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The 45-Kilodalton Protein of Cytomegalovirus (Colburn) B-Capsids Is an Amino-Terminal Extension Form of the Assembly Protein

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Intranuclear B-capsids from cytomegalovirus (strain Colburn)-infected cells contain an abundant 37-kDa assembly protein, thought to be involved in capsid formation, and three minor protein constituents (i.e., 45, 39, and 38 kDa) that are immunologically and structurally related to the assembly protein. In the experiments reported here, antisera produced against synthetic peptides were used in conjunction with chemical protein cleavage to examine the structural relationship of these proteins in more detail. Results of these experiments verify that the carboxyl end of the 39-kDa assembly protein precursor is lost during maturation and suggest that the 38-kDa protein may be a processing intermediate. It is shown that the 45-kDa protein is coterminal with the mature assembly protein at its carboxyl end but differs by a predicted 115-amino-acid extension at its amino terminus. In addition, evidence is presented that the 45-kDa protein has a 48-kDa precursor and a 47-kDa putative processing intermediate which have the same carboxy-terminal sequences and undergo the same maturational events as those of the assembly protein. A working model considering the structural relationship of these proteins is presented.

Herpesvirus-infected cells typically contain an intranuclear capsid form (e.g., B-capsid) that is distinguished by the presence of an abundant 35- to 40-kDa phosphoprotein, referred to as the assembly protein (7, 12). This herpesvirus group-common protein, which is not found in the mature virion, is thought to be involved in capsid assembly and perhaps DNA packaging and may be analogous to the bacteriophage scaffolding protein (10, 14, 20, 21, 26). Several immunologically related forms of the assembly protein have been detected in herpes simplex virus- and varicella-zoster virus-infected cells and capsids (2, 6, 21), and there are reports that small amounts of these proteins are also present in virions of herpes simplex virus (22, 24).

The 37-kDa assembly protein of cytomegalovirus (CMV; strain Colburn) is derived by proteolytic cleavage from a precursor (10), corresponding to the 39-kDa B-capsid protein. In addition to the assembly protein, small amounts of its precursor, and a suspected processing intermediate referred to as the 38-kDa protein (10, 12), B-capsids of strain Colburn contain a third structurally and immunologically related species referred to as the 45-kDa protein (7, 10). Because the 45-kDa protein is larger than either the assembly protein or its precursor, it must be the product of a larger open reading frame (ORF) or undergo posttranslational processing different from that of the assembly protein.

Recent nucleotide sequencing data indicated that the ORF encoding the assembly protein of strain Colburn is the 3′ end of a larger ORF, itself predicted to encode a 42.5-kDa protein (18a, 23). To determine whether the 45-kDa, B-capsid protein may be a translation product of this larger ORF and to further investigate the relatedness of the 45-, 39-, and 38-kDa B-capsid proteins to the assembly protein, the predicted amino acid sequence of the larger ORF was used to design specific immunological and chemical cleavage assays.

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MATERIALS AND METHODS

Cells, virus, and B-capsid isolation. CMV strain Colburn (7, 8) was grown in human foreskin fibroblast cells (7); B-capsids were recovered from the nuclear fraction of infected cells following sonication as described before (17).

Antisera. Peptides that represented the carboxy terminus (N′-PPKDMVKLNRRLFVAALNKME-C′) and amino terminus (N′-SHPMSAVATPAAS-C′) of the CMV (Colburn) assembly protein (23) were synthesized. An amino-terminal cysteine was added to each peptide during synthesis to enable it to be coupled (5) to keyhole limpet hemocyanin. Coupled peptide was combined with Freund's adjuvant and injected into rabbits subcutaneously and intramuscularly. Approximately 5 mg per rabbit was used in the primary injections, and comparable amounts were injected in two boosters approximately 6 and 12 weeks later. Sera prepared from the same animals prior to immunization showed no specific reactivity.

Gel electrophoresis, immunoassays, and peptide analyses. Electrophoretic separation of proteins in sodium dodecyl sulfate (SDS)-containing polyacrylamide gels was done essentially as described before (15). Immunoassays were done essentially as previously described by Towbin et al. (28): (i) SDS-polyacrylamide gel electrophoresis (PAGE)-separated proteins or peptides were electrotransferred to nitrocellulose (BA 83; Schleicher and Schuell), (ii) 5% bovine serum albumin (BSA) in 10 mM Tris-HCl (pH 7.4)–0.15 M sodium chloride was used for blocking overnight at 4°C, (iii) antisera were used at dilutions ranging from 1:20 to 1:150, and (iv) bound antibody was visualized by using either ^{125}I-protein A (3) or a second antibody-alkaline phosphatase colorimetric assay (4, 24). Peptide comparisons were done essentially as described before (1, 18): proteins were separated by SDS-PAGE, the relevant portion of the gel was excised and treated with 0.025 M *N*-chlorosuccinimide (NCS; Aldrich Chemical Co., Inc., Milwaukee, Wis.), and the resulting fragments were separated by second-dimension SDS-PAGE in 16% diallyltartardiamide cross-linked gels. Peptides were visualized in the second gel by fluorography (16) or electro-

transferred to nitrocellulose (BA 83) and visualized by fluorography and by the colorimetric immunoassay referenced above.

Cleavage at cysteine. B-capsids were reduced and cyanylated (13, 27). The modified proteins were cleaved at cysteine residues by increasing the pH to 9 with 1 M Trizma base (Sigma, St. Louis, Mo.), pH 11, and then incubating the preparation for 15 to 30 min at ambient temperature (~25°C). Preparations were boiled in sample buffer (2% SDS, 5% β-mercaptoethanol, 5% glycerol, 0.1 M Tris [pH 7.0]), subjected to SDS-PAGE in a 10% gel, immunoassayed with Anti-N1 followed by ^{125}I-protein A, and visualized by fluorography as outlined above.

RESULTS AND DISCUSSION

The hypothetical relationship of the 45-kDa protein to the assembly protein and to other B-capsid proteins is presented for reference in Fig. 1. Specific landmarks include (i) cysteines, tryptophans, and translational start methionines; (ii) the putative maturational cleavage site; and (iii) the regions recognized by the antipeptide sera, Anti-N1 and Anti-C1 (Fig. 1A). Additionally, the peptide fragments expected from the chemical cleavage experiments described below are shown in Fig. 1B and C.

Three experiments were done to test the relationships shown in Fig. 1A. In the first, rabbit antisera to synthetic peptides representing the amino and carboxy termini (Anti-N1 and Anti-C1, respectively) of the assembly protein precursor were produced and then used to test for the presence of corresponding sequences in the other known (i.e., 45- and 38-kDa) and predicted (i.e., pre-45 and 47-kDa) proteins shown in Fig. 1A. A third antiserum prepared against SDS-PAGE-purified assembly protein, previously shown to recognize the 45-, 39-, and 38-kDa proteins in addition to the assembly protein (10), was used as a reference. These sera were used in Towbin immunoassays (Fig. 2). Anti-N1 was found to react with the assembly protein in nonlabeled B-capsids (Fig. 2, B Cap.) and with its 39-kDa precursor and the 38-kDa protein. Anti-N1 also reacted with the 45-kDa protein and two larger bands, corresponding to the 47- and 48-kDa proteins shown in Fig. 1A. Thus, all six proteins indicated in Fig. 1 contain the amino terminus of the assembly protein precursor as evidenced by their reactivity with Anti-N1. Anti-C1, in contrast, reacted with the 39-kDa assembly protein precursor in nonlabeled B-capsids (Fig. 2, B Cap.) but did not react with either the mature assembly protein or the 38-kDa protein, thereby confirming that the carboxyl end of the precursor is absent from the processed forms (10). In apparent correspondence, the 45- and 47-kDa proteins were not recognized by Anti-C1, but their 48-kDa putative precursor was reactive. This indication that the precursor of the 45-kDa protein and the assembly protein have the same carboxy-terminal sequence, which is removed during maturational processing, suggested that the extension distinguishing the 45-kDa protein from the assembly protein is at the amino end (Fig. 1). In agreement with earlier results obtained from pulse-chase radiolabeling experiments (7, 10), the mature assembly protein partitioned more strongly with the nuclear fraction than with the cytoplasmic fraction (cell fractions labeled with [^{35}S]methionine), whereas its precursor was relatively more abundant in the cytoplasmic fraction (Fig. 2, Nuc. and Cyto. lanes of Anti-N1 and Anti-C1 panels, respectively).

The second experiment was done to examine the structure of these related proteins by peptide comparison and took

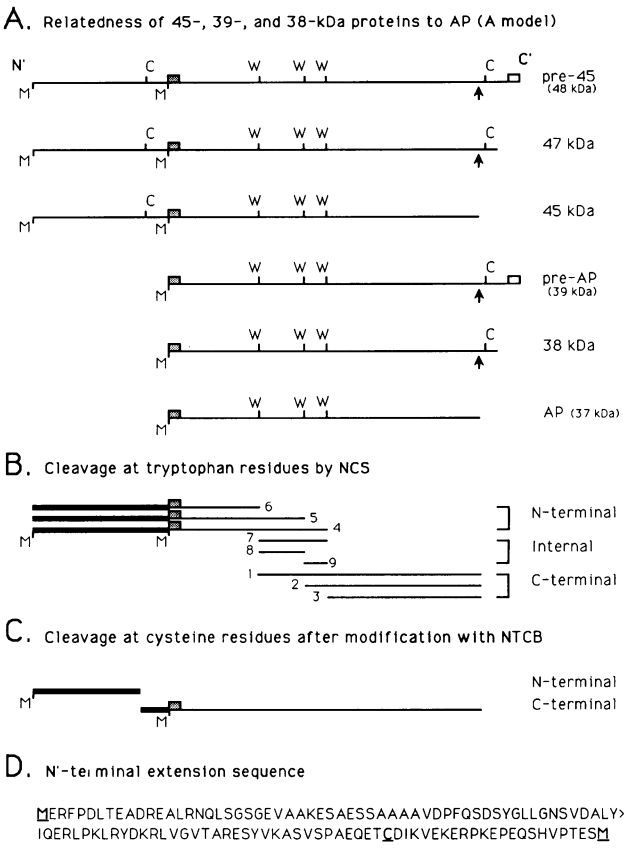

FIG. 1. Model depicting the hypothetical relationship between the 37-kDa assembly protein and the closely related, lower-abundance 45-, 39-, and 38-kDa proteins. (A) Schematic based on the deduced amino acid sequence of the CMV strain Colburn assembly protein (10, 23) and its in-frame amino-terminal extension (18a), showing the positions of all predicted cysteine (C) and tryptophan (W) residues in the assembly protein (AP) and 45-kDa protein (45 kDa) and in their respective precursors, i.e., 39 kDa (pre-AP) and 48 kDa (pre-45), and putative processing intermediates, i.e., 38- and 47-kDa proteins. The translational start site methionines (M) are indicated for reference. The putative maturational cleavage site in the precursor molecules (10) (↑), the amino-terminal peptide used to prepare Anti-N1 (▥), and the carboxy-terminal peptide used to prepare Anti-C1 (□) are indicated. (B) Fragments of the assembly protein and 45-kDa protein generated by cleavage at tryptophan residues with NCS. All fragments are predicted to contain at least one tyrosine (23). The carboxyl and internal fragments of both proteins are predicted to have corresponding relative sizes, but the amino-terminal fragments of the 45-kDa protein are predicted to be elongated by a 115-amino-acid sequence (indicated by thickened lines). Fragments are numbered 1 (largest) to 9 (smallest) and can be seen in Fig. 3. (C) Fragments of the 45-kDa protein generated by cleavage at cysteine following treatment with 2-nitro-5-thiocyanobenzoic acid. Thickened line shows that the predicted amino-terminal extension of the 45-kDa protein is cleaved to generate a short fragment and an elongated assembly protein sequence containing the N1 peptide (▥). Thickened lines represent the 115-amino-acid sequence distinguishing the 45-kDa protein from the assembly protein. (D) Predicted amino acid sequence of the in-frame amino-terminal extension that distinguishes the 45-kDa protein from the 37-kDa assembly protein. Methionine residues (M) represent the predicted translational start sites of the 45-kDa protein and the assembly protein. The similarly highlighted cysteine residue (C) represents the predicted 2-nitro-5-thiocyanobenzoic acid cleavage site indicated in panel C.

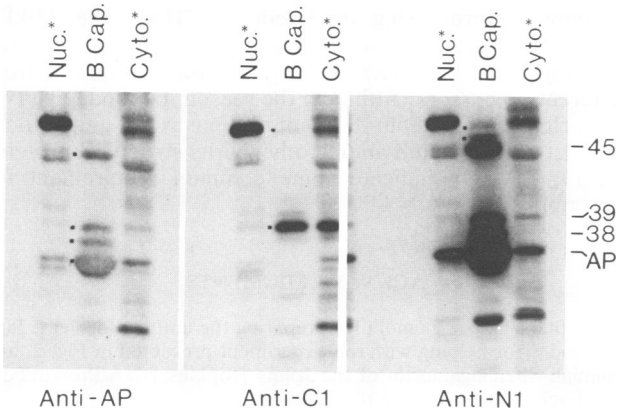

FIG. 2. Maturational processing removes carboxyl but not amino ends of 45-kDa and assembly protein precursors. Nonlabeled intranuclear B-capsids (B Cap.) were isolated, solubilized, subjected to SDS-PAGE, electrotransferred to nitrocellulose, and probed with rabbit antisera to the whole assembly protein (Anti-AP) or to synthetic peptides representing the carboxyl (Anti-C1) and amino ends (Anti-N1) of the assembly protein precursor (see boxes in Fig. 1A). [^{35}S]methionine labeled (*) proteins from the nuclear (Nuc.) and cytoplasmic (Cyto.) fractions of Colburn CMV-infected cells were separated in adjacent lanes to provide size markers (e.g., DB51, the heavily labeled ~50-kDa protein in nuclear fraction). The pre-45 and 47-kDa proteins are best seen (indicated by dots) above the 45-kDa band in the B Cap. lane of the Anti-N1 panel. The assembly protein precursor (pre-AP, 39 kDa) and putative 45-kDa protein precursor (pre-45, very faint 48-kDa band) are indicated by dots to the left of the B Cap. lane in the Anti-C1 panel. The 45-, 39-, and 38-kDa proteins and the assembly protein are indicated by dots in the Anti-AP panel.

advantage of the small number of tryptophan residues for cleavage and the fortuitous location of at least one tyrosine per fragment for radioiodination (18a, 23). ^{125}I-labeled B-capsid proteins were separated by SDS-PAGE and treated with NCS to cleave after tryptophan residues, and the resulting peptides were separated by second-dimension SDS-PAGE. Autoradiograms prepared from the second-dimension gel (Fig. 3A and C) showed that the assembly protein was cleaved into nine fragments, as predicted in Fig. 1B. The largest of these (i.e., fragments 1 to 3), representing the three carboxy-terminal fragments (Fig. 1A and B), comigrated with three fragments in the 45-kDa protein. The intermediate size fragments (i.e., fragments 4 to 6) of the assembly protein, representing its three amino-terminal fragments (Fig. 1B and 3A), had no size counterparts in the 45-kDa protein. However, the 45-kDa protein did contain three larger fragments that were not present in the assembly protein and that were immunoreactive with Anti-N1 (Fig. 3C). These findings are consistent with the immunological results presented above and establish that the 45-kDa protein has the same carboxyl end as the assembly protein but differs at its amino terminus. It can also be seen in the original autoradiogram used to prepare Fig. 3A that the 39- and 38-kDa proteins (indicated by open circles to the left of AP) contain fragments that comigrate with the three amino-terminal fragments of the assembly protein (i.e., fragments 4 to 6) but have slightly larger carboxy-terminal fragments which reflect the M_rs of the three proteins, i.e., 39 kDa > 38 kDa > assembly protein. This result is presented diagrammatically in Fig. 3B and supports the conclusion that the 38- and 39-kDa proteins differ from each other and the assembly protein only at their carboxyl ends (Fig. 1A). Several larger proteins appeared to have some as-yet-undetermined structural relatedness to the 45-kDa protein (circles and arrow to the left of 45K, Fig. 3A).

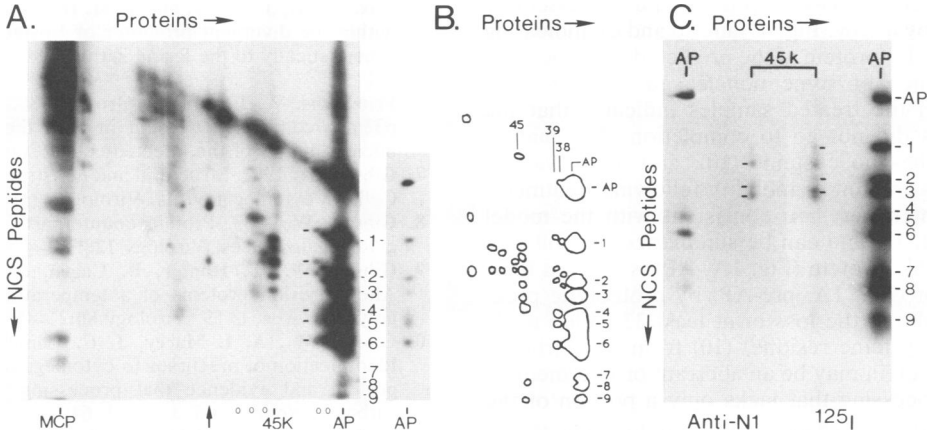

FIG. 3. Assembly protein, 38-kDa protein, and 39-kDa assembly protein precursor differ at their carboxyl ends, and assembly protein and 45-kDa protein differ at their amino ends. (A) B-capsid proteins were ^{125}I labeled, separated by SDS-PAGE, and cleaved in situ at tryptophan residues by NCS as shown in Fig. 1B, and the resulting fragments were resolved in a second-dimension gel and visualized by autoradiography. AP lane at right of panel A is lighter exposure of the same gel made to show the assembly protein fragments, which are indicated numerically as follows: 1 to 3, carboxyl; 4 to 6, amino; and 7 to 9, internal. The internal fragments are better seen in panel B. Some variation in the relative mobility of amino-terminal fragment 5 of the 45-kDa protein was noted and attributed to compositional differences in the second-dimension gel but has not been further investigated. MCP, Position of major capsid protein. (B) Diagram made by tracing the relevant spots in the autoradiogram shown in panel A. (C) ^{125}I-labeled B-capsid proteins were separated by SDS-PAGE, cleaved in situ with NCS, separated in a second-dimension gel, electrotransferred to nitrocellulose, and probed with Anti-N1 followed by a second antibody coupled to alkaline phosphatase to visualize the immunoreactive fragments. The immunoimage (left side of the panel) shows the amino-terminal NCS fragments (i.e., no. 4 to 6) of the assembly protein and three larger fragments (lines) from the 45-kDa protein. The positions of all NCS fragments on the same blot (mirror image) is shown in the right portion (^{125}I) of the panel. The three carboxy-terminal fragments of the 45-kDa protein (i.e., no. 1 to 3, indicated by lines) are the same sizes as those in the assembly protein (see Fig. 1B) and were resolved from the three immunoreactive fragments.

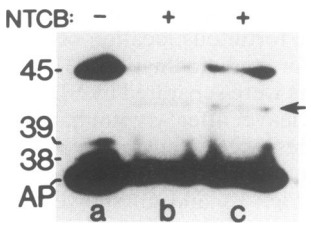

FIG. 4. Presence and location of cysteine residue in 45-kDa protein are consistent with model shown in Fig. 1. Purified B-capsids were treated with 2-nitro-5-thiocyanobenzoic acid followed by alkaline pH for 15 min (lane c) or 30 min (lane b) to cleave proteins at cysteine residues or neutral pH for 30 min as a noncleaved control (lane a). The proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose, and probed with Anti-N1 followed by ^{125}I-protein A. Positions are indicated for the assembly protein (AP) and 45-, 39-, and 38-kDa proteins, as well as the cleavage product of the 45-kDa protein (←).

The purpose of the third experiment was to verify the amino acid sequence of the extension portion of the 45-kDa protein. It was expected that the 115-amino-acid extension (Fig. 1D) would contain the sole cysteine residue in the mature protein (18a). Cleavage at that cysteine would be predicted to yield a large fragment containing the entire assembly protein plus a 22-amino-acid extension and a small 93-amino-acid fragment representing the amino terminus of the 45-kDa protein (Fig. 1C). To test this prediction, B-capsid proteins were cleaved at cysteine residues in mild base following cyanylation, subjected to SDS-PAGE, and then immunoassayed with the Anti-N1 serum. Nontreated B-capsids (Fig. 4, lane a) were compared in parallel and served to identify the positions of the assembly protein and the 45-, 38-, and 39-kDa proteins, all of which are reactive with Anti-N1 (Fig. 2). The treated B-capsid preparations showed a new band between the 45-kDa protein and the assembly protein (indicated by arrow, Fig. 4, lanes b and c), indicating cleavage of the 45-kDa protein at the predicted cysteine (Fig. 1C). The persistence of some noncleaved 45-, 39-, and 38-kDa proteins in the treated samples indicates that the chemical reactions did not go to completion. The mature assembly protein has no cysteine (10), and there was no change noted in its amount or mobility following treatment.

The data presented here are consistent with the model diagrammed in Fig. 1A and can be summarized as follows. The 37-kDa assembly protein (Fig. 1A, AP) is derived from a 39-kDa precursor (Fig. 1A, pre-AP) by proteolytic processing, which results in the loss of at least 32 amino acids (i.e., through the cysteine residue) (10) from the carboxyl end. The 38-kDa protein may be an aberrant or intermediate product of this processing that lacks only a portion of the sequence missing from the assembly protein (i.e., at least the terminal 22 residues that react with Anti-C1). If the 38-kDa protein is such an intermediate, a multistep processing pathway rather than a single endoproteolytic cleavage event would be indicated. The 45-kDa protein differs from the assembly protein by an amino-terminal extension, which includes a single cysteine residue that presumably accounts for dimerization of the protein in B-capsids (10). At their carboxyl ends, however, the 45-kDa protein and the assembly protein are more similar, if not identical. Both appear to be derived from 2- to 3-kDa-larger precursors, the pre-45 and pre-AP, by a proteolytic event that eliminates the carboxyl end of the precursor and that may involve the 47- and 38-kDa proteins as processing intermediates. Thus, the 37-kDa assembly protein and the 45-kDa protein are closely related and appear to be derived by a common mechanism from different precursors. Although the reason for producing two such closely related but different proteins is not understood, the fact that it occurs among other virus structural proteins (9, 19, 25) may indicate some common requirement for assembly.

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