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Characterization of Two Conformational Forms of the Major DNA-Binding Protein Encoded by Herpes Simplex Virus 1

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We have resolved two electrophoretic species of the major DNA-binding protein, infected cell polypeptide 8 (ICP8), encoded by herpes simplex virus 1. In pulse-chase experiments, we observed the conversion of the ICP8a form, the slower migrating species, to the faster migrating form, ICP8b. Thus, the two species appear to be related as precursor-product. The conversion was not due to proteolytic cleavage, because higher concentrations of reducing agents in the sample buffer shifted the faster moving form to the slower moving species. Also, the two forms have identical peptide patterns as analyzed by partial proteolysis in sodium dodecyl sulfate. Thus, the faster moving species appears to be a conformational isomer containing intramolecular disulfide bonds. The functional significance of the two forms of the protein is discussed.

Herpes simplex virus (HSV) encodes a major DNA-binding protein (2, 14, 15, 18) as a beta or delayed early gene product (8, 9). This protein has been called ICP8 (infected cell polypeptide 8), VP130 (viral protein of molecular weight 130,000), or ICSP 11/12 (infected cell-specific protein 11/12) (2, 8, 9, 11, 12, 14, 15, 18). Two lines of evidence indicate that the protein plays a role in the replication of viral DNA: (i) mutant viruses which encode a defective DNA-binding protein or whose temperature-sensitive lesions map in or near the ICP8 gene are defective for DNA replication (4, 14); and (ii) monospecific antiserum to the HSV type 2 (HSV-2) protein inhibits DNA replication in vitro but does not neutralize the viral DNA polymerase activity (14). The HSV-2 protein ICSP 11/12 has been reported to bind more tightly to single-stranded DNA than to double-stranded DNA (16), and the purified HSV-1 and HSV-2 DNA binding proteins can enhance the denaturation of a polydeoxyadenylic acid-polydeoxythymidylic acid duplex (14). In infected cells, the protein is localized through a series of stages to the nucleus, where it finally binds to viral DNA (6, 10, 13). Thus, ICP8 appears to be involved in the replication of HSV DNA in the cell nucleus.

No posttranslational modifications of this protein have been reported. It appears to lack phosphorylation (18). Others have reported two closely migrating protein bands near the size of ICP8 in the bound fraction from a DNA-cellulose column when extracts from HSV-2-infected cells were used (16, 18). In this communication, we define the relationship between two electrophoretic forms of the HSV-1 ICP8 protein molecule.

We observed a pair of closely migrating bands in the region of a polyacrylamide gel where ICP8, the major DNA-binding protein encoded by HSV, migrates (Fig. 1). The two bands were present in extracts prepared from cells labeled at 4 h postinfection. The molecular weights of the two bands were determined to be 127,000 and 125,000 by comparison with the migration of proteins of known molecular weight (not shown). Because we believe these two bands to be two different forms of ICP8, we have named the slower migrating band ICP8a and the faster migrating band ICP8b.

The two bands were both present in extracts of cells immediately after a 5-min pulse label (Fig. 1). During the 2-h chase period, there was a progressive conversion to the ICP8b form so that by 2 h of chase, the ICP8b form was the predominant form. Figure 2 shows the microdensitometer tracing of the ICP8 region of a gel from a similar pulse-chase experiment. During the chase period, the amount of the ICP8a form decreased, and the amount of the ICP8b form increased. Thus, it appeared that the ICP8a form was the precursor to the ICP8b form, although the conversion was never complete.

The time of expression of the two bands corresponded to that for a beta or delayed early gene in that both were labeled at maximal rates at 3 to 6 h postinfection (not shown). Also, the period of expression of the two species was extended to at least 10 h postinfection by the incubation of infected cells in medium contain-

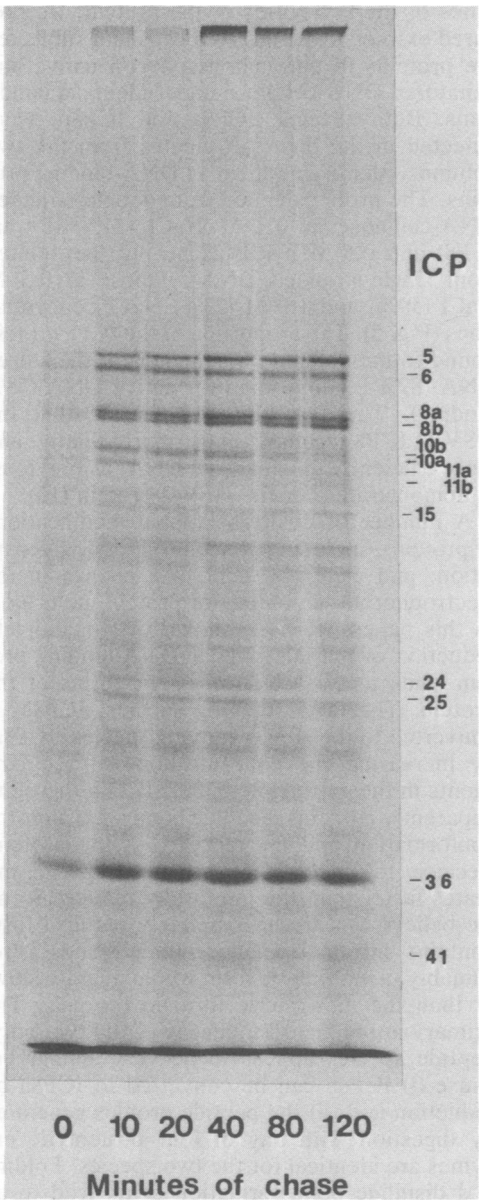

FIG. 1. Pulse-chase labeling of the two forms of ICP8. Cultures of Vero cells infected with HSV-1 strain mP (multiplicity of infection, 20; reference 5) were pulse-labeled for 5 min at 4 h postinfection with 12 μCi of [^{35}S]methionine per ml in methionine-free minimal essential medium supplemented with 1% dialyzed calf serum. Unlabeled methionine was added to 1 mM, and cultures were harvested at the times shown during the chase period. At the times indicated, the flasks were immersed in an ice bath, and the monolayers were washed with phosphate-buffered saline and dissolved in gel sample buffer (8) containing 0.5% mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride. The extracts were subjected to electrophoresis in a 9.25% polyacrylamide gel. An autoradiogram of the dried gel is shown.

ing phosphonoacetate (not shown). This drug blocks viral DNA replication, lowers or completely inhibits the expression of late proteins, and prolongs the expression of beta proteins (4). The alpha or immediate early viral protein ICP0, known to migrate in the same region of the gel, migrated slightly faster than this doublet and was not labeled with these kinetics. Thus, the two protein species, ICP8a and ICP8b, possess the properties of a beta protein.

Because we had prepared the cellular extracts in the previous experiments in sample buffer containing 0.5% mercaptoethanol before electrophoresis, we determined whether the two species might be due to incomplete reduction of ICP8. We treated extracts with increasing levels of dithiothreitol (DTT) in an attempt to ensure complete reduction of ICP8. The extract prepared in 0.5% mercaptoethanol largely contained the ICP8b form (Fig. 3, lane a). However, when DTT was added to the extracts before boiling, the ICP8b form was converted to the ICP8a form. The conversion was also more complete as higher levels of DTT were added (Fig. 3, lanes b through e). These results argue that the ICP8b form is an incompletely reduced form of ICP8 and migrates faster due to a folded structure held by intramolecular disulfide bonds.

To further examine the relationship between the two polypeptide species, we excised the bands from unfixed, dried gels and subjected the proteins to partial proteolysis in sodium dodecyl sulfate by the method of Cleveland et al. (3). Cleavage of ICP8a and ICP8b by the *Staphylococcus aureus* V8 protease yielded identical patterns of peptides (Fig. 4, lanes 1 to 8). Digestion with elastase yielded a variety of sizes of peptide products, and again the profiles with the

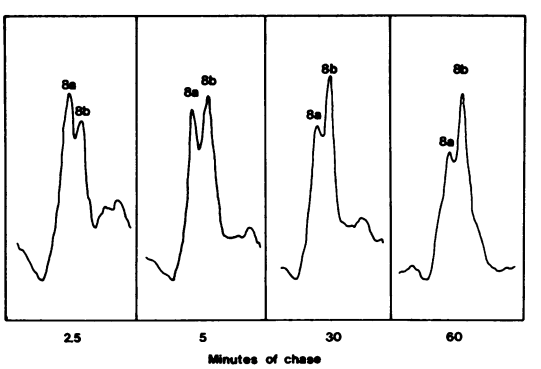

FIG. 2. Conversion of ICP8a to ICP8b during a chase period. Cultures of infected cells were labeled and subjected to chase conditions as described in the legend to Fig. 1. The cultures were harvested at the times shown, and extracts were prepared and subjected to electrophoresis. The microdensitometer (Joyce-Loebl) tracing of the ICP8 species from the resulting autoradiogram is shown.

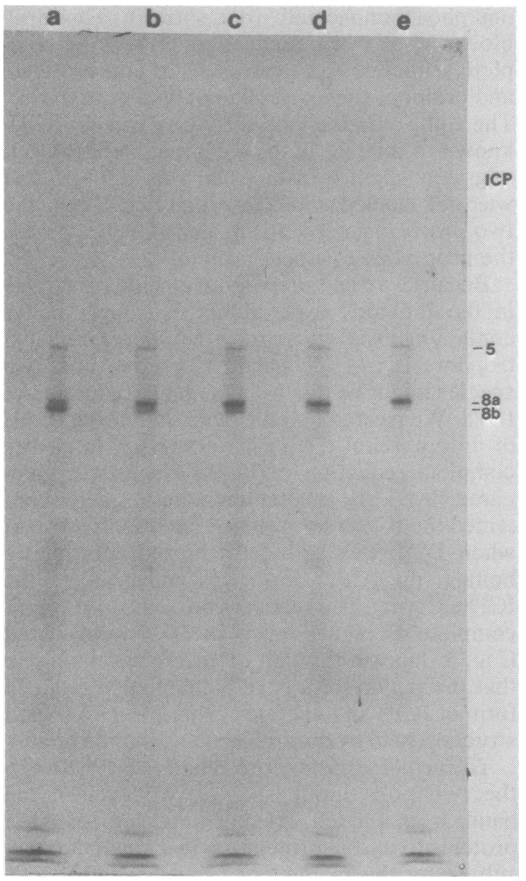

FIG. 3. Conversion of ICP8b to ICP8a by reduction. A nuclear preparation was prepared from infected cells by Dounce homogenization followed by washing of the nuclei with 1% Nonidet P-40–0.5% deoxycholate. The nuclei were dissolved in gel sample buffer containing 0.5% mercaptoethanol. The extract was divided into 5 aliquots, and DTT was added to the following concentrations before boiling of the samples and electrophoresis: a, no additions; b, 10 mM; c, 20 mM; d, 40 mM; e, 100 mM. An autoradiogram of the dried gel is shown.

ICP8a and ICP8b species were identical (Fig. 4, lanes 9 to 12). The profiles generated by digestion of the two species with chymotrypsin and papain were also identical (not shown). The two species therefore appeared to be very similar in their primary amino acid sequence. These results support the idea that the ICP8a form represents the unfolded form of the protein and that by oxidation it is converted to the disulfide-bonded, folded form (ICP8b).

We have previously reported that both forms can be detected in both the cytoplasm and nucleus of infected cells (10). Thus, the conversion was not specific to one compartment of the infected cell. To further test the functional properties of the two forms of the protein, we prepared extracts from infected cells and subjected the proteins to chromatography on native and denatured DNA-cellulose under identical conditions. Both species, ICP8a and ICP8b, were detected in the high salt eluates from the two columns, identifying them as DNA-binding proteins. The proteins eluted from double-stranded DNA-cellulose at 0.3 M NaCl (34% of total ICP8) and 0.5 M NaCl (22%), but they eluted from single-stranded DNA-cellulose at 0.5 M NaCl (59%) and 1.0 M (23%) NaCl concentrations (Fig. 5). The proportion of the ICP8 protein bound to the columns was higher for denatured DNA (89% binding) than for native DNA (58% binding). These results demonstrate that the HSV-1 ICP8 protein binds more tightly and more efficiently to denatured DNA, as previously demonstrated for the HSV-2 protein (16).

A number of posttranslational modifications of proteins, including phosphorylation, glycosylation, and cleavage, lead to a change in the electrophoretic mobilities of protein molecules. In this paper, we have shown that incomplete reduction of the HSV major DNA-binding protein leads to two electrophoretic forms of the protein. The faster moving species, ICP8b, is converted to the slower moving species, ICP8a, by increasing the concentrations of reducing agents in the gel sample buffer. ICP8b therefore apparently contains disulfide bonds that alter the conformation and the mobility of the protein. Because the incompletely reduced species migrates faster than the more fully reduced form, we believe that the incompletely reduced form contains intramolecular disulfide bonds. Presumably, a multimeric form would migrate slower than the monomeric form in the gels. The primary amino acid sequences of the two polypeptide species appear to be very similar because (i) ICP8b can be converted to ICP8a by reduction and (ii) the peptide profiles generated by digestion with any of four proteolytic enzymes are identical for the two species. Folding and disulfide bond formation could lead to an altered conformation that allows the protein to move faster through the gel matrix or alters the binding of sodium dodecyl sulfate to the protein.

Several types of evidence suggest that both forms may exist in the infected cell. (i) We observed a progressive conversion of the ICP8a form to the ICP8b form, the extent of which was dependent on the time of chase. Thus, within one polyacrylamide gel, the state of the protein was different when extracted from cells at different times. (ii) Our preparation of extracts in sample buffer containing 0.5% mercaptoethanol probably precluded the spontaneous formation of disulfide bonds (7). (iii) We detected a similar doublet in the ICP8 region of gels in which the

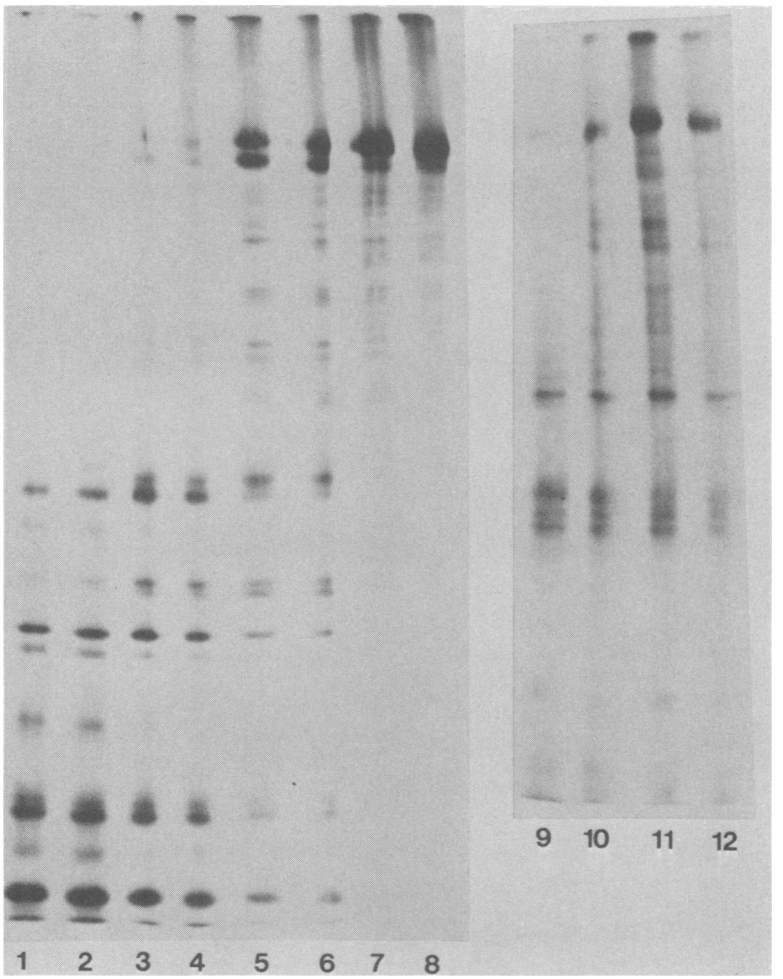

FIG. 4. Partial proteolysis of ICP8a and ICP8b species. ICP8a and ICP8b in labeled nuclear extracts were resolved on 9.25% polyacylamide gels. The gels were dried and subjected to autoradiography without fixation because fixation of gels led to precipitation of ICP8 in a form that could not be electroeluted (M. Quinlan, unpublished observation). The autoradiogram was used as a template to cut out the a and b bands from the dried gel. The remainder of the procedure was similar to that of Cleveland et al. (3), except that the dried gel bands were soaked for 30 min in a solution containing 12.5 mM Tris-hydrochloride (pH 6.8), 0.1% sodium dodecyl sulfate, 1 mM EDTA, and 30 mM DTT, as described by Smith and Hightower (17). DTT was included in an attempt to completely reduce the two proteins and make them equally susceptible to proteases. Shown is a fluorogram of a 15% gel in which these two proteins were incubated with various concentrations of protease. Odd-numbered lanes contained ICP8a. Even-numbered lanes contained ICP8b. The amounts of protease added to each well were as follows: 1 and 2, 0.5 μg of *S. aureus* V8 protease; 3 and 4, 0.1 μg of V8 protease; 5 and 6, 0.025 μg of V8 protease; 7 and 8, 0.005 μg of V8 protease; 9 and 10, 0.1 μg of elastase; 11 and 12, 0.025 μg of elastase.

residual ammonium persulfate had been removed by preelectrophoresis or in which extracts reacted with *N*-ethyl maleimide were run (not shown). Thus, at least a portion of the oxidized form was not due to oxidation of the protein within the gel.

The conversion from the ICP8a form to the ICP8b form appears not to be dependent on the cellular location of the protein. Furthermore, our experiments suggest that both forms of the protein can bind to single-stranded DNA. At this time, we cannot correlate this conformational change with any stage of maturation or function of the protein. We envision the conversion as a progressive oxidation of the ICP8 molecule, leading to increased disulfide bonding during its lifetime within the infected cell. The disulfide bonding may be facilitated by the tertiary struc-

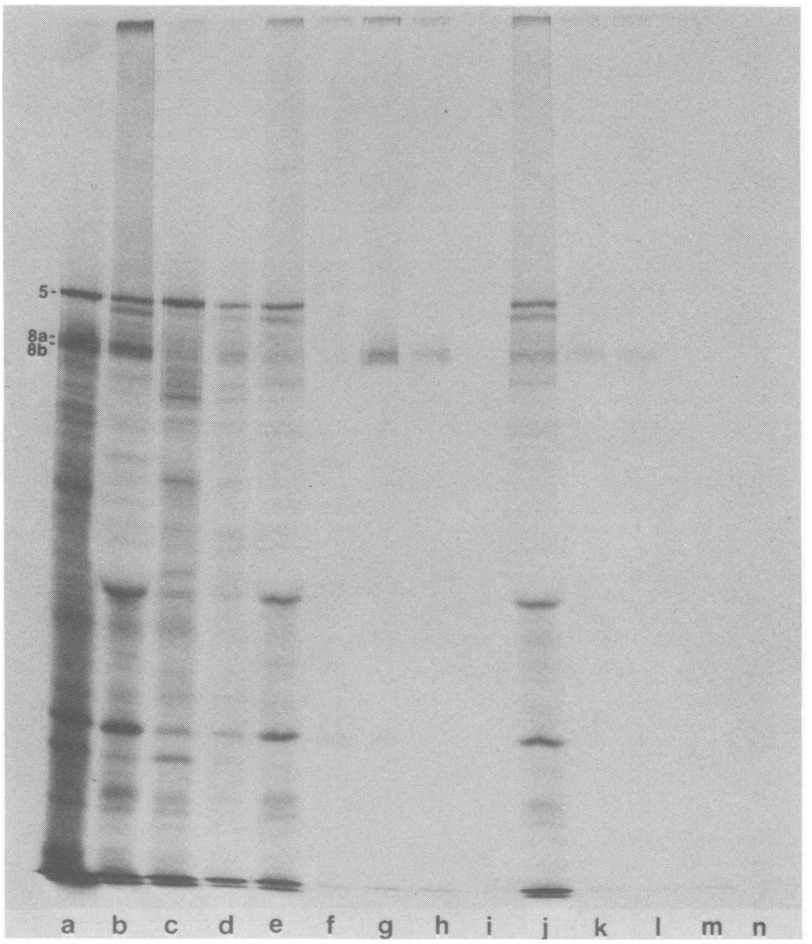

FIG. 5. DNA-cellulose chromatography of infected cell extracts. Labeled infected cell extracts were prepared by a modification of the methods of Alberts and Herrick (1) and Powell and Purifoy (16). Flasks (25 cm^2) of infected Vero cells were labeled from 4 to 5 h postinfection with [^{35}S]methionine and chased for 1 h with cold methionine. The monolayers were washed with phosphate-buffered saline, scraped from the flasks, and frozen at −80°C. The cells were thawed in buffer containing 20 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM EDTA, 0.5 M NaCl, 1 mM 2-mercaptoethanol, and 500 μg of bovine serum albumin per ml and were sonicated for 15 s. The lysates were incubated with 50 μg of DNase I per ml for 1 h at 20°C. The extract was clarified by centrifugation at 27,000 × *g* for 15 min. EDTA was added to 20 mM, and the extracts were dialyzed extensively against D buffer (150 mM NaCl, 20 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA, 1 mM mercaptoethanol, 10% glycerol). The extract was again clarified by centrifugation at 27,000 × *g* for 15 min. The supernatant was loaded onto 0.75-ml DNA-cellulose columns, washed with D buffer, and eluted with D buffer containing various concentrations of NaCl. Bovine serum albumin was added to 5 μg/ml in all eluates, and each eluate was dialyzed against a solution of 0.15 M NaCl. Proteins were recovered from all fractions by precipitation with 9 volumes of acetone. Native and denatured calf thymus DNA-celluloses were prepared by the procedure of Alberts and Herrick (1) and kindly supplied by William Ruyechan, Uniformed Services University of the Health Sciences, Bethesda, Md. The native DNA-cellulose contained approximately 1 mg of nucleic acid per g of powder, whereas the denatured DNA-cellulose contained approximately 0.75 mg of nucleic acid per g of powder. Shown is an autoradiogram of a gel in which various protein fractions were subjected to electrophoresis. a, Total cellular lysate; b, extract put on DNA columns; c, pellet from high salt DNase extraction; d, pellet following dialysis. Samples e to i are from denatured DNA-cellulose: e, flow through; f, 0.3 M NaCl eluate; g, 0.5 M NaCl eluate; h, 1.0 M NaCl eluate; i, 4.0 M NaCl eluate. Samples j to n are from native DNA-cellulose: j, flow through; k, 0.3 M NaCl eluate; l, 0.5 M NaCl eluate; m, 1.0 M NaCl eluate; n, 4.0 M NaCl eluate.

ture of the protein. Further work is needed to determine whether the folded structure suggested by these experiments is indeed present in the protein in the infected cell and whether it is required for any of the interactions of this protein.

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