Capsids form in the nuclei of cells infected with all herpesviruses. Herpes simplex virus (HSV) capsid pentons and hexons form spontaneously from five and six molecules of ICP5, respectively; these capsomeres are linked by triplexes consisting of two molecules of VP23 and one molecule of VP19C to form a porous procapsid (23, 36, 43). ICP5 is also associated with ICP35, which forms an internal shell or scaffold within the procapsid. The procapsid is believed to give rise to the three other types of capsids seen in HSV-infected cells, designated types A, B, and C. All of these capsids differ internally but contain identical outer shells, as determined by cryoelectron microscopy (21, 35, 49). Type B capsids retain the scaffold internal to the outer shell, type A capsids contain only the outer shell, and type C capsids lack the internal scaffold but contain viral DNA (14). Type C capsids then bud from the nuclear membrane in a reaction termed primary envelopment (19, 32).

One of the vertices of A, B, and C capsids is biochemically and structurally unique and has been designated the portal vertex. Thus, the U16-encoded protein (pU16) forms a dodecameric ring with an internal diameter of at least 65 Å, i.e., sufficiently wide to accommodate DNA as it is packaged into the capsid (44). Critical to the discovery of the portal was the observation that an antibody to the C terminus of pU16 is located at the external surface of the viral capsid (45).

Recent immunogold analysis of pU17 and pU15 supports their location on the external surface of the viral capsid on more than one vertex (24, 41). Although it is also required for DNA packaging, the precise function of U17 is unknown (34). Analysis of a U17 deletion mutant revealed an alteration of the normal intranuclear distributions of capsids and a number of viral proteins including pU6, ICP35, and ICP5 (39). These observations suggest that the U17 protein is involved in ensuring proper capsid assembly, the reorganization of the infected cell nucleus, or, directly or indirectly, capsid or protein transport within the nucleus. Relevant to this last possibility is the observation that HSV capsids are actively transported in the nucleus and that this transport is both energy and actin dependent (13). U17 is believed to enhance the stability of capsids and is required for the retention of full-length genomic DNA in the capsid (18, 24, 37, 41).

The hypotheses that pU16 serves as the portal, the U15, U17, and U133 proteins form the HSV terminase, and pU17 and pU18 contain viral DNA (22, 39).
Immunization of chickens with the purified fusion protein was approximately 1.3
multiplicity of infection of 5 PFU per cell. Lysates from ap-
Tris-HCl (pH 7.8), 150 mM NaCl, and 1 mM EDTA (TNE).

Capsids were either attached to Formvar carbon-coated electron microscopic grids or placed into microdialysis tubes (200-μm diameter), subsequently embedded in LRWhite, and sliced with a diamond knife into 60-nm sections that were then placed onto electron microscopy grids.

Previously described rabbit antisera directed against the C terminus of pUL6, the C terminus of pUL15, full-length pUL28, and full-length pUL33 were prepared by adsorption against capsids purified from Vero cells infected with 5.0 PFU/ml of the appropriate viral null mutant (4, 5, 27, 30, 38–40). The adsorbed antisera were then diluted 1:50 in PBS supplemented with 1% Triton-100 and 1% fish gelatin and applied directly to the electron microscopy grids, followed by extensive washing. Experiments performed with the pUL17-specific chicken IgY were similar except that the antibody was not preadsorbed and was diluted 1:5,000 for reactions with capsids. As a control, the capsid samples were also reacted separately with a polyclonal antiserum directed against the internal scaffold protein ICP35 (10) (NC 3-4) (kindly provided by Roselyn Eisenberg and Gary Cohen). Bound immunoglobulins remaining after the washing were recognized by goat anti-rabbit immunoglobulin conjugated to 12-nm gold beads or goat anti-chicken IgY conjugated to 12-nm gold beads. After further washing, the grids were viewed using a Philips 201 electron microscope after counterstaining with 2% aqueous uranyl acetate and 0.5% Reynold’s lead citrate. Only capsids that were visually verified as intact B capsids were included in the data. The B capsids were scored as positively immunolabeled only when a gold bead was observed in direct association with the capsid shell or interior.

The results are summarized in Table 1, and representative examples of immunostained capsids are shown in Fig. 2.

Examination of at least 400 capsids in each treatment group and data from multiple experiments revealed the following information.

(i) Background levels of immunostaining with the pUL15-, pUL17-, pUL28-, and pUL33-specific antisera, as revealed by the number of appropriate mutant capsids bearing gold beads, were significantly below similarly stained wild-type HSV-1(F) capsids. (All P values were <0.001 as assessed by Fisher’s exact t test.)

(ii) As shown previously (22), pUL6-specific epitopes were recognized on the surface of the capsid inasmuch as significantly more (P < 0.001) gold beads were present in intact wild-type capsids reacted with the pUL6-specific antiserum than in capsids lacking pUL6. These epitopes were detected more frequently in intact capsids than in sectioned capsids (P < 0.001), suggesting that the bulk of the epitopes were available primarily for reaction at the capsid surface rather than internal to the capsid shell. Approximately 16.3% (53 of 325) of capsids were labeled, suggesting either that the immunogold staining was insensitive and did not detect portal protein in many capsids or that many capsids lacked portals. Biochemical studies showing that populations of B capsids average 14.8 ±

FIG. 2. Digital image of representative electron micrographs of capsids labeled with various antibodies. Capsids were purified from cells infected with HSV-1(F). These were attached to copper mesh grids (left column) or were embedded in Lowicryl and sectioned (right column). Each row shows intact capsids and thin sections that reacted with antisera directed against the indicated proteins. Bound immunogold was identified by reactions with appropriate antiserum conjugated to 12-nm gold beads. Arrowheads indicate gold beads associated with capsids. Electron micrographs of immunogold analyses performed with pUL33- and pUL28-specific antibodies were similar to those of pUL17 (data not shown). A comprehensive analysis of the data is presented in Table 1.
2.6 copies of pUL6 per capsid (22), coupled with the high likelihood that the portal ring contains 12 copies of pUL6 (44), argue against the latter possibility.

(iii) As expected, the ICP35-specific antiserum did not recognize the external surface of capsids to an appreciable extent inasmuch as only 18 capsids of 1,007 capsids examined (0.018%) were labeled with the NC 3-4 antibody. Upon sectioning of the capsids, however, ICP35-specific epitopes were rendered significantly more immunoreactive with the antisera (P < 0.001), as revealed by increased labeling of sectioned capsids (115 [28%] of 413 sectioned capsids). These observations indicated that, as expected, ICP35 was present in the capsid interior rather than the capsid surface and verified that the inner surfaces of unsectioned capsids were sequenced from the applied antibodies under the experimental conditions used.

(iv) Epitopes from pUL17, pUL28, and pUL33 localized at the surface of the capsid, as revealed by immunoreactivity of intact capsids, which was significantly above background levels obtained upon reaction with the corresponding deletion virus capsids. (All P values were less than 0.001 by Fisher's exact test.) In all three cases, although immunoreactivity was present in sectioned HSV-1(F) capsids, the level of immunoreactivity was significantly lower than that obtained using intact capsids, presumably because a given thin section contains only a limited portion of the capsid surface. Another possibility is that embedding capsids could reduce the immunoreactivity of pUL17, pUL28, and pUL33. More capsids (13%) were labeled with the pUL17-specific antibody than with either the pUL28- or pUL33-specific antibody (8.7% and 8.9%, respectively). This could be a consequence of increased affinity of the pUL17-specific antibody relative to the other antibodies or increased amounts of pUL17 in association with capsids. Given the observation that only around two copies of pUL28 are present per B capsid, and the observation that pUL17 can localize to multiple vertices, it seems likely that more pUL17 is associated with capsids than pUL28 (7, 41). In any case, these data are consistent with other studies of pUL17 in HSV capsids showing that the protein is on the external capsid surface but are in contrast with the localization of the pUL17 homolog of pseudorabies virus that has been reported to associate with packaged DNA (16, 41).

(v) Antisera directed against C-terminal epitopes of pUL15 were recognized on the external surface of capsids, as revealed by the increased immunoreactivity of intact capsids compared to that of pUL15-negative capsids. Unlike the case with pUL6, pUL33, pUL28, and pUL17-specific antibodies, immunoreactivity of the pUL15-specific antibody remained high in sectioned capsids. A slight increase in immunoreactivity in sectioned capsids compared to that obtained with unsectioned capsids was not statistically significant (P = 0.06). On the other hand, because less external surface area of the capsid is represented in a 40- to 60-nm thin section, the preservation of immunoreactivity in sectioned capsids suggests that more pUL15 C-terminal epitopes were present within the capsid interior than were epitopes of pUL28, pUL17, or pUL33. It is unclear whether these observations represent the possibilities that (a) pUL15 extends from the external surface to the internal surface of the capsid, (b) the pUL15 epitopes are masked less efficiently upon embedding than pUL28, pUL17, or pUL33 epitopes, or (c) multiple copies of pUL15 are present at different locations within the capsid. Assuming that all B capsids are biochemically identical (an assumption that has not been tested), the observation that each B capsid contains only 1.2 copies of pUL15 (7) argues against the latter possibility.

(vi) Very few capsids that reacted with any of the antibodies contained more than one gold bead. This is in contrast to results reported previously by others (41) and may reflect the respective affinities of the different antibodies in the two studies.

Comparative resistance of capsid-associated proteins to protease digestion. To confirm the results obtained by immunogold labeling, B capsids were purified on continuous sucrose gradients as described above and divided into four equal pools of 250 μl. The aliquots were incubated in the absence of trypsin or in the presence of 10, 25, or 50 μg/ml trypsin (MP Biomedical) for 45 min at 37°C. The digested capsids were then diluted into 14 ml of ice-cold TNE containing protease inhibitors (1 tablet Complete protease inhibitors [Roche] per 50 ml TNE), and the digested capsids were pelleted in an SW41 rotor at 35,000 rpm for 2 h. Pelleted capsids were solubilized in approximately 50 μl denaturing buffer containing SDS, mercaptoethanol, and bromophenol blue, and 25 μl of each sample was electrophoretically separated on a single lane of an SDS-polyacrylamide gel, followed by immunoblotting with the indicated antibodies as described above, except that the primary antibodies were diluted as follows: rabbit anti-pUL6, 1/1,000; rabbit anti-ICP35, 1/1,000; rabbit anti-pUL15, 1/1,000; chicken anti-pUL17, 1/50,000; rabbit anti-pUL28, 1/5,000; and rabbit anti-pUL33, 1/500. Anti-rabbit and anti-chicken secondary antibodies conjugated to horseradish peroxidase were diluted 1/5,000, and bound immunoglobulin was revealed by ECL (Amersham). The results are shown in Fig. 3.

Unlike all the other proteins examined, ICP35 was not significantly affected by incubation with 10 μg/ml trypsin. Upon digestion with 25 μg/ml trypsin, however, the amounts of full-length ICP35 species were decreased, and a band that ran faster than the 27,000-Mr marker became apparent. In contrast, upon incubation with 10 μg/ml trypsin, pUL6 was partially cleaved to proteins with approximate Mr's of 40,000 and 30,000, and these bands remained detectable even upon digestion with up to 50 μg/ml trypsin.

In contrast to the results obtained with ICP35 and pUL6, immunoreactivity of pUL15 was completely eliminated upon digestion with 10 μg/ml trypsin, whereas digestion at this concentration significantly reduced but did not eliminate reactivity with pUL17-specific and pUL33-specific antibodies. Incubation with concentrations higher than 10 μg/ml of trypsin completely eliminated pUL17 and pUL33 immunoreactivity. Digestion of capsids with 10 μg/ml trypsin cleaved pUL28 into a prominent band containing a protein with an apparent Mr of 60,000, whereas concentrations of trypsin higher than 10 μg/ml precluded the detection of any pUL28-specific immunoreactivity. These data indicate that ICP35, a protein located within the capsid interior, is more resistant to trypsin digestion than pUL6, pUL15, pUL17, pUL28, and pUL33 located at the external surface of the viral capsid. These observations are consistent with the hypotheses that
pUL15, pUL28, and pUL33 represent the viral terminase inasmuch as their external location would facilitate an interaction with DNA as it is being packaged. One model that is also supported by the presence of some pUL15 epitopes in sectioned capsids (Table 1) is that pUL15 is more intimately associated with the capsid, whereas pUL28 is located more peripherally. This is consistent with the observation that empty capsids that are believed to have engaged but not retained DNA (A capsids) contain approximately 12 copies of pUL15 but less than 1 copy of pUL28 on average (7). Thus, consistent with its DNA binding activity (2), pUL28 may associate with DNA as it is expelled and may thereby become lost from the A capsid. In contrast, pUL17 may be located externally to either stabilize the capsid or capsomeres or engage molecular motors for capsid transport in the nucleus or cytoplasm. As shown previously by others, the presence of pUL17 at multiple vertices is consistent with these possibilities (41).

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