Distinct monoclonal antibodies separately label the hexons or the pentons of herpes simplex virus capsid

B. L. TRUS*[†], W. W. NEWCOMB[‡], F. P. BOOY*, J. C. BROWN[‡], AND A. C. STEVEN*[§]

*Laboratory of Structural Biology Research, National Institute of Arthritis, Musculoskeletal and Skin Diseases, and [†]Computer Systems Laboratory, Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD 20892; and [‡]Department of Microbiology and Cancer Center, University of Virginia Health Sciences Center, Charlottesville, VA 22908

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ABSTRACT The surface shell of the capsid of herpes simplex virus type 1 (HSV-1) is 15 nm thick and 125 nm in outer diameter and has the form of an icosahedral ($T = 16$) surface lattice, composed of 150 hexons and 12 pentons. Hexons are traversed by axial channels and have six-fold symmetric external protrusions, separated by triangular nodules ("triplexes"). Pentons resemble hexons morphologically, apart from their different order of symmetry. To localize VP5, the major capsid protein, in the shell structure and to investigate whether pentons are composed of the same molecules as hexons, we have performed cryo-electron microscopy and three-dimensional image reconstructions of control HSV-1 B capsids and of B capsids immunoprecipitated with two monoclonal antibodies raised against purified VP5 and purified capsids. The results clearly map the epitope of the anti-VP5 monoclonal antibody to the distal tips of the hexon protrusions. In contrast, no detectable labeling of pentons was observed. We conclude that the hexon protrusions are domains of VP5 hexamers, other parts of these molecules forming the basic matrix of the capsid shell to which the other proteins are attached at specific sites. Conversely, the anti-capsid monoclonal antibody decorates the outer rim of pentons but does not bind to hexons. These observations imply that either pentons are composed of some other protein(s) or that they also contain VP5, but in a conformation sufficiently different from that assumed in hexons as to transform its antigenic character. Other evidence leads us to favor the latter alternative.

Although considerable progress has now been made toward characterizing the molecular composition of herpesvirus nucleocapsids, and their overall structures, assignment of the various capsid proteins to specific structural features has, for the most part, still to be achieved. The major capsid protein, VP5 (150 kDa), accounts for about 70% of the mass of its surface shell (1), which also contains significant amounts of three other proteins—VP19 (50 kDa, ≈ 330 copies), VP23 (34 kDa, ≈ 660 copies), and VP26 (12 kDa, 1000–1300 copies). Trace amounts of several other proteins may also be present (2–5).

The shell structure is seemingly common to all three species of capsids that have been purified from infected cells: A capsids, an empty, abortive, form; B capsids, which are related to a maturable precursor (6); and C capsids, which are fully packaged with DNA. Visualized at ≈ 3.5 nm resolution in three-dimensional reconstructions calculated from cryo-electron micrographs (7–10), the capsid shell has several distinctive features. The hexons have hollow external protrusions that extend ≈ 5 nm beyond a middle layer of density, whose most conspicuous features are the 320 "triplexes," triangular nodules located at the local three-fold sites between hexon protrusions and between pentons and hexons.

The innermost layer, or "floor," is closely knit and bounded by an inner surface that is smooth and featureless, apart from the openings of the axial channels that traverse each capsomer.

Hexons have six-fold symmetry, as demonstrated from negatively stained projections (11, 12) and confirmed in three dimensions from cryo-reconstructions (7–10). The latter data have also revealed that the six-fold symmetric aspect of the hexon in projection is due primarily to its external protrusions. Mass measurements effected by scanning transmission electron microscopy have established that the capsid has a complement of about 900 copies of VP5 (1). Taken together, these data make a strong case that its surface lattice capsid is based on a $T = 16$ icosahedral packing of hexamers of VP5. However, it has not yet been settled whether the pentons also consist of VP5 or whether—as with other large icosahedral double-stranded DNA viruses such as adenovirus (13) and T-even bacteriophages (14)—they are composed of different molecule(s) from those that make up the hexons. Nor has it been determined which structural features of the shell are contributed by VP5 and which represent the other capsid proteins.

Previously, antibody decoration has been shown to be an effective technique for identifying morphological features within a supramolecular structure, visualized either by negative staining or shadowing (15, 16) or by cryo-electron microscopy and three-dimensional image reconstruction (17, 18). Here, we have used the latter approach to localize the epitopes of two monoclonal antibodies (mAbs)—raised against purified VP5 and purified B capsids, respectively—on the capsid surface.

MATERIALS AND METHODS

Generation of mAbs. A slice containing 140 μ g of VP-5 was cut out of an SDS/PAGE gel of purified B capsids and emulsified with an equal volume of Freund's complete adjuvant. Half of the sample was injected subcutaneously in the nape of the neck of an 8-week-old BALB/c female mouse, and the remainder was injected intraperitoneally. Four weeks later, the same procedure was followed, except that Freund's incomplete adjuvant was used. Twenty-four days later, 40 μ g (20 μ l) of purified B capsids was injected into the spleen, and 160 μ g was injected intraperitoneally. Four days later, the spleen cells were harvested and fused with Sp2/0-Ag14 myeloma cells (19), as described (20). A similar protocol was followed using similar amounts of purified B capsids as antigen.

Abbreviations: mAb, monoclonal antibody; HSV-1, herpes simplex virus type 1.

[§]To whom reprint requests should be addressed at: Laboratory of Structural Biology Research, National Institute of Arthritis, Musculoskeletal and Skin Diseases, Building 6, Room 114, National Institutes of Health, Bethesda, MD 20892.

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Cloned hybridoma cells resulting from the fusions were screened for the production of antibodies by an ELISA (21) in which purified B capsids were employed as antigen. ELISA-positive clones were further screened by an immunoprecipitation assay. Ten microliters of hybridoma cell supernatant was mixed with 150 μ l of B capsids (at 0.2 mg/ml in 10 mM Tris/1 mM EDTA/0.25 M NaCl, pH 7.5) and incubated overnight to allow precipitation to take place. Cell lines whose supernatants precipitated B capsids were subcloned to ensure that only a single cell line was present and then retested by the ELISA and immunoprecipitation assay described above.

Characterization of mAb 8F5. Experiments were carried out with antibodies produced by hybridoma clones 8F5 and 3B; these were grown as an ascites in BALB/c mice and in cell culture, respectively. Antibodies were purified from the ascites fluid or culture supernatant by adsorption and elution from protein A-Sepharose (22). mAb 8F5 and mAb 3B were found to be IgG3 and IgG1, respectively. The specificity of mAb 8F5 for VP5 was confirmed by means of an ELISA carried out with purified VP5, in the form of residual capsids depleted of all other capsid proteins by extraction in 2.8 M guanidine hydrochloride, as antigen. The preparation and characterization of these particles will be described in full elsewhere (W.W.N., unpublished data). SDS/PAGE analysis showed them to consist exclusively of VP5.

Production and Purification of Herpes Simplex Virus Type 1 (HSV-1) B Capsids. The 17MP strain of HSV-1 was grown in monolayer cultures of BHK-21 cells as described (23). B capsids were purified from the infected cells according to ref. 10. Although A capsids, B capsids, and C capsids have seemingly identical outer surfaces (refs. 7 and 10; this study), B capsids were used in these experiments because they are obtained in greater quantities from the nuclei of infected cells under the conditions used.

Cryo-Electron Microscopy of Immunoprecipitated B Capsids. Equal amounts (mg) of purified B capsids and mAbs, both at 1 mg/ml of protein, were mixed at room temperature. This mixture corresponds to a molar ratio of 3:2 for IgG to VP5. The immunoprecipitate was allowed to settle out of solution, the supernatant was decanted, and the precipitate was then dispersed by sonication. Thin films of B capsid suspensions or immunoprecipitates were rapidly frozen in a Reichert KF-80 cryostation (Reichert) and observed in a Philips EM400T electron microscope equipped with a Gatan model 626 cryoholder (Gatan, Warrendale, PA). The procedures followed to prepare and observe these specimens have been described (9).

Image Processing and Reconstruction. Micrographs recorded at 36,000 $\times$ or 60,000 $\times$ were screened by optical diffraction to identify images whose defocus condition corresponded to the first zero of the phase-contrast transfer function being at ≈ 2.4 nm $^{-1}$. Micrographs were digitized on a Perkin-Elmer 1010MG microdensitometer, at a sampling rate corresponding to ≈ 0.83 nm per pixel. These data were analyzed as described (10), using the PIC program (24) for preprocessing operations and Fourier-based techniques (25–28) to determine the particles' orientations and to perform the icosahedral reconstructions. Each reconstruction was based on data from a single micrograph: 40 particles were included in the unlabeled reconstruction, 25 particles in the 8F5-labeled reconstruction, and 20 particles in the 3B-labeled reconstruction. The radial limit of the circular mask applied to the capsid images before reconstruction was increased by ≈ 8 nm to 71 nm in the case of the labeled capsids.

RESULTS

Properties of mAbs. mAb 8F5 was raised against purified VP5 eluted from an SDS/polyacrylamide gel. Although 8F5

did not react positively in a conventional Western blot assay (29), its specificity for VP5 was demonstrated in an ELISA with VP5 as antigen. In this reaction, purified mAb 8F5 gave an optical density reading of 0.30 compared with a background (control mAb reaction) of 0.07.

mAb 3B was raised against purified B capsids. Like mAb 8F5, it readily precipitates purified B capsids from solution (see below). However, this mAb does not recognize denatured capsid proteins, as evidenced by the negative outcomes of Western blots and radioimmunoprecipitation assays (data not shown). Thus, we infer that it recognizes a "conformational" epitope (30) presented by the native, but not the denatured, antigen. The immunochemical characteristics of mAb 8F5 indicate that it shares this property, at least in part. We have not been able to demonstrate directly the molecular specificity of mAb 3B; however, current evidence suggests that it also binds to VP5 (see *Discussion*).

To estimate the binding stoichiometry of these mAbs on purified B capsids, immunoprecipitates were formed and collected by low-speed centrifugation. This material was washed in buffer to flush out unbound antibodies, pelleted again, and analyzed by SDS/PAGE. From quantitation of the dye-binding capacity of the VP5 band relative to that of the IgG light chain, we estimate an average binding stoichiometry of 0.65–0.70 IgG molecules per VP5 monomer for mAb 8F5, corresponding to 590–670 IgGs per capsid. A much lower retention level, <50 IgGs per B capsid, was recorded for mAb 3B.

Cryo-Electron Microscopy. Micrographs of immunoprecipitated and control B capsids are presented in Fig. 1. B capsids cross-linked by mAb 8F5 (Fig. 1*B*) seldom come closer to making contact than about 15 nm. Moreover, compared with unreacted controls (Fig. 1*A*), flares of density extend from the surfaces of the 8F5-labeled capsids, connecting them together. In contrast, the immunoprecipitates produced by mAb 3B (Fig. 1*C*) are much more lightly labeled, consistent with the quantitation data (above), although occasional tufts of density are discernible around the particles' peripheries.

To characterize the binding of these antibodies to B capsids in greater detail, three-dimensional reconstructions were calculated. The outer surface of the B capsid viewed down a two-fold axis of symmetry is depicted in stereo in Fig. 2*A*. Its structure is indistinguishable from those of A capsids or C capsids (see ref. 10). In comparison, the 8F5-labeled B capsid (Fig. 2*B*) has hexon protrusions that are massively extended in a radial sense, by a hollow cylindrical distribution of density, while the molecular topography of its pentons is quite unchanged. The situation is entirely reversed in the case of mAb 3B (Fig. 2*C*): here, the hexons are essentially unchanged in appearance (apart from the somewhat lower resolution achieved in this reconstruction), whereas five finger-like protuberances extend outward from the tips of each penton.

The same aspects of mAb binding are conveyed in thin sections (two-fold view) in Fig. 2*D–G*. Compared to the control capsid shell (Fig. 2*D*), all of the hexons but none of the pentons are labeled by mAb 8F5 (Fig. 2*E*). Conversely, with mAb 3B, the only labeling is seen at the pentons [compare Fig. 2*F* and *G* (arrow)].

With mAb 8F5, we observe no significant difference between the respective labeling densities of hexons located in different parts of the icosahedral lattice—i.e., the P hexons, C hexons, and E hexons (see figure 2 of ref. 12 for nomenclature). In each case—see Fig. 2*E*—the hexon radial extensions are somewhat less dense than the shell—i.e., their peak density above background (buffer) is about 60–70% of that of the shell. This factor reflects the average occupancy of all of the symmetry-related antibody-binding sites that were averaged in the reconstruction. As such, it gives an estimate of the average binding stoichiometry that agrees well with the figure

FIG. 1. Cryo-electron micrographs of purified HSV-1 B capsids (A) and of B capsids immunoprecipitated with mAbs 8F5 (B) and 3B (C). The immunoprecipitates in B and C appear to be two-dimensional—i.e., only one particle thick. Most likely, this effect results from the restructuring of three-dimensional aggregates formed in solution, upon being compressed into thin films on the electron microscope grids. (Bar = 100 nm.)

obtained by SDS/PAGE. With mAb 3B, the corresponding measurement indicates a somewhat lower occupancy of the pentons ($\approx 40\%$, equivalent to ≈ 25 IgGs per capsid), compared with mAb 8F5 on the hexons, and is also consistent with the biochemical estimate of its binding stoichiometry.

DISCUSSION

mAbs 8F5 and 3B Recognize Conformational Epitopes on B Capsids. mAb 8F5 was raised against VP5 purified by preparative SDS/PAGE. Since its epitope has been found to be present in 900 copies per capsid, and there are no other capsid proteins of this abundance that migrate in the same part of the gel as VP5, there can be little doubt that it is indeed labeling this protein. This conclusion is in keeping with 8F5's specificity, as demonstrated by the positive outcome of an ELISA reaction with purified VP5.

mAb 3B was raised against purified B capsids. This antibody is penton-specific, and its epitope is present in 60 copies per native B capsid. Its epitope is conformation-dependent, in the sense that its antigenic specificity is destroyed when the protein is denatured in SDS. That mAb 8F5 shares this property, to some extent, is evidenced by the contrast between its ready reactivity with native B capsids in solution and the requirement for a sensitive immunochemical assay to detect its binding to purified VP5. Noting the apparent paradox that the antigen used to produce this antibody was prepared by SDS/PAGE, we infer that the VP5 must have renatured sufficiently, during or after inoculation, to generate an immune response against its native hexon conformation.

mAb 8F5 Binds to Hexons but Not to Pentons. Negatively stained immuno-electron microscopy with polyclonal anti-VP5 antibodies has previously been used to confirm that this, the major capsid protein, is distributed all over the capsid surface (31). Our more detailed findings with mAb 8F5 establish that the hexon protrusions are made, at least in part, of VP5.

In the averaged density distribution represented in our reconstruction (Fig. 2 B and E), the hexon decoration takes the form of a hollow cylindrical extension of its protruding

domain(s). The 15-nm-long axial channel through the hexon center is thereby extended by ≈ 10 nm. This feature does not resemble the characteristic T shape of an IgG molecule: it is, in fact, a barrel of six Fab fragments. The six-fold rotational symmetry of the barrel, and hence the epitope copy number per hexon, is evident from transverse thin sections through it, close to its point of contact with the hexon tip (data not shown). On average, four of the six epitopes per hexon were occupied in this experiment.

The epitope is located close to the distal tip of the hexon protrusion, one of the most exposed sites on the entire capsid surface. The binding configuration of the Fab fragment is such that it is directed almost exactly radially, relative to the capsid center. This geometry is ideal for cross-linking capsids (Fig. 1B). There is an apparent diminution in density of the Fab barrel toward the outer radial limit of our reconstruction (Fig. 2E), which probably results from two effects: (i) the tapering of the Fab arm as it meets the Fc stalk of the IgG (32) and (ii) stresses imposed in the binding and possibly also when the immunoprecipitate is flattened into a thin film. The latter effect probably causes some play in the Fabs' orientations relative to their binding sites, resulting in smearing of the averaged density at outer radii.

Labeling Pattern of mAb 3B: Binding to Pentons but Not to Hexons. Our reconstruction of B capsids labeled with mAb 3B extends to somewhat lower resolution than the other two reconstructions presented here (≈ 4.3 nm vs. ≈ 3.5 nm), because a smaller number of usable particles was obtained from this micrograph and because their orientations were less uniformly distributed over the icosahedral asymmetric unit. Moreover, the fractional occupancy of the exposed epitopes by mAbs is somewhat lower than in the experiment with mAb 8F5. Nevertheless, the reconstruction clearly demonstrates binding of mAb 3B at five-fold sites around the protruding rim of each penton, with no detectable binding to hexons (Fig. 2 C and G). This finding explains the relatively low amount of these IgGs that bound to B capsids, since there are only 60 copies of this epitope per capsid as compared to 900 for 8F5. We note that even a much smaller number of occupied epitopes per capsid would still allow for efficient

FIG. 2. Stereo pairs of HSV-1 B capsid three-dimensional reconstructions. Surface representations are viewed along a two-fold axis of symmetry for unlabeled B capsids (A) and B capsids labeled with mAb 8F5 (B) or mAb 3B (C). The unlabeled capsid (A) is 125 nm in diameter. Also shown are thin sections (0.8 nm) through several three-dimensional reconstructions (D–G). In each case, the capsid orientation corresponds to the view along a two-fold axis of symmetry. (D and E) Central sections through unlabeled A capsids and B capsids labeled with mAb 8F5, respectively. p, Penton; hE, E hexon; hP, P hexon. No mAb attachment is observed in E at the penton sites. (F and G) Sections parallel to but displaced by 4.2 nm relative to the central section (mAb 3B binds in such a way that the central section does not pass through the bound mAbs; compare C). The only capsomers showing significant extensions of density are the pentons (arrow). (Bar = 25 nm.)

immunoprecipitation: because their major capsid proteins are present in such high copy numbers, HSV capsids may be readily precipitated under conditions where only vestigial binding of IgGs is detected by biochemical quantitation.

Implications for Capsid Structure. Recently, we have obtained a reconstruction with a second anti-VP5 mAb, called mAb 5C. Like mAb 8F5, it gives no detectable labeling of pentons but binds efficiently to hexons at a site that is quite different from that of the mAb 8F5 epitope. Quantitative characterization of the interface between the capsid surface and the bound antibodies suggests that epitopes may be localized on this surface to within a resolution of the order of 1 nm, even though the nominal resolution of the reconstructions is only at the level of 3.5–4.0 nm (unpublished data). This conclusion is consistent with the observations of Wang *et al.* (18) on the binding of monoclonal Fabs to cowpea

mosaic virus, whose structure had previously been determined to high resolution by x-ray crystallography.

Of the three mAbs whose interactions with the HSV-1 capsid have been characterized to date, one is penton-specific, two are hexon-specific, and none of the three recognizes both kinds of capsomers. These observations establish that the antigenic character of the hexon is quite different from that of the penton. It follows, therefore, that either hexons and pentons are composed of different proteins or that the conformational adjustments that accompany accommodation of VP5 in the penton, as compared to the hexon state (33), are substantial in effect and distributed over the molecular surface. Recently, we have obtained results that support the latter alternative (W.W.N., unpublished data): treatment of purified capsids with appropriate concentrations of urea leads to quantitative extraction of the pentons,

detected by electron microscopy, accompanied by solubilization of $\approx 7\%$ of the VP5. Less plausible, but not entirely eliminated, is the possibility that the pentons are composed of some other viral protein, electrophoretically indistinguishable from VP5. In favor of the "VP5 as penton" scenario, earlier studies have documented conformational dependence of immunoreactivity, for instance, of a calmodulin epitope (34) in response to calcium binding.

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