DNA Poised for Release in Bacteriophage φ29

Jinghua Tang,1 Norman Olson,1 Paul J. Jardine,2 Shelley Grimes,2 Dwight L. Anderson,2,3,* and Timothy S. Baker1,4,*
1Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093, USA
2Department of Diagnostic and Biological Sciences and Institute for Molecular Virology
3Department of Microbiology
University of Minnesota, Minneapolis, MN 55455, USA
*Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093, USA
*Correspondence: tsb@ucsd.edu (T.S.B.), diander@umn.edu (D.L.A.)
DOI 10.1016/j.str.2008.02.024

SUMMARY

We present here the first asymmetric, three-dimensional reconstruction of a tailed dsDNA virus, the mature bacteriophage φ29, at subnanometer resolution. This structure reveals the rich detail of the asymmetric interactions and conformational dynamics of the φ29 protein and DNA components, and provides novel insight into the mechanics of virus assembly. For example, the dodecameric head-tail connector protein undergoes significant rearrangement upon assembly into the virion. Specific interactions occur between the tightly packed dsDNA and the proteins of the head and tail. Of particular interest and novelty, an ~60Å diameter toroid of dsDNA was observed in the connector-lower collar cavity. The extreme deformation that occurs over a small stretch of DNA is likely a consequence of the high pressure of the packaged genome. This toroid structure may help retain the DNA inside the capsid prior to its injection into the bacterial host.

INTRODUCTION

Double-stranded DNA (dsDNA) bacteriophages are remarkably efficient genome delivery machines. They have long served as model systems for investigating fundamental principles of virus structure and macromolecular assembly (Johnson and Chiu, 2007). Electron cryo-microscopy (cryo-EM) and three-dimensional (3D) image reconstruction provide a powerful means to study the structure, complexity, and dynamics of a wide range of macromolecular complexes (Jiang and Ludtke, 2005). Additionally, these methods complement atomic resolution techniques, such as X-ray crystallography, especially when crystals of an entire functional complex are lacking. When atomic models of components or subassemblies are accessible, they can be fitted into reconstructed density maps to produce informative pseudoatomic models (Mitra and Frank, 2006). Advances in cryo-EM and 3D image processing yielded the first subnanometer structure determinations of highly symmetric (icosahedral) viruses, including hepatitis (Bottcher et al., 1997; Conway et al., 1997) and papilloma (Trus et al., 1997) viruses, providing significant biological insights that could not have been gleaned from lower resolution reconstructions (Baker et al., 1999).

Recently, a number of asymmetric viruses, including the tailed dsDNA bacteriophages T7, φ15, P22, and φ29, have been characterized using single-particle, cryo-reconstruction methods (Agirrezabala et al., 2005; Chang et al., 2006; Jiang et al., 2006; Lander et al., 2006; Xiang et al., 2006). These phages are asymmetric structures because they exhibit symmetry mismatches between head (five-fold) and tail (six-fold) components. Studies of the phased phages yielded structures at ~17Å resolution, which is insufficient to distinguish secondary structural features in proteins and DNA. This contrasts with the subnanometer resolution that is often now achieved with highly symmetric icosahedral particles (e.g., Jääinoja et al., 2007; Morais et al., 2005; Zhang et al., 2005; Zhou et al., 2001). Limitations in particle homogeneity and stability, the need for large numbers of images, and barriers in computational resources and algorithms have collectively hindered attempts to achieve higher resolution with large, asymmetric structures like these phages.

The Bacillus subtilis bacteriophage φ29, a tractable machine of considerable complexity, is well suited for unraveling the mechanisms of viral assembly and infection because of the richness and depth of information on φ29 morphogenesis, the nearly complete inventory of gene products encoded by the 19.3 kbp dsDNA, and the extensive physiological study of gene functions (Anderson and Reilly, 1993; Grimes et al., 2002; Meijer et al., 2001; Peterson et al., 2001). Moreover, determination of the structure of φ29 is well advanced (Morais et al., 2001, 2003, 2005; Simpson et al., 2000; Tao et al., 1998; Xiang et al., 2006), as is study of its dynamic assembly mechanisms using integrated genetic, biochemical, and biophysical approaches (Anderson and Reilly, 1993; Chemla et al., 2005; Grimes et al., 2002; Meijer et al., 2001; Smith et al., 2001). Briefly, the mature, fiberless φ29 virus particle has a prolate head and a small, non-contractile tail, containing 235 and about 80 protein molecules, respectively. The prolate head shell is composed of gene product 8 (gp8) and encapsidates the linear dsDNA genome, which has a copy of the terminal protein, gp3, covalently bound to each 5’ end (Meijer et al., 2001). The head-tail connector, a dodecamer of gp10, is embedded within a unique five-fold vertex of the head (Tao et al., 1998). The connector serves as the site of assembly of the transient DNA packaging machine that translocates the dsDNA genome into a precursor head shell, or prohead. Compacting the DNA to near crystalline density requires a force generating capacity exceeding 100 pN (Rickgauer et al., 2008). After packaging, the ATPase and prohead RNA components of the packaging machine are displaced by assembly of the lower collar/tail tube (gp11), followed in sequence by the
tail knob (gp9) and twelve appendages (gp12*) (Hagen et al., 1976).

In our quest to detail molecular conformational changes that regulate ø29 assembly and the propulsive events during infection and DNA injection, we have employed asymmetric cryo-EM 3D reconstruction to examine the secondary, tertiary, and quaternary structures of all phage components. Concurrently, the fitting of atomic models derived from X-ray crystal structures into these reconstructions continues to provide a complementary approach to vigorously explore the ø29 system (Morais et al., 2003, 2005; Simpson et al., 2000; Xiang et al., 2006). Here we report asymmetric 3D reconstructions of full- and empty-fiberless particles of bacteriophage ø29 at resolutions finer than 10 Å. Details of protein subunit secondary structures, DNA packing and conformation, symmetry mismatches, and the response of DNA to high compression force in the confines of the tail have not been described before. Among the many insights to be gleaned from the new structures, here we focus primarily on the structure of the DNA and demonstrate that a short stretch of it bends sharply into a toroid inside the phage tail.

RESULTS AND DISCUSSION

3D Reconstructions of the Virion and Empty Particle

Low dose images of vitrified samples were used to compute 3D reconstructions of fiberless ø29 virions and empty particles (Figure 1) at resolutions calculated to be 7.8 and 9.3 Å, respectively (see Experimental Procedures). At these resolutions it is possible to assign structural components of the phage, reveal how they interact, and visualize features of secondary structure. In both reconstructions, tubular density features, ascribed to α helices, were resolved in all 235 copies of gp8 in the prolate head and in all 12 copies of gp10 in the connector (see below). The stability and uniformity of the virion and empty particles required to permit reconstruction at this resolution are quite remarkable. The stoichiometrically distinct head, connector, and tail components, despite their asymmetric distribution, must be in nearly identical register in all particles.

Similar analysis of stable, asymmetric structures of the ribosome at subnanometer resolutions by cryo-TEM has greatly expanded our understanding of its functions (Allen and Frank, 2007; Berk and Cate, 2007). As a point of reference, it is noteworthy that the ø29 particles detailed here are more massive by an order of magnitude than the ribosome. Unlike the ribosome, ø29 has fewer unique components, but all (except DNA-gp3) are present in multiple copies as symmetric or quasi-symmetric oligomers whose different stoichiometries lead to symmetry-mismatched interactions believed to impart important functional properties (Hendrix, 1978; Johnson and Chiu, 2007).

The 235 copies of gp8 in the ø29 capsid are arranged in a Q = 5 lattice (Aebi et al., 1974; Figure 1A) similar to that seen in earlier ø29 reconstructions (Morais et al., 2005; Tao et al., 1998; Xiang et al., 2006). The core of each gp8 subunit, as first revealed in symmetrized reconstructions of isomeric variants of the ø29 capsid (Morais et al., 2005), has a structure that resembles the HK97 fold (Wikoff et al., 2000), seemingly common to all tail, dsDNA phage capsids (Baker et al., 2005). The exterior domain of gp8, which is reported to be analogous to the bacterial immunoglobulin-like BIG2 domain (Morais et al., 2005), forms a prominent feature on the exterior surface of the head (Figure 1A). The twelve-fold tail is attached to the head at a five-fold vertex, where the symmetry mismatch between head and tail is accommodated.

The tail is attached to the head via the dodecameric head-tail connector (Figures 1 and 2). The wide end of the connector, with its twelve copies of gp10, is embedded among five gp8 hexamers in the head shell. The lower collar/tail tube, comprised of twelve copies of gp11 (Carrascosa et al., 1983), is attached to the narrow end of the connector. The tube and gp9 knob at the end of the tail display smooth, cylindrical morphologies and no recognizable substructures, perhaps indicating that subunits in these regions are packed tightly and contain little, if any, surface protrusions. Twelve appendages that function in viral adsorption, each shown to be a trimer of gp12* (a gp12 cleavage product) (Xiang et al., 2006), contact the interface between gp10 and gp11 via twelve narrow, connecting arms.

Appendages adopt up or down orientations that correlate with the location of each appendage relative to the five-fold symmetry of the head. A thin, longitudinal cross-section from the virion reconstruction (Figure 2A) captures one appendage in the up (at left side) and another in the down (right side) orientation. A similar pattern of appendage orientations was observed in asymmetric reconstructions of fibered φ29 phage and was suggested to be a consequence of interaction of gp12* with the accessory gp8.5 head fibers (Xiang et al., 2006). However, this is not the case here, since the particles we imaged are a fiberless variant. Instead, our density maps show that the connecting arms of gp12* contact the gp8 head shell, thereby suggesting that gp12* conformation is dictated by symmetry mismatched interactions with the shell. Detailed analyses of gp8-gp12* interactions and those between other symmetry-mismatched subunit pairs such as gp8-gp10, are revealing additional insights about φ29 and are part of ongoing studies (authors’ unpublished data).

Conformation of the Connector In Situ Differs from that in Crystals

Density corresponding to the gp10 dodecamer in the virion (Figure 3A) and empty particle (data not shown) reconstructions display a large number of tube-like features that are aligned at about a 30° angle to the long axis of the particles. These tubes of density correspond to the φ helices of the connector revealed by high-resolution X-ray crystallography (Guasch et al., 2002; Simpson et al., 2000; Figure 3B), not previously seen in situ. However, conspicuous differences between φ29 connectors in crystals and connectors in virions, as well as empty particles, reveal that the connector is remodeled by its environment in the phage.

As observed in the crystals of the dodecamer (Guasch et al., 2002; Simpson et al., 2000), each gp10 subunit consists of three primary domains (Figure 3B): an αφ-rich top domain at the wide end of the connector, an αz-rich central domain, and an αβ bottom domain that resembles a paper clip. The atomic model of the entire gp10 dodecamer, treated as one rigid body, clearly did not fit into the cryo-EM density maps (correlation coefficient = 0.55). The top and central domains of all gp10 subunits, when tilted by about 10° toward the connector axis (Figure 3C), did fit the density nicely (Figure 3A), as evidenced by a correlation coefficient of 0.70. These fits were aided by the presence and
arrangement of several prominent helices. Conversely, the bottom domain could not be fit satisfactorily as a rigid body into the remaining connector density. This density lies much further from the connector axis than does the bottom domain in the crystal structure, and features within this region are not yet well enough resolved to determine whether any “paper clip” structure is retained. Therefore, the bottom domain likely undergoes significant rearrangement in phage particles. Changes in the angle of the connector core subunit and the remodeling of the paper clip are presumably induced by interactions made with DNA, gp8, gp11, and gp12*, none of which are present in connector crystals. In empty particles, the bottom domain also loses its paper clip conformation and is folded back, but to a lesser extent as seen in virions (data not shown). With just three snap shots of the connector (one at near atomic resolution in crystals and two at subnanometer resolution in situ), it is impossible to accurately define, in precise detail, the sequence of changes in connector that accompany various stages of virus assembly. Detailed analysis of these events will require additional structural studies of assembly intermediates at comparable resolutions.

**DNA Path from Head to Tail**

Comparison of cutaway views of virions and empty particles emphasizes that the primary difference between them is the presence of DNA-gp3 inside the virion (**Figures 1B and 1C**). DNA is densely packed in the virion head and extends into the tail tube after passing through the connector and lower collar. The bulk DNA inside the prolate head appears to be organized in a series of layers, four of which are clearly evident inside the virion (**Figure 4A**). A radial density profile, computed from a planar section perpendicular to the phage axis in the virion 3D map (data not shown), reveals about eight total layers, with a 23Å spacing that is consistent with previous measurements (Subirana et al., 1979). The layered nature of the bulk φ29 DNA is a common feature observed in all previous reconstructions of asymmetric phages (Agirrezabala et al., 2005; Chang et al., 2006; Jiang et al., 2006; Lander et al., 2006), including φ29 (Comolli et al., 2008; Xiang et al., 2006), and has been predicted on the basis of modeling studies (Petrov and Harvey, 2007; Petrov et al., 2007). Unlike previous reconstructions, the subnanometer φ29 virion reconstruction reveals an extensive network of protein-DNA interactions between the DNA and capsid shell (**Figure 4B**). These interactions, which likely generate the characteristic circular rings and pattern of striations in the outer layer of the bulk φ29 DNA (**Figure 4C**), involve contacts at similar or identical locations in most of the gp8 subunits, and hence their arrangement follows the Q = 5 head protein lattice. The most prominent contact seen in the virion reconstruction occurs at one end of the long tubular density on the capsid-interior side of gp8. This feature is modeled, for clarity, as an α helix in **Figure 4B**. Lack of an atomic structure of gp8 precludes specific assignment of this DNA-interacting domain. Fitting of an HK97 homology model to the gp8 density (authors’ unpublished data) suggests that this end of the modeled helix corresponds to the N-terminal end of the HK97 long helix (Wikoff et al., 2000).
Figure 2. Density Sections from the Tail Regions in the Fiberless ø29 Full, A–C, and Empty, D–F, Particles

Features with highest or lowest densities appear darkest and lightest, respectively.

(A) Longitudinal section of the virion reconstruction labeled to highlight the locations of some phage components.

(B and C) Density sections normal to the tail axis at the locations depicted by dashed lines in (A) intersect two portions of the top of the gp10 connector in (B) and the middle of the gp11 lower collar in (C).

(D, E, and F) Same as (A–C) for the empty particle. The predominant density features, seen in the virion but not in the empty particle reconstructions, are identified as belonging to DNA and gp3. These include (1) the ring of ~190 Å diameter that encircles the gp10 dodecamer inside the capsid (B), (2) linear columns positioned along the phage axis and traversing the connector (A and B) and upper tail tube (A), (3) the ~60 Å diameter toroid in the connector-collar cavity (A and C), and (4) more diffuse density in the lower portion of the tail tube, terminating at the entrance to the tail knob cavity (A). The twelve asymmetrically arranged copies of gp10 are labeled in (E) but not in (B). Scale bar in (F) applies to all panels.

Whether these regular DNA-shell contacts organize the DNA while it is being packaged or stabilize it in the mature virion has yet to be determined.

Additional views of the connector and its surroundings are revealed in two cross-sections made perpendicular to the tail axis, which intersect the top of the connector (Figures 2B and 2E) and the middle of the lower collar (Figures 2C and 2F), respectively. A circular ring of density ascribed to DNA and that follows a sinusoidal path in three dimensions (data not shown) encircles the top of the connector (Figure 2B). Ring-like DNA structures may be a universal component of dsDNA phages, as they have been observed in λ15 and P22 (Jiang et al., 2006; Lander et al., 2006), as well as ø29 (Xiang et al., 2006), and have also appeared in model simulations of λ15 (Petrov et al., 2007). However, unlike in P22 (Lander et al., 2006), this portion of the ø29 DNA does not appear to contact the connector but instead appears to be pressed up against the inner wall of the capsid immediately adjacent to the connector. It has been suggested that the DNA ring in P22 controls termination of DNA translocation (Lander et al., 2006). However, ø29 packages a unit length chromosome and does not require such a sensor (Xiang et al., 2006). A ring-like DNA structure may simply arise when portions of the bulk DNA are compressed into the groove between the connector and capsid as simulated in λ15 (Petrov et al., 2007) and hence may have no defined role in packaging in ø29.

In our subnanometer reconstruction of the ø29 virion it is possible to identify and trace the path of the right end of the DNA-gp3 molecule, starting from where it emerges from the bulk of the packaged DNA in the head and ending near the distal portion of the tail (Figure 5). Definitive location in virions of the right end of the ø29 DNA and the covalently attached, gp3 terminal protein is now possible for two primary reasons. We have reconstructions at similar subnanometer resolutions of both the empty particle and the full, mature virion. In addition, a column of high density, with a diameter (~20 Å), pitch, and helical morphology characteristic of suitably resolved dsDNA, is visible...
Figure 3. Connector Structure in the Fiberless ø29 Virion and in Crystals
(A) Stereo view of a portion of the connector region in the reconstructed virion density map (gray wire mesh; ~2σ threshold) into which an atomic model (magenta ribbon diagram) of the X-ray crystal structure of the connector (Guasch et al., 2002) was docked. The gp10 subunits of the model were adjusted, essentially as a set of rigid bodies, to best fit the prominent α helices into the corresponding tubes of density in the reconstructed 3D map.
(B) Tertiary structure of the gp10 monomer in crystals of the symmetric dodecamer (Guasch et al., 2002). Top and central (in red), and bottom (cyan) domains in gp10 are identified. Highly flexible regions, invisible in the gp10 X-ray structure, are indicated by dashed curves. Helices (α1–α6) are labeled according to their location in the primary amino acid sequence (α6 is kinked, with short and long segments).
(C) Stereo view showing a ribbon diagram of the gp10 crystal structure (red and cyan) superimposed with a fitted virion model (magenta). The bottom domain (B) was not modeled in the virion density map (see text).

for the first time in an infectious virus (Figures 2 and 5). Indeed, these characteristic features are a consequence of the DNA being similarly oriented and occupying the same location in most phages.

In the virion reconstruction, a cylindrical column of high density emerges from the bulk DNA inside the head, slightly above the connector, and traverses the connector along its axis for ~80 Å (Figures 2A and 5). We assign this feature to be

Figure 4. DNA Organization in ø29
(A) Cutaway view of the ø29 virion head, showing the compact layers in the bulk DNA (red). Four layers are clearly visible, with the outermost one closely following and interacting with the capsid (blue) inner surface in several places (e.g., arrows). (B) Near axial view of the virion, looking toward the tail with most of the capsid density removed. This highlights numerous contacts between the capsid and bulk DNA. These contacts, which are colored blue but may include protein, DNA or a mixture, predominantly occur near the same end of the long α helix in each gp8 (green helical ribbons) and cluster near the Q = 5 lattice edges (yellow lines).

(C) Structure of DNA, segmented from the mature phage (Figure 1B), viewed from below the tail toward the top of the head. The outermost layer of the bulk DNA contains numerous latitudinal striations as well as a ring of density that encircles but does not contact the connector at the base of the head. Two linear columns of density, each ~20 Å in diameter, and the ~60 Å diameter toroid sandwiched between them lie along the tail axis. Density thresholds set to ~1σ (A and B) and ~2σ (C). The higher threshold in (C) was chosen to emphasize the DNA packing.
DNA rather than protein, as was recently suggested elsewhere (Xiang et al., 2006). Just below the connector-lower collar junction, a second, ~80 Å long column of high density traverses the narrow section of the lower collar and continues into the tail tube to a position ~100 Å above the tail knob (Figures 1B, 2A, and 5). The diameter, intensity, and location of this column also led us to identify the density as being dsDNA as well. However, density below and contiguous with this column abruptly weakens in intensity, compared with the surrounding tail tube, continues another ~150 Å along the tail tube channel, and then tapers to a point at the top of the knob cavity formed by gp9 (Figures 2A and 6A). We identify this low-density feature as belonging to gp3 on the basis of its weaker intensity, its location at the end of the DNA to which it is covalently attached (Mejer et al., 2001), and because it extends over a distance roughly equivalent to the length of an extended gp3 (Kamtekar et al., 2004; Xiang et al., 2006).

The right end of the φ29 DNA interacts with portions of the connector, lower collar, and tail tube in its path from head to tail (Figure 6). This segment of the φ29 DNA first appears to contact the C terminus of gp10, where twelve termini form a crown-like structure at the top of the wide end of the connector. Next, density corresponding to connector channel loops, which are disordered and hence not resolved in the gp10 crystal structure, appear to provide multiple contacts with the DNA, one of which is identified with an arrow in Figure 6A. Similar loops found in the SPP1 phage connector are believed to play a role in DNA translocation (Isidro et al., 2004; Lebedev et al., 2007). In the second...
Structure

Subnanometer Asymmetric Reconstruction of ø29

column, which lies below the gp10-gp11 junction, the DNA appears to be clamped at the constricted entrance of the tail tube. Finally, the terminal gp3 appears to form a plug that anchors the DNA near the end of the tail tube. The role these DNA-protein interactions play in retaining and subsequently releasing the highly pressurized genome is unknown.

Most notably, the columns of DNA density that traverse the connector and the lower collar in the virion are interrupted by a toroid-like structure in the center of the cavity formed at the connector-lower collar junction (Figures 2C and 5). The ~20 Å thick toroid has outer and inner radii of about 29 and 9 Å, respectively, with no detectable density in its center. The corresponding region in empty particles has no density (Figure 2F).

On the basis of its dimensions and its close juxtaposition to the high-density columns above and below it that must connect in some way, the toroid appears to represent a novel dsDNA structural motif.

DNA under Pressure

The DNA toroid is arguably the most intriguing part of the ø29 structure revealed in the virion reconstruction. We questioned whether this feature might arise as an averaging artifact, generated when compressed DNA adopts a consistent (but randomly oriented in different particles) bulge as it passes through the connector-lower collar cavity. However, the doughnut-like morphology, coupled with the observation that the toroid density is comparable in intensity to that in the columns of density above and below it, strongly argues that the toroid is a genuine feature in ø29. The most plausible interpretation of our 3D density map is that, under compression and in the confines of the connector-lower collar cavity, the DNA responds to the high compressive forces exerted upon it (Rickgauer et al., 2008; Smith et al., 2001) by sharply bending into a toroid, with no evidence for extensive contacts with gp10 or gp11 (Figures 5B and 6A).

Detailed inspection and analysis of the virion map showed thinner density features entering and exiting the top and bottom of the toroid, respectively (Figures 3 and 5). Apparently, with surprising regularity, the entry and exit points in the toroid occur at the same sites in most virions. Consistent with this finding, the axis of the toroid is tilted slightly away from the tail axis. If instead the entry and exit points were random, an untitled doughnut would result, and little or no density would connect it to the columns of high density above and below it. The well-defined character of the toroid and initial modeling experiments (authors’ unpublished data) suggest that it contains 30 to 40 DNA bases.

Can DNA in fact form such a sharply curved, toroid-like structure? The persistence length of dsDNA is generally reported as ~500 Å (Purohit et al., 2005), and hence, energy is required to bend DNA over shorter radii. Though bending required to produce DNA curvature like that predicted for an ~60 Å diameter toroid might seem extreme, it has recently been noted that “Tightly bent DNA is a fact of life” (Garcia et al., 2007). It is also known that, in the presence of DNA-binding proteins such as integration host factor (IHF) (Rice et al., 1996), DNA can be bent as tightly as seen here in ø29.

The toroid structure we observe in ø29 appears to represent a novel mode of DNA bending, possibly driven by compression of the DNA in a confined volume. During packaging, a force of ~100 pN is required to insert the last of the DNA into the head (Rickgauer et al., 2008). Even if some of this stored energy dissipates after packaging is complete, the DNA molecule remains under significant tension (Gonzalez-Huici et al., 2004; Purohit et al., 2005). To our knowledge, no experimental system has probed how dsDNA responds to compression when it is tightly constrained, such as in the confines of the ø29 phage tail. Under high compression and bounded by the connector-collar cavity, the DNA might behave like a stiff, yet flexible string, that tends to naturally fold into a toroid when two fixed points on it are brought together. What role, if any, the ø29 DNA toroid serves remains a mystery. This structure might assist in holding the pressurized DNA in the tail while it awaits ejection during infection. Analysis of the toroid by cryo-EM at higher resolution, coupled with demonstration of its presence in other phages, will help determine whether the toroid is a key factor in making ø29 and counterparts such efficient and precise DNA delivery machines.

EXPERIMENTAL PROCEDURES

Fiberless ø29 virions and empty particles were prepared and purified as previously described for fibered phage (Tao et al., 1998). Briefly, empty particles were made by incubating virions in 1 M sodium perchlorate in TMS buffer for 16 hr at 37°C, followed by repurification by isopycnic centrifugation in CsCl. Samples of ø29 were vitrified over holey, carbon-coated grids and imaged at 200 keV and at a nominal magnification of 38,000X on a Kodak SO163 electron image film at low electron doses (~20e/Å²) in an FEI CM200 FEG microscope.

Micrographs exhibiting minimal astigmatism and specimen drift and underfocused 1–5 µm were digitized at 7 µm intervals on a Zeiss PHODIS scanner and bin averaged to yield an effective pixel size of 3.68 Å. A total of 74 and 122 micrographs of the full and empty particles, respectively, were selected for particle boxing using the program RobEM (http://cryoem.ucsd.edu/programs.shtml).

Asymmetric 3D reconstructions of the fiberless ø29 particles were computed using a modified, model-based scheme (Baker and Cheng, 1996; Baker et al., 1999; Ji et al., 2006). First, we used EMAN (Ludtke et al., 2004) and a ø29 prehead reconstruction (Morais et al., 2002) to generate a five-fold symmetric reconstruction of the fiberless ø29 capsid. Several cycles of refinement, with images repeatedly remasked to include progressively more of the tail in each image, led to a hybrid model, with five- and twelve-fold symmetrized head and tail components, respectively. We then used this model in EMAN to produce an asymmetric reconstruction of the fiberless virion at about 30 Å resolution. With this reconstruction, we next used FREEALIGN (Grigorieff, 2007) to assign origin and orientation parameters to each image and to produce an asymmetric phage reconstruction at ~25 Å resolution. This then served as the starting model for extensive, iterative refinement at higher resolution using modified versions of our parallelized origin and orientation refinement program, POOR (Ji et al., 2006), and parallelized 3D reconstruction program, PSDR (Marinescu and Ji, 2003). Both of these programs were implemented without any symmetry constraints being imposed and yielded a 3D map of the virion from 12,682 particle images at a resolution estimated to be reliable to 7.8 Å, using Fourier Shell Correlation criteria (Van Heel and Harauz, 1986) (0.5 threshold). The protocol outlined above was similarly used to produce a 9.3 Å resolution map of the fiberless ø29 empty particle from 12,829 particle images.

For visualization purposes, a segmentation program (Yu and Bajaj, 2005) was used to help delineate the ø29 DNA in the virion 3D map. The molecular envelopes of the protein components were estimated manually in CHIMERA (Goddard et al., 2005) as guided by existing knowledge of ø29 (Grimes et al., 2002). However, the precise delineations of these envelopes, especially at intersubunit boundaries, are not known.

The atomic model of the gp10 dodecamer crystal structure (PDB code: 1H5W) was manually fitted and subsequently computationally adjusted to best fit the cryo-EM density maps with a modified version of RSREF (Tang et al., 2008).
et al., 2001). Illustrations were composed using the CHIMERA (Goddard et al., 2005) and RobEM (http://cryoem.ucsd.edu/programs.shtml) programs.

**ACCESSION NUMBERS**

The 3D density maps of the fiberless φ29 empty and virion particles have been deposited in the EBI-MSD EMD database with accession codes EMD–1419 (empty) and EMD–1420 (virion).

**ACKNOWLEDGMENTS**

We thank Dr. Zeyun Yu for advice on the use of his volume segmentation program, the San Diego Supercomputer Center for access to TeraGrid computing, and Drs. Wei Xu, Witold Grochulski, and Michael Sherman for their help in initiating these φ29 cryoEM studies. This work was supported in part by the National Institutes of Health (grants GM-033050 to T.S.B. and DE-003606 to D.L.A. and S.G.), and NSF shared instrumentation grant BIR-041921, support from the University of California-San Diego, and the Agouron Foundation (all to T.S.B.).

Received: January 10, 2008
Revised: February 15, 2008
Accepted: February 19, 2008
Published: June 10, 2008

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Native 3D intermediates of membrane fusion in herpes simplex virus 1 entry

Ulrike E. Maurer*, Beate Sodeik†, and Kay Grünewald‡‡

*Department of Molecular Structural Biology, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany; and †Institute of Virology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

Edited by Patricia G. Spear, Northwestern University Feinberg School of Medicine, Chicago, IL, and approved May 14, 2008 (received for review February 20, 2008)

The concerted action of four viral glycoproteins and at least one cellular receptor is required to catalyze herpes simplex virus 1 entry into host cells either by fusion at the plasma membrane or intracellularly after internalization by endocytosis. Here, we applied cryo electron tomography to capture 3D intermediates from Herpes simplex virus 1 fusion at the plasma membrane in their native environment by using two model systems: adherent cells and synaptosomes. The fusion process was visualized as a series of structurally different steps. The incoming capsid separated from the tegument and was closely surrounded by the cortical cytoskeleton. After entry, the viral membrane curvature changed concomitantly with a reorganization of the envelope glycoprotein spikes. Individual glycoprotein complexes in transient conformations during pore formation and dilation revealed the complex viral fusion mechanism in action. Snapshots of the fusion intermediates provide unprecedented details concerning the overall structural changes occurring during herpesvirus entry. Moreover, our data suggest that there are two functional “poles” of the asymmetric herpesvirus: one related to cell entry, and the other formed during virus assembly.

cryo electron tomography | herpesvirus | synaptosomes | glycoproteins

Membrane fusion is essential for cell–cell fusion during development, intracellular membrane trafficking, exocytosis as well as virus entry into and egress from cells. Herpes simplex virus type 1 (HSV-1) enters host cells via membrane fusion. HSV-1 is the prototype of the family Herpesviridae, which includes a number of other human pathogens like varicella zoster virus, cytomegalovirus, Epstein–Barr virus, and Kaposi’s sarcoma-associated herpesvirus. It has a complex structure consisting of an icosahedral, DNA-containing capsid, which is asymmetrically located within the virion and surrounded by an amorphous protein layer called the tegument, and a membrane envelope heterogeneously studded with morphologically distinct spikes formed by 12 different glycoprotein species (1). Five of these glycoproteins (gB, gC, gD, and the complex of gH and gL) mediate the entry of HSV-1 into host cells (2). The specific mode of entry is cell-type dependent. Neurons and Vero cells (an African monkey kidney cell line) are infected by direct fusion at the plasma membrane and subsequent release of the capsid into the cytosol, whereas in keratinocytes and HeLa cells, the primary step in entry involves endocytosis (3). After initial attachment by a nonessential interaction of the viral glycoprotein gC with heparan sulfate proteoglycans on the plasma membrane, entry proceeds by binding of gD to one of its three known cellular receptors: herpesvirus entry mediator (HVEM), nectin-1, or modified heparan sulfate (4). The heterodimer gH/gL and the homotrimer gB are required for membrane fusion (2). So far, it is unclear how the interactions between the four essential entry-associated viral glycoproteins are coordinated to mediate membrane fusion (4, 5) and what role the recently discovered gB receptor PILRα (6) has.

Here, we applied cryo electron tomography (cryo-ET) to probe the early events of HSV-1 infection comprehensively and in unprecedented detail. Using several adherent cell lines and synaptosomes as host systems, we addressed the structural reorganization of pathogen and host upon interaction. In particular, a systematic study addressing the intermediate states, i.e., the native dynamics of the fusion process in three dimensions at close to macromolecular resolution was missing. We characterized (i) entry sites at the plasma membrane, including changes in the membrane curvature during entry, and the fate of the glycoprotein spikes; (ii) the orientation of the asymmetric virion in relation to the plasma membrane during fusion; (iii) structural clues as to how the virus traverses the dense cortical actin barrier underneath the plasma membrane; and (iv) the details of the functional “gymnastics” of the herpesvirus glycoprotein spikes during fusion, that have been suggested (4) based on the available crystal structures (7, 8) and similarities to other viral fusion systems (9).

Results

HSV-1 Enters Vero, PTK2, and HFF Cells by Fusion at the Plasma Membrane. Cells grown on a holey carbon support film of an electron microscopy grid were inoculated with HSV-1 and vitrified in growth medium. These native samples were directly examined in the frozen hydrated state by electron microscopy. We initially chose Vero cells as an established model system for HSV-1 fusion at the plasma membrane (10, 11). These adherent cells are not especially thin. Cellular cryo-ET (12), however, is limited to specimen areas with a maximal thickness of ~1 μm (13), and therefore we could only analyze the outer rims of Vero cells. Two other cell types susceptible to HSV-1 infection, human foreskin fibroblasts (HFF) and rat kangaroo kidney cells (PTK2) (11, 14), provided an extended, sufficiently flat cell periphery, and thus were also included in our study, although their mode of entry was unknown. The cells were pretreated with nocodazole, a microtubule depolymerizing drug, to prevent dynin driven transport of incoming capsids from the periphery toward the cell nucleus (10, 11).

Entry events were captured in the cell lines Vero, PTK2, and HFF. All three were found to be entered by fusion of the HSV-1 envelope with the plasma membrane (Fig. 1 for PTK2; data for HFF and Vero cells not shown) as demonstrated by the presence of nonenveloped cytosolic capsids 2 minutes after the onset of infection, and recognition of the corresponding fusion sites at the plasma membrane. Taking advantage of the 3D tomographic information, we identified the sites of virus entry at the plasma


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

†To whom correspondence should be addressed. E-mail: gruenewald@biochem.mpg.de.

This article contains supporting information online at www.pnas.org/cgi/content/full/0801674105/DCSupplemental.

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membrane by virtue of the unique structural signature of the viral glycoproteins and the tegument (Fig. 1 D–G).

The glycoprotein spikes protruded in dense clusters from the plasma membrane at the entry site. The majority of the tegument density—the so-called outer tegument (15)—formed a dense layer just underneath the plasma membrane, clearly corresponding in shape and extent to the patch of glycoprotein spikes on the outer face of the plasma membrane. The volume of this tegument density was consistent with the volume of the tegument in complete virions (1). The capsid was typically separated from most of the tegument density and did not show evidence for any densities that could be ascribed to the tegument (Fig. 1 C–G and J).

Capsids released into the cell were located close to actin filaments [Fig. 1C and supporting information (SI) Movie S1]. To exclude the possibility that the actin organization had been modified by nocodazole, which might affect the cortical cytoskeleton, we performed experiments in the absence of nocodazole. The overall actin network organization in untreated cells near the viral entry sites resembled that of nocodazole treated cells (Fig. 1 J and K), but the number of capsids in the peripheries of untreated cells was lower (14).

**HSV-1 Entry into Synaptosomes.** Due to the specimen thickness, tomography of virus entry events was restricted to the cell periphery, resulting in a low number of analyzable events. Viruses were rarely observed entering at the outer rim of adherent cells, hence there was a shortage of side-views of the glycoprotein patches on the cell membrane and the underlying tegument (Fig. 1 J and K). To analyze the structural dynamics of entry by fusion more extensively, we therefore shifted to synaptosomes, a smaller host system that was completely accessible by cryo-ET. Synaptosomes are physiologically active endings of neurons prepared by homogenization and fractionation of brain tissue (16, 17). Neurons are authentic host cells for herpesvirus, and they have been characterized as a model host system for HSV-1 cell entry (C. H. Nagel, A. Binz, S. Borzsutzky, R. Bauerfeind, and B.S., unpublished data). Moreover, presynaptic regions of neurons express nectin-1, an HSV-1 entry receptor, at high levels at the plasma membrane (18).

Viruses entered exclusively into the presynaptic part (Fig. 2A and B and Movie S2). The overall morphology of glycoprotein spikes and tegument at the entry site was analogous to our observations in intact cells. The tomograms from synaptosomes have higher quality due to the thinner specimens; therefore, we exploited them for a more detailed analysis. To trap different intermediate stages of the fusion process and thereby reveal its structural dynamics, we analyzed tomograms of synaptosomes inoculated with HSV-1 at a variety of infection times and temperatures ranging from 1 to 60 min and from 10 to 37°C, respectively (for details see Materials and Methods and Table S1). The actual fusion at the plasma membrane occurred within seconds. We detected cytosolic capsids inside synaptosomes after only 1 min of incubation at 37°C. Surprisingly, the mem-
brane fluidity at 10°C was sufficient to enable entry, whereas no fusion intermediates were detected at 4°C. Capsids were located close to the entry site, judged by glycoprotein spikes and tegument at the plasma membrane, even after 60-min incubation at 37°C.

**Structural Dynamics of HSV-1 During Entry.** Summarizing the data based on 51 tomograms, we present a gallery of characteristic slices through the tomograms and corresponding schematic drawings for the six major steps into which we divided the entry process (Fig. 3A–F and A’–F’). Of the 66 viral entry intermediates, we allocated 45 structures to the steps A–F, comprising 15, 1, 4, 5, 9 and 11 events, respectively. These numbers support the notion that the fusion pore represented the most short-lived intermediate. Twenty-one structures represented heterogeneous intermediates between state A and B (Fig. S1).

The first morphological stage was virus attachment to the plasma membrane (Fig. 3A). We observed a prominent gap between the viral and the cellular membrane. The width of this gap (~20 nm) corresponded to the length of the longest glycoprotein spikes protruding from the virus. This primary interaction of the virus with the host cells involved a large contact area, apparent as an indent of the plasma membrane. Next, the viral and synaptoosomal membranes came close together, and a fusion pore formed (Fig. 3B). The pore enlarged and allowed mixing of the viral content with the cytosol (Fig. 3C). The viral envelope, which had been integrated into the cell membrane, initially maintained its curvature. Subsequently, and consistent with earlier reports (10, 19), the capsid and potentially some bound inner tegument advanced into the cytosol, whereas the outer tegument remained attached to the membrane (Fig. 3D). Akin to entry into cells, the capsid was not surrounded by any densities assignable to tegument proteins. The curvature of the former viral membrane decreased (Fig. 3E) and the number of glycoproteins and the amount of tegument at the entry site declined (Fig. 3F) until no respective densities remained.

**Intermediate Glycoprotein Conformations During Pore Formation and Dilatation.** Three-dimensional renderings of the short-lived fusion pore (Fig. 3B) are presented in Fig. 4 and in Movie S3. The virion pole where the membrane is in close proximity to the capsid (1) was located close to the pore opening and can be seen through the pore neck from inside the cell (Fig. 4B), whereas most of the tegument mass was distant to the fusion pore. The pore diameter of ~25 nm (Fig. 5C) reflected a state after the frequent reversions to the hemifusion state known as “flickering” (20), and thus the beginning of lateral pore expansion. Detailed analysis of the region between the viral and cell membranes next to the pore revealed bent V- or Y-shaped densities connecting the two membranes and possibly represented glycoprotein complexes undergoing conformational changes (Fig. 5 A, C, and D). In HSV-1 inoculated Vero cells, another example of V/Y-shaped structures was captured next to an early contact site just before pore formation, perhaps corresponding to hemifusion as indicated by the elongated neck shape and a pointed membrane contact (Fig. 5 E–H). In both stages, early contact and pore dilation, the V/Y-complexes had an arm length of up to ~15 nm. The component of the V/Y-complex proximal to the viral membrane was in both cases curved, whereas the segment
connecting to the plasma membrane was straight. The major difference between early contact and pore dilation was that the angles between the glycoprotein segment and the plasma membrane decreased from ≈50° to ≈40° concomitant with a change from a tapered contact of the viral and plasma membrane to a more parallel arrangement. Moreover, the shape of the “hinge” connecting the two components of the complex changed from a globular structure of ≈5 nm in diameter to an elongated one of ≈8 nm in length. A small structure close to the potential lipid mixing region connecting the two different membranes was observed in both states.

Discussion

In this study, we monitored the native structural dynamics during HSV-1 entry into adherent host cells and synaptosomes in unprecedented detail. We captured several intermediates, including the very short-lived events of pore formation and dilation by imaging single virions during the entry process in the frozen hydrated state. By only using high quality virus inocula (see Materials and Methods), we consider it most reasonable that the virions captured here at the plasma membrane represented functional intermediates in the cell entry of HSV-1 that were suspended from further progression into the cytosol by sudden freezing. Thus, they provide valuable, physiologically relevant in situ snapshots of the fusion dynamics.

In adherent cells, cytosolic capsids were located between actin bundles, which appeared to enclose the capsids (Fig. 1). Viruses are known to modulate the host’s cytoskeleton and associated pathways (21, 22). Rearrangement of the actin cytoskeleton during entry via endocytosis was reported for HSV-1 (23). Thus, we suppose that the actin bundles had reorganized upon viral stimulation to facilitate capsid entry and to remove the dense cytoskeletal barrier. The morphological appearance of the cortex—bundles and filaments—implies that there was no local actin depolymerization around the capsid. However, the actin network from which the adherent cells at this resolution can only be visualized in intact filopodia (24). A direct control of the cytoskeleton organization from a cell at exactly the same area before virus entry is not feasible with the technique used here. To evaluate this aspect in full detail, the potential players in the respective signaling pathways need to be identified.

The viral membrane dynamics changed its curvature into extremely bended structures during the entry process (Fig. 3). We consider this a native feature because in cryo electron microscopy, in contrast to electron microscopy of fixed samples, membranes are preserved in their native state. The curvature was possibly stabilized by the distinct lipid contents of this domain (25) and by clusters of the glycoprotein spikes, which are present in the highly curved regions (Fig. 3 C–E) and which interact with the tegument.

Some of the glycoprotein spikes must represent the postfusion state of the glycoproteins gH/gL and gB. However, most glycoproteins were not recruited to the fusion site, and resembled those seen on the virus (1). Our data (Figs. 3–5) support the notion that only a small number of glycoprotein spikes is sufficient to catalyze HSV-1 cell entry. We did not detect any particular structure indicative for a large multisubunit complex formed by numerous spikes and receptors. Glycoproteins were densely clustered on both extracellular virions and the plasma membrane after fusion so that any postfusion conformation complexes would have been difficult to detect.

The glycoproteins and the tegument remained at the plasma membrane after the capsid was released into the cytosol (Figs. 1–3). This implies a continued contact of the tegument proteins with the cytoplasmic tails of the viral glycoproteins after fusion. During the subsequent events (Fig. 3), the membrane curvature flattened and the amount of glycoproteins and the tegument density decreased. The glycoproteins perhaps diffused away and were recycled or degraded until finally no glycoproteins remained at the entry site; the tegument possibly dissociated.

The HSV-1 virion has an asymmetric structure, characterized by a noncentric localization of the capsid. We propose that this asymmetric structure of the virion is related to different functions and results in two functional poles (Fig. S2). One pole of the virion is associated with virus assembly during secondary envelopment at cytoplasmic membranes and is characterized by a dense “cap” of tegument proteins (1). Some of these tegument proteins interact with the cytosolic tails of various glycoproteins (26, 27). These interactions impede lateral movement of glycoproteins in the membrane and maintain dense clusters of glycoprotein spikes on this pole. Bulky, non-entry-associated glycoprotein spikes in close proximity to gD may act as spacers and prevent binding of gD to its cellular receptor (Fig. 6A). On the opposite virion pole, where the capsid almost contacts the viral
envelope, tegument material is sparse and, as such, are glycoprotein spikes. The larger spacing between the latter prevents sterical hindrance, and this pole may present the entry-associated glycoproteins in a functional arrangement (Fig. 6B).

Such a preference for the capsid proximal side of the viral membrane to participate in the fusion pore was, in fact, observed in our study (Figs. 4 and 5). Therefore, we assign the entry function to this pole, thereby introducing the concept of a functional bipolar and structurally asymmetric virion.

Based on the current view (4, 5) of the sequence of events in HSV-1 fusion, we suggest a refined model for the fusion mechanism of HSV-1, which is compatible with the observed glycoprotein intermediates during pore formation and dilation (Fig. 5). At the “entry pole,” gD may bind to its cellular receptor (Fig. 6C) and subsequently undergo the necessary conformational changes (28) that activate gH/gL (Fig. 6D). Like viral class I fusion proteins, gH contains hydrophobic alpha-helices (29). The heterodimer gH/gL perhaps changes conformation, and one segment binds to the plasma membrane. This connects the two membranes, pulls them together and enables lipid mixing as shown for mutants lacking gB (30). Based on the lower molecular weight of the gH/gL heterodimer compared with the gB homotrimer, we suggest allocating the small structure close to the lipid mixing region of the viral and the plasma membrane (Fig. 5D and H) to a gD-gH/gL complex (31, 32). The conformational switch of gH/gL then recruits gB to the gD-gH/gL complex. The observed V/Y-complexes in Fig. 5 had an arm length of ≈15 nm, thus possibly representing gB (33). The gB, a class III fusion protein, has features of both class I and class II fusion proteins and shares structural homologies with the class III fusion protein glycoprotein G of vesicular stomatitis virus (7). The x-ray structures of the pre- and postfusion states of the latter lead to an assignment of putative conformational changes and intermediates (34, 35). Accordingly, we suggest analogous to the V/Y-shaped structures (Fig. 5) that gB might flip out one segment that binds to the plasma membrane forming the elongated neck like structure (Fig. 6E). Finally, the fusion pore forms and expands facilitated by gB (Fig. 6F).

The suggested assignments of glycoproteins are mainly based on single structures derived from the tomograms presented in Fig. 5. Because cryo electron microscopy of biological material provides low-contrast images with a low signal to noise ratio, but with the advantage of avoiding artifacts caused by fixation, dehydration, and staining, these structures do not stand out very strongly against the background. However, the detected glycoprotein intermediate structures were very similar, although they originated from experiments with two different host systems, synaptosomes and Vero cells. Therefore, we think that these intermediates are representative for the HSV-1 fusion mechanism.

In the case of one fusion effector, crystal structures of truncated viral glycoproteins at different pH have revealed distinct pre- and postfusion conformations, and suggested a fusion mechanism by deducing hypothetical intermediate structures and molecular dynamics for all three classes of viral fusion proteins (9). However, membrane fusion during the entry of herpesviruses or poxviruses and during intracellular membrane traffic requires the interaction of several effectors (36, 37). Here, we present the first direct observation of several intermediates in virus fusion during cell entry in a native environment at close to molecular resolution, i.e., snapshots of a multicomponent fusion machinery in action.

Materials and Methods

Purification of HSV-1. Extracellular HSV-1 virions (wild type strain 17+) were produced from African green monkey kidney (Vero) cells (CCL-8; American Type Culture Collection) or human foreskin fibroblasts (HFF) cells (kindly provided by Prashant Desai, University of Baltimore, Baltimore, MD) and purified and characterized by plaque titration and real-time PCR with previous DNase treatment as described (1, 14, 38). Viruses produced from Vero cells had a genome/pfu ratio of 6 and viruses from HFF cells of 11.

Infection of Cells Grown on Grids. Au-grids with holey carbon films [either Quantifoil (Quantifoil) or C-flat (Protochips)] were glow-discharged for 20 s and preincubated with growth medium at 37°C and 5% CO2. Rat kangaroo kidney cells (PtK2; CCL-56; American Type Culture Collection), HFF cells, or Vero cells were grown on the grids in their respective growth medium: alpha-minimum essential medium for PtK2 and HFF and D-MEM for Vero (both Invitrogen) supplemented with 10% FCS (Sigma-Aldrich), 100 units/ml peni-
cillin, and 100 μg/ml streptomycin (both Invitrogen) for 15 h. After pretreatment with 50 μM nocodazole (Sigma-Aldrich) for PtK2 and 7 μM nocodazole for HFF and Vero for 2 h, the cells were cooled on ice and infected with 10^6 pfu of HSV-1 produced from Vero cells per 25 μl of medium and grid. Virus binding to the cells was allowed for 2 h on ice; the samples were then warmed up to 37°C for 2–5 min. After adding 3 μl of colloidal gold particles (15 nm, protected with BSA), excess fluid was absorbed with filter paper. The samples were vitrified by plunge freezing in liquid ethane and stored in liquid nitrogen. Cells incubated without nocodazole were treated likewise.

Preparation of Inoculated Synaptosomes. Isolation of synaptosomes from the forebrain of a 6-week-old male Wistar rat was adapted from Leenders et al. (39). After harvesting the F4 fraction from the 15%/23% interface of a four step (23%, 15%, 10%, 3%) Percoll gradient (Sigma-Aldrich), the synaptosomes (typical diameter ~0.5 μm) were centrifuged for 15 min at 15,000 × g and 4°C and resuspended in 1 ml of RPMI 1640 wiGlut 25 mM Hepes medium (Invitro-
gen) containing 0.1% BSA (Sigma–Aldrich). The synaptosomes from 1 brain were activated at 37°C for 30 min and inoculated on ice with 10^6 pfu HSV-1 produced from HFF cells. Binding was allowed for 60 min on ice. Washing was carried out by centrifugation steps of 5 min at 3,500 × g and 4°C. The subsequent incubation times (1, 2, 5, 10, 30, or 60 min) and temperatures (10°C, 18°C, 25°C, 37°C or on ice) were varied for individual experiments. Finally, 3 μl of sample were applied to holey carbon films on Cu-grids (Quan-
tifoil; glow-discharged for 20 s), 3 μl of colloidal gold particles (10 nm, protected with BSA) were added, and vitrified as described above.

Cryo electron Tomography. Data were collected on a Philips CM300 FEG and a Tecnai Polara (FEI) transmission electron microscope, both equipped with a GATAN GIF 202 postcolumn energy filter (Gatan), and images were collected with a 2k × 2k Multiscan CCD camera (Gatan). The microscopes were operated at 300 kV and a final magnification of 43,974 and 37,248 giving a pixel size of 0.68 nm and 0.81 nm at the specimen level, respectively. Tilt series were collected covering a minimum angular range of 120°, with an angular incre-
ment of 2°, 2.5°, or 3°. Defocus was measured along the tilt axis after each tilt and automatically maintained at ~12 μm ± 0.5 μm. The total electron dose received at the specimen level was kept between 50 and 100 electrons². The exposure time was adjusted to the tilt angles by a 1/icos scheme.

Image Processing. Alignment, reconstruction, extraction of subtomograms, and further image processing were performed using the EM (40) and TOM (41) software packages implemented in MATLAB (Mathworks). Tilted images were aligned using gold beads as fiducial markers. Three-dimensional reconstruc-
tions were calculated using weighted back projections. Visualization and segmentation was performed using Amira 4.1 (TGS) and BioSof (42). Nonlinear anisotropic denoising (43) and Gaussian filtering was applied before segmen-
tation. Final figure preparation and drawings were performed in Photoshop CS2 (Adobe) and Canvas X (ACD Systems International).

ACKNOWLEDGMENTS. We thank C.H. Nagel for sharing his experience on inoculating synaptosomes with HSV-1; V. Lucic, G.K.W. Kong, and R. Fernán-
dez-Busnadiego for discussions and practical help with setting up of the synapto-
some preparation protocol; K. Dönherr for many discussions on HSV-1 cell entry experiments; I. Ibiricu and C. Hagen for support; A.C. Steven for stimulating discussions; W. Baumeister for support and encouragement; and J. A. G. Briggs and A. Leis for critical reading of the manuscript. This work was supported by Deutsche Forschungsgemeinschaft Emmy-Noether-Programm Grant GR1990/1-2 (to K.G.), Deutsche Forschungsgemeinschaft Priority Pro-
gramme 1155 Grants GR1990/1-2 (to K.G.) and SO403/3 (to B.S.), and European Commission New Emerging Science and Technologies Contract 012702 (to B.S.).

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