of only 3% of the total molecular weight of the virion. Such a small difference in the virion capsid apparently did not change its size, shape, or density sufficiently to manifest itself in an altered sedimentation rate on a 10 to 40% velocity gradient.

FHV eluted particles are pH sensitive. In contrast to eluted picornavirus particles, FHV eluted particles exhibited striking sensitivity to pH. At pH 7.0, FHV eluted particles sedimented like wild-type FHV on a velocity gradient, but when the pH was incrementally lowered to pH 5.0, eluted particles aggregated. If eluted FHV particles, like their picornavirus counterparts, represent aborted infection intermediates (20), their acid sensitivity might reflect a response to conditions normally encountered after receptor-mediated endocytosis. Within cells the pH decreases from 7.0 to 5.0 as ligands make their way through the endocytotic pathway and eventually to lysozomes. It is plausible that at low pH eluted particles undergo further structural transitions that eventually lead to uncoating of the viral genome. In support of this notion, preliminary evidence from our laboratory indicates that FHV entry does indeed require low pH (unpublished results).

Significance of eluted particle formation. Eluted particles may be intermediates in the FHV infection pathway that were unable to successfully carry out a productive infection. Events associated with receptor binding and invagination of the cell membrane for receptor-mediated endocytosis may induce a capsid conformation with a substantially decreased receptor binding affinity. This could cause a fraction of the particles to dissociate from the cells as eluted particles. Alternatively, some of the early endosomal compartments recycle back to the cell surface, perhaps sometimes before virus has detached from the receptor or been passed to a late endosome (31). It is well established that low-density lipoprotein dissociates from its receptor at the mildly low pH of early endosomes before recycling of the low-density lipoprotein receptor to the plasma membrane (7). Inability of virions to quickly disassociate from their viral receptors would result in a recycling to the cell surface, possibly as eluted particles.

The reproducible ratio of eluted particles to cell-associated particles has obvious implications for the efficiency of the viral life cycle. For FHV, the particle-to-PFU ratio is 300:1. Previously it was suggested that such high ratios, also observed for some picornaviruses, may be caused by the presence of lethal mutations in the viral genome (34). While this is a possibility, an additional explanation is that not all virus particles successfully complete the steps required for cell entry due to elution and subsequent inability to reattach to the cellular receptor.

The loss of a fraction of gamma protein from eluted particles is intriguing in light of previous results showing that gamma is membrane active in vitro and spontaneously partitions into and permeabilizes liposomes (4, 5, 21). We propose that a particle conformation similar, if not identical, to the eluted particle is formed during receptor-mediated endocytosis of FHV and that low endosomal pH induces further structural transitions in the virion. These additional transitions permit membrane permeabilization by gamma peptides and uncoating of the viral RNA. Formation of the eluted particle intermediate would thus allow FHV to tightly regulate when and how the genome is delivered into the cytosol.

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REFERENCES

1. **Abraham, G., and R. J. Colonno.** 1988. Many rhinovirus serotypes share the same cellular receptor. J. Virol. **51:**340–345.
2. **Ball, L. A., and K. L. Johnson.** 1998. Nodaviruses of insects, p. 225–267. *In* L. K. Miller and L. A. Ball (ed.), The insect viruses. Plenum Press, New York. N.Y.
3. **Belnap, D. M., D. J. Filman, B. L. Trus, N. Cheng, F. P. Booy, J. F. Conway, S. Curry, C. N. Hiremath, S. K. Tsang, A. C. Steven, and J. M. Hogle.** 2000. Molecular tectonic model of virus structural transitions: the putative cell entry states of poliovirus. J. Virol. **74:**1342–1354.
4. **Bong, D. T., A. Janshoff, C. Steinem, and M. R. Ghadiri.** 2000. Membrane partitioning of the cleavage peptide of flock house virus. Biophys. J. **78:**839–845.
5. **Bong, D. T., C. Steinem, A. Janshoff, J. E. Johnson, and M. R. Ghadiri.** 1999. A highly membrane-active peptide in Flock House virus: implications for the mechanism of nodavirus infection. Chem. Biol. **6:**473–481.
6. **Breindl, M.** 1971. VP4, the D-reactive part of poliovirus. Virology **46:**962–964.
7. **Brown, M. S., J. Herz, and J. L. Goldstein.** 1997. LDL-receptor structure. Calcium cages, acid baths and recycling receptors. Nature **388:**629–630.
8. **Bubeck, D., D. J. Filman, N. Cheng, A. C. Steven, J. M. Hogle, and D. M. Belnap.** 2005. The structure of the poliovirus 135S cell entry intermediate at 10-angstrom resolution reveals the location of an externalized polypeptide that binds to membranes. J. Virol. **79:**7745–7755.
9. **Canady, M. A., M. Tihova, T. N. Hanzlik, J. E. Johnson, and M. Yeager.** 2000. Large conformational changes in the maturation of a simple RNA virus, nudaurelia capensis omega virus (NomegaV). J. Mol. Biol. **299:**573–584.
10. **Conner, S. D., and S. L. Schmid.** 2003. Regulated portals of entry into the cell. Nature **422:**37–44.
11. **Crowell, R. L., and L. Philipson.** 1971. Specific alterations of coxsackievirus B3 eluted from HeLa cells. J. Virol. **8:**509–515.
12. **Curry, S., M. Chow, and J. M. Hogle.** 1996. The poliovirus 135S particle is infectious. J. Virol. **70:**7125–7131.
13. **De Sena, J., and B. Mandel.** 1977. Studies on the in vitro uncoating of poliovirus. II. Characteristics of the membrane-modified particle. Virology **78:**554–566.
14. **Fenwick, M. L., and P. D. Cooper.** 1962. Early interactions between poliovirus and ERK cells: some observations on the nature and significance of the rejected particles. Virology **18:**212–223.
15. **Fernandez-Tomas, C. B., and D. Baltimore.** 1973. Morphogenesis of poliovirus. II. Demonstration of a new intermediate, the proviron. J. Virol. **12:**1122–1130.
16. **Fisher, A. J., and J. E. Johnson.** 1993. Ordered duplex RNA controls capsid architecture in an icosahedral animal virus. Nature **361:**176–179.
17. **Fricks, C. E., and J. M. Hogle.** 1990. Cell-induced conformational changes in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. J. Virol. **64:**1934–1945.
18. **Friesen, P. D., and R. R. Rueckert.** 1981. Synthesis of black beetle virus proteins in cultured *Drosophila* cells: differential expression of RNAs 1 and 2. J. Virol. **37:**876–886.
19. **Gallagher, T., and R. R. Rueckert.** 1988. Assembly-dependent maturation cleavage in provirions of a small icosahedral insect ribovirus. J. Virol. **62:**3399–3406.
20. **Huang, Y., J. M. Hogle, and M. Chow.** 2000. Is the 135S particle an intermediate during cell entry? J. Virol. **74:**8757–8761.
21. **Janshoff, A., D. T. Bong, C. Steinem, J. E. Johnson, and M. R. Ghadiri.** 1999. An animal virus derived peptide switches membrane topology: possible relevance to nodaviral transfection process. Biochemistry **38:**5328–5336.
22. **Joklik, W. K., and J. E. Darnell.** 1961. The adsorption and early fate of purified poliovirus in HeLa cells. Virology **13:**439–447.
23. **Knipe, D. M., B. Roizman, P. M. Howley, T. P. Monath, S. E. Straus, R. M. Chanock, J. L. Melnick, and B. N. Fields (ed.).** Fields virology, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, Pa.

24. **Korant, B. D., K. Lonberg-Holm, F. H. Yin, and J. Noble-Harvey.** 1975. Fractionation of biologically active and inactive populations of human rhinovirus type 2. Virology **63:**384–394.
25. **Krishna, N. K., D. Marshall, and A. Schneemann.** 2003. Analysis of RNA packaging in wild-type and mosaic protein capsids of flock house virus using recombinant baculovirus vectors. Virology **305:**10–24.
26. **Kumar, U., R. Sasi, S. Suresh, A. Patel, M. Thangaraju, P. Metrakos, S. C. Patel, and Y. C. Patel.** 1999. Subtype-selective expression of the five somatostatin receptors (hSSTRI-5) in human pancreatic islet cells: a quantitative double-label immunohistochemical analysis. Diabetes **48:**77–85.
27. **Lonberg-Holm, K., and B. D. Korant.** 1972. Early interaction of rhinoviruses with host cells. J. Virol. **9:**29–40.
28. **Lonberg-Holm, K., and J. Noble-Harvey.** 1973. Comparison of in vitro and cell-mediated alteration of a human rhinovirus and its inhibition by sodium dodecyl sulfate. J. Virol. **12:**819–826.
29. **Lonberg-Holm, K., and F. H. Yin.** 1973. Antigenic determinants of infective and inactivated human rhinovirus type 2. J. Virol. **12:**114–123.
30. **Marshall, D., and A. Schneemann.** 2001. Specific packaging of nodaviral RNA2 requires the N terminus of the capsid protein. Virology **285:**165–175.
31. **Maxfield, F. R., and T. E. McGraw.** 2004. Endocytic recycling. Nat. Rev. Mol. Cell Biol. **5:**121–132.
32. **Noble, J., and K. Lonberg-Holm.** 1973. Interactions of components of human rhinovirus type 2 with HeLa cells. Virology **51:**270–278.
33. **Philipson, L.** 1963. The early interaction of animal viruses and cells. Prog. Med. Virol. **71:**43–78.
34. **Racaniello, V. R.** 2001. *Picornaviridae*: the viruses and their replication, p. 685–722. *In* B. N. Fields, D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, Pa.
35. **Schneemann, A., R. Dasgupta, J. E. Johnson, and R. R. Rueckert.** 1993. Use of recombinant baculoviruses in synthesis of morphologically distinct virus-like particles of flock house virus, a nodavirus. J. Virol. **67:**2756–2763.
36. **Schneemann, A., and D. Marshall.** 1998. Specific encapsidation of nodavirus RNA is mediated through the C terminus of capsid precursor protein alpha. J. Virol. **72:**8738–8746.
37. **Schneemann, A., V. Reddy, and J. E. Johnson.** 1998. The structure and function of nodavirus particles: a paradigm for understanding chemical biology, p. 381–446. *In* K. Maramorsch, F. A. Murphy, and A. J. Shatkin (ed.), Advances in virus research, vol. 50. Academic Press, San Diego, Calif.
38. **Tosteson, M. T., and M. Chow.** 1997. Characterization of the ion channels formed by poliovirus in planar lipid membranes. J. Virol. **71:**507–511.
39. **Tosteson, M. T., M. L. Nibert, and B. N. Fields.** 1993. Ion channel induced in lipid bilayers by subvirion particles of the nonenveloped mammalian reoviruses. Proc. Natl. Acad. Sci. USA **90:**10549–10552.
40. **Venter, P. A., N. K. Krishna, and A. Schneemann.** 2005. Capsid protein synthesis from replicating RNA directs specific packaging of the genome of a multipartite, positive-strand RNA virus. J. Virol. **79:**6239–6248.
41. **Zlotnick, A., V. S. Reddy, R. Dasgupta, A. Schneemann, W. J. Ray, R. R. Rueckert, and J. E. Johnson.** 1994. Capsid assembly in a family of animal viruses primes an autoproteolytic maturation that depends on a single aspartic acid residue. J. Biol. Chem. **269:**13680–13684.