J Microbiol Immunol Infect
2000;33:141-148

Different forms of HSV-1 VP22a within purified virion and infected cells

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Received: December 15, 1999 Revised: January 27, 2000 Accepted: March 24, 2000

Utilizing the monoclonal antibody MCA406, our experimental data suggest that the herpes simplex virus type 1 (HSV-1) protein, VP22a, is present in the purified virus in a different form from that present within infected cells, namely the virion and infected-cell form, respectively. It seems reasonable to suggest that two different forms of VP22a are synthesized during the HSV-1 productive cycle. Using varying quantities of reducing agents, both inter- and intramolecular disulfide linkages were demonstrated in this protein family. Moreover, the VP22a-virion form could not be detected under nonreducing conditions by monoclonal antibody, even in the presence of proteolysis inhibitors, i.e. aprotinin, phenyl-methane-sulfonyl fluoride (PMSF) and soybean trypsin inhibitor. Varying temperature had little effect on the breakdown of VP22a disulfide bonds. A higher molecular-weight band, present in the nonreduced gel tracks, clearly indicates the presence of intermolecular disulfide bonds. Similarly, the appearance of bands of lower apparent molecular weight in the nonreduced tracks suggests the presence of intramolecular disulfide bonding. The VP22a infected-cell form may be modified to the virion form during the capsid-assembly process, prior to full capsid formation.

Key words: Herpes simplex virus type 1 (HSV-1), VP22a, capsid, assembly

The herpes simplex virus type 1 (HSV-1) gene,UL26, consists of two open reading frames (ORF), each encoded by its own transcript, the larger encodes a protease which cleaves itself and ICP35 (VP22a). Given the overlap with the more abundant ICP35, it is conceivable that this protease assembles with, and is dedicated to, the processing of ICP35. The UL26 ORF encodes a protein approximately 80 kDa in molecular weight. A remarkable property of this protease is that the coding region and promoter of the substrate ICP35 are entirely contained within the C-terminal half of the protease-coding region [1], the substrate sharing amino acids with the protease. The protease itself contains a processing site at its C-terminus which is susceptible to proteolytic cleavage. For HSV-1, the cleavage sites are between residues 247 and 248, and at the C-terminus, between residues 610 and 611. VP24 is encoded by the first 247 codons of UL26, and VP21 starts at codon 248, probably extending to the end of the ORF (codon 635) [2].

The VP22a (ICP35) protein is involved in packaging viral DNA into capsids, and in capsid maturation [3]. Unlike other capsid proteins, VP22a is highly processed, comprising many phosphorylated and nonphosphorylated forms which resolve into numerous spots visible when examined using two-dimensional gel electrophoresis. Evidence obtained using a temperature-sensitive mutant suggests that VP22a is necessary for DNA insertion and encapsidation [4].

Whether VP22a binds to the surface of full or empty capsids is uncertain, Gibson and Roizman predicting that, inasmuch as VP22a is present in full nucleocapsids but not empty capsids, this polypeptide would be discovered on the surface of capsids [5]. Braun *et al* also supported this conclusion, their results demonstrating that surface labeling reveals the presence of ICP35 on the surface of full capsids, but not empty ones [6]. In 1988, Sherman and Bachenheimer published a contradictory finding, revealing the presence of this protein in empty capsids [7], a finding also confirmed in 1988 by Rixon *et al* [3]. These contradictory results may be due to differences in techniques used, and/or incomplete separation of the different capsid forms. Further, ICP35 (VP22a) changes structure in the transition from empty to full capsids, and consists of a family of highly heterogeneous proteins which form numerous bands in both one- and two-dimensional separations [6]. There is continuing uncertainty, however, concerning the relationship between the occurrence of these proteins on capsids and packaging of viral DNA. In this study, we

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demonstrate that HSV-1 VP22a is present in different forms in purified virus and infected cells, and that both these forms may contain intramolecular, and probably intermolecular, disulfide bonding.

Materials and Methods

Virus strains and cell culture

The HSV-1 17^+ strain was grown in baby-hamster kidney (BHK-21) cells in the autoclavable form of Dulbecco's medium (Sigma, MO, USA) supplemented with 2 mM glutamine, 10% (w/v) tryptose phosphate broth, 0.1% (w/v) sodium bicarbonate, 0.12 mg/mL penicillin, 0.1 mg/mL streptomycin and 10% (w/v) calf serum.

Virus purification

Virus stocks were prepared by infecting confluent monolayers of BHK-21 cells in roller bottles (1.5×10^8 cells/roller bottle), at a low multiplicity of infection (MOI) - 0.02 to 0.05 plaque-forming units per cell (pfu/cell) and left for 2 to 3 days at 37 °C, or 3 to 4 days at 34 °C. The cells were scraped into the medium and centrifuged at 1,600 x *g* for 20 min. The supernatant was centrifuged for a further 90 min at 16,000 x *g*, and the pellet (extracellular) resuspended in phosphate-buffered saline (PBS). After disruption by sonication, the virus was layered onto a 10-40% (w/v) sucrose gradient. The gradient was then centrifuged for 45 min at 30,000 x *g* at 4 °C. Under a strong light the virus could be seen as a dense band in the middle. This was removed using a syringe, diluted fourfold in PBS and then centrifuged for 1 h at 80,000 x *g* at 4 °C. The pellet was resuspended in 200 mL PBS per gradient and stored at -70 °C.

Virus-infected cell antigen

Roller bottles containing confluent BHK cells were incubated with virus at an MOI between one and two. The cells were harvested after 24 h at complete cytopathic effect (CPE) and centrifuged at 1,600 x *g* for 20 min on a GSA rotor. The infected cell pellet was washed twice in PBS and centrifuged again. The pellet was solubilized by resuspending in radioimmune precipitation assay (RIPA) buffer (1% NP-40, 0.1% SDS, 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4) then protein solubilizing buffer [0.125 M Tris/HCl, pH 6.8; 4% (w/v) glycerol and a drop of bromophenol blue]. The suspension was collected and sonicated using a probe sonicator and then centrifuged at 40,000 rpm for 1 h in a Sorvall OTD-50 ultracentrifuge using a T1270 fixed angle rotor. The solubilized fraction was aliquoted and store at -70 °C.

Sodium dodecylsulfate polyacrylamide gel electrophoresis

Proteins to be analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were solubilized in an equal volume of protein solubilizing buffer [0.125 M Tris/HCl, pH 6.8; 4% (w/v) glycerol and a drop of bromophenol blue], with the addition of 0.01 M dithiothreitol (DTT) to the sample buffer immediately prior to use, unless otherwise stated. Protein samples were boiled in the presence of protein solubilizing buffer for 3 to 5 min prior to loading onto a gel. Electrophoresis was carried out at either 40 mA constant current for approximately 4 h, or overnight at 40 volts constant voltage.

Western blotting

Viral polypeptides separated by SDS-PAGE were transferred and immobilized onto nitrocellulose paper in 0.025 M Tris/0.192 M glycine, 20% methanol buffer at 30 to 40 volts overnight. The paper was then blocked using 10% calf serum in PBS for 1 h at 37 °C. This was followed by washing three times with PBS and the addition of culture-medium supernatant or antibodies (1/100 or 1/200 dilution) in 10% calf serum in PBS at 37 °C for 1 to 2 h, followed by another three PBS washes and the addition of horseradish-peroxidase conjugated rabbit antimouse antiserum (1/1000 dilution) in 10% calf serum in PBS. After 1 h at 37 °C, enzyme substrate was added and allowed to react for 10 to 20 min.

Antisera

Monoclonal antibody MCA406 (anti-ICP35, IgG) was obtained from Bioproducts for Science-Serotec [8].

Visualizing disulfide bonding by one-dimensional SDS-PAGE

One-dimensional SDS-PAGE was performed according to the method modified from Allore and Barber [9]. Prior to electrophoresis all samples were incubated for 3 min in a boiling-water bath. Samples to be heat reduced were made with either 5% (w/v) 2-ME (Sigma, stock solution 14.29 M) or 5 mM DTT. Electrophoresis was carried out at 100 volts through the stacking gel and at 200 volts through the separating gel. The electrophoresis was continued until the tracking dye, bromophenol blue, reached the end of the gel (approximately 6 h).

Results

In this study, there were two different patterns for VP22a

consistently seen on Western blots, the virion form and the infected-cell form, which range in molecular weight from 52 to 36 kDa. The virion pattern revealed a major band and several minor bands; the infected-cell pattern showed two major bands and, similarly, several minor bands. The ICP35 family, as has been demonstrated in all previous reports, revealed the infected-cell pattern [2,5,6,10,11]. By probing specimens with monoclonal antibody under different study conditions, two interesting findings were observed. The first was that VP22a could not be detected using the monoclonal antibody MCA406, under nonreducing conditions, if purified HSV-1 was used as the antigen. The second finding was that two different patterns of VP22a, virion and infected-cell patterns, were consistently seen on Western blots.

Antigen samples were supplemented with aprotinin (10 mg/mL), phenyl-methane-sulfonic fluoride (PMSF) (1 mM), and soybean trypsin inhibitor (1 mg/mL; for inhibition of proteolysis) before boiling under reducing and nonreducing conditions and testing using Western blotting (Fig.1). The data showed that virion form VP22a could not be detected under the nonreducing condition using the monoclonal antibody MCA406, even in the presence of proteolysis inhibitors, i.e. aprotinin, PMSF and soybean trypsin inhibitor. Notably, the different forms of VP22a are shown in Fig. 1 (closed and open triangles). The effect of temperature on the detection of VP22a was also investigated with both the purified HSV-1/17$^+$ and HSV-1/17$^+$-infected-cell extracts left at 37 °C in a water bath for 5 min then heated at 100 °C for 1, 2, 3, 4 or 5 min, with or without the reducing agent, DTT (data not shown). No difference was observed.

In an attempt to further evaluate the role of disulfide bond formation in the structure of VP22a in the virion, a method described by Allore and Barber [9] was modified and used to examine the shift among the bands. A gradient of reducing agent was established between adjacent slab gel tracks, one containing 2-mercaptoethanol (2-ME) electrophoresing identical protein samples. This method has been used to visualize the change in mobility of disulfide bond-containing proteins during the transition from a reducing to a nonreducing environment. In our study it was used to

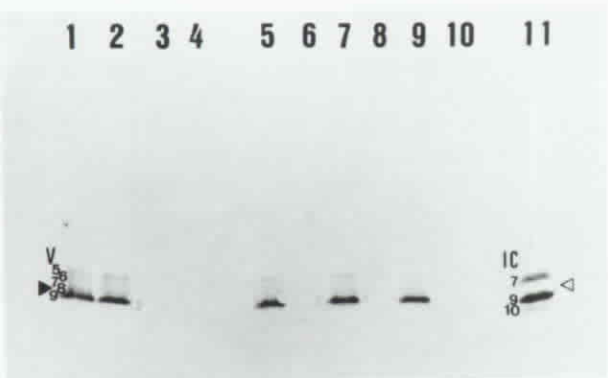

Fig. 1. Western blot analysis of different antigen samples on a 15% DATD-linked gel, probed with monoclonal antibody MCA406. The antigen samples are indicated below. Lanes 1 and 2: purified HSV-1/17$^+$ heated/100 °C (lane 1) and unheated/37 °C (lane 2); Lanes 3 and 4: purified HSV-1/17$^+$ heated (lane 3) and unheated (lane 4) under nonreducing conditions; Lanes 5 and 6: purified HSV-1/17$^+$ with (lane 5) and without (lane 6) aprotinin (10 mg/mL); Lanes 7 and 8: purified HSV-1/17$^+$ with (lane 7) and without (lane 8) PMSF (1 mM); Lanes 9 and 10: purified HSV-1/17$^+$ with (lane 9) and without (lane 10) soybean trypsin inhibitor (1 mg/mL); Lane 11: HSV-1/17$^+$-infected cells. The reactive species are indicated. V = virion form; IC = infected-cell form

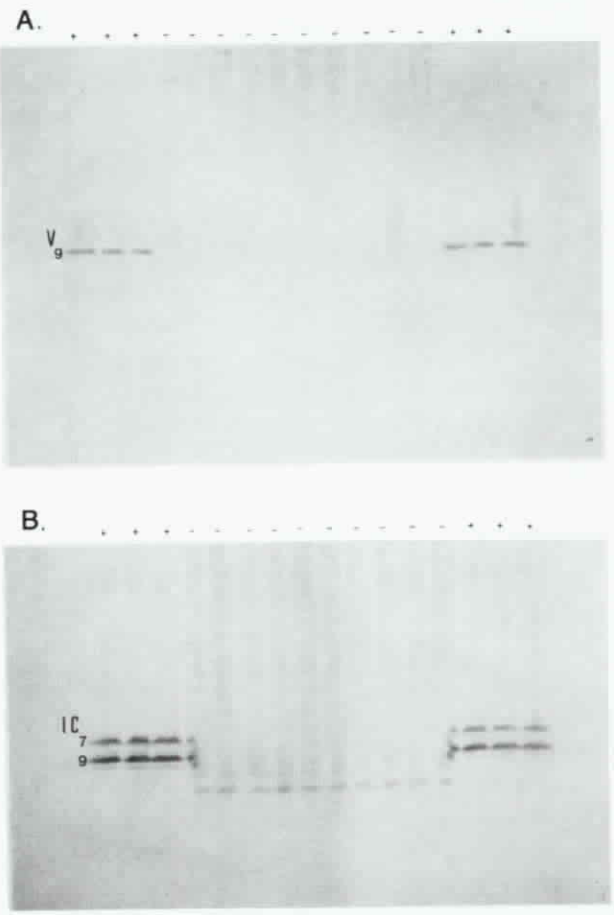

Fig. 2. Visualization of VP22a disulfide bonds on a one-dimension SDS-PAGE gel. Western blot analysis of (**A**) purified HSV-1/17$^+$ and (**B**) HSV-1/17$^+$-infected cells with (+) or without (-) reducing agent, 5 mM DTT on a 15% DATD-linked gel and probed with monoclonal antibody 406. V = virion form; IC = infected cell form

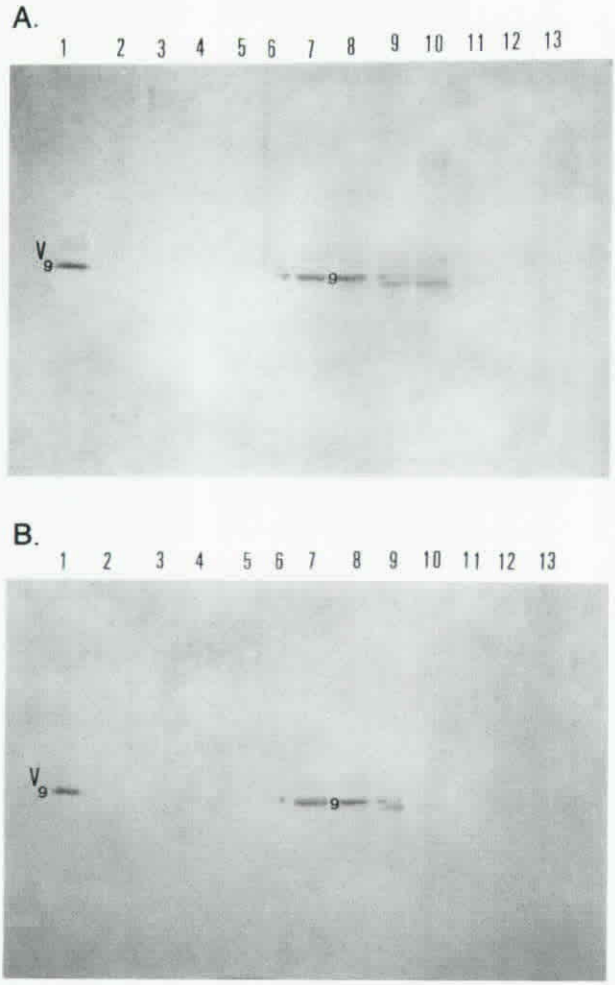

Fig. 3. Disulfide bonding of VP22a-virion form. Western blot analysis of (**A**) heated/100 °C and (**B**) unheated/room temperature purified HSV-1/17$^+$ prior to loading onto a 15% DATD-linked gel with different amount (10 mM DTT or 5% MSH) of reducing agents, DTT (lanes 1-6 diluted 1/1, 1/10, 1/50, 1/100, 1/500, and 1/1000 respectively, lane 7 with 10 mM DTT), or 2-ME (lanes 8-13 diluted 1/1, 1/10, 1/50, 1/100, 1/500, and 1/1000 respectively), probed with monoclonal antibody MCA406. V = virion form; IC = infected cell form

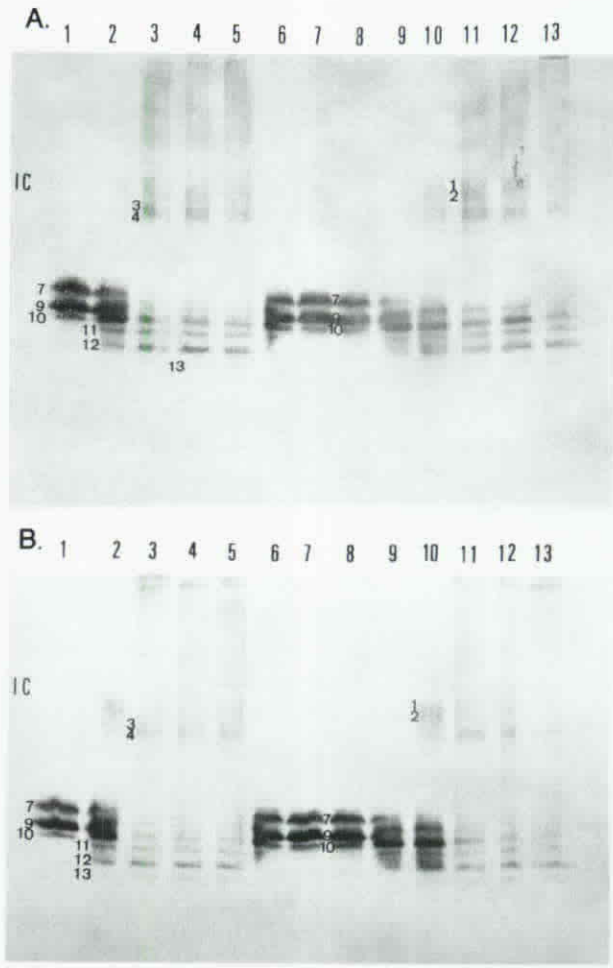

Fig. 4. Disulfide bonding of VP22a infected-cell form. Western blot analysis of (**A**) heated/100 °C and (**B**) unheated/room temperature HSV-1/17$^+$-infected-cell extracts prior to loading onto a 15% DATD-linked gel with different quantities (10 mM DTT or 5% MSH) of reducing agents, DTT (lanes 1-6 diluted 1/1, 1/10, 1/50, 1/100, 1/500, and 1/1000 respectively, lane 7 with 10 mM DTT), or 2-ME (lanes 8-13 diluted 1/1, 1/10, 1/50, 1/100, 1/500, and 1/1000 respectively), probed with monoclonal antibody MCA406. V = virion form; IC = infected-cell form

facilitate the positive identification of proteins containing intrachain disulfide bonds.

The intermediate status of reduction observed in the interfacial tracks, during the course of the electrophoresis, was presumably due to the lateral diffusion of reducing agent. As the reducing agent spread from its track of origin to the adjacent track, increasing numbers of molecules were reduced. As some of the molecules had traveled part-way down the gel in the nonreduced state and part-way under reducing conditions, a "diagonal" effect was observed at the interface between the fully reduced and the nonreduced states. Figure 2 shows the Western blot analysis for purified HSV-1/17$^+$ (Fig. 2A) and HSV-1/17$^+$-infected cells (Fig. 2B), with or without reducing agent on a 15% one-dimensional SDS-PAGE gel, probed with monoclonal antibody MCA406. The VP22a-family bands could not be distinguished in this experiment due to comigration. Pulse-chase immunoprecipitation (pulsed for 15 min at 6 h postinfection and chased for 20, 40, and 80 min) also failed to demonstrate the relationship between the individual bands (data not shown).

Different reducing agents were used for the

detection of disulfide bonds for HSV-1 VP22a. Attempts were made to further break down the VP22a disulfide bonds using different reducing agents, DTT and 2-ME, in various concentrations (5 mM DTT or 10% 2-ME, diluted 1/10, 1/50, 1/100, 1/500, 1/1000). While the disulfide bonds were completely broken down in higher concentrations (5 mM DTT or 10% 2-ME) of reducing agents at room temperature (Fig. 3A) or 100 °C using purified HSV-1/17$^+$ (Fig. 3B) or HSV-1/17$^+$-infected cells (Fig. 4), some disulfide bonds remained intact in lower concentrations (diluted 1/10-1/1000) of reducing agents. Varying temperature had little effect on the breakdown of VP22a disulfide bonds. Usually, the presence of a higher molecular weight band in the nonreduced gel track provided a clear indication of the existence of intermolecular disulfide bonds. Similarly, the appearance of bands of lower apparent molecular weight in the nonreduced track suggested the presence of intramolecular disulfide bonding.

In order to specify these bands, which appeared under different conditions, the VP22a band family members were classified according to the increasing electrophoretic mobility. The VP22a infected-cell form bands were designated as VP22a-IC1 to VP22a-IC13 (Figs. 1-4). The VP22a virion-form bands were designated as VP22a-V5 to VP22a-V10. The molecular weights of the first four bands of VP22a infected-cell forms, i.e. IC1-IC4, were in the range from 82 kDa to 68 kDa while the first four bands of VP22a-virion form are blanked. For other close-spaced bands, i.e. V5-V10 and IC5-IC13, their molecular weights were ranged from 52 to 36 kDa. The major bands revealed within the virion and infected-cell forms were VP22a-V5, IC7 and IC9, respectively. The shift assays depicted in Figs. 3A and B suggest that polypeptide V9 contains intramolecular disulfide bonding. Nonreduced polypeptide V9 had a similar molecular weight to reduced polypeptide V10 (1/10-1/50 dilution of reducing agents; lanes 7-10), but no bands could be detected at increased dilutions (1/100-1/1000; lanes 2-6 and 11-13). For the VP22a infected-cell form, it was demonstrated that polypeptides IC7 and IC9 migrated faster than IC8 and IC10, respectively, at a 1/10-1/50 dilution of reducing agents (Figs. 4A,B, lanes 1-2 and 7-10). Eventually IC7 and IC9 demonstrated the same molecular weight as IC10 and IC12, respectively, at high dilutions (1/100-1000) of reducing agents [Figs. 4A,B, lanes 3-5 and 11-13]. Figure 4 demonstrates that polypeptides IC7 and IC9 both exhibit intramolecular disulfide bonding since the highly reduced polypeptides IC7 and IC9 reveal the same molecular weight as the nearly nonreduced polypeptides IC10 and IC12. The polypeptides IC11, IC12 and IC13 were detected only under nonreducing conditions, and were therefore described as nonreduced polypeptides.

Discussion

In 1984, Braun *et al*, designated the six bands of the ICP35 family as ICP35a to ICP35f [6]. Work by Liu and Roizman offered evidence that the product of UL26 is an 80 kDa protease which cleaves itself [1,12]. The translational product of the ORF was designated as the protease Pra. Pra cleaves itself and ICP35c and ICP35d at exactly the same position, 25 amino acids from the C-terminus, to yield Prb and the ICP35 e and f bands, respectively. The cleavage of Pra to Prb occurs after the translation of the UL26 ORF and incubation of the translated protein *in vitro*, the protease activity mapped at the amino-terminal domain of the UL26-gene product, i.e., in a region not shared with ICP35. Further, it was noted that HSV-1 protease was autoprocessed to a greater extent during coexpression with ICP35 [13]. This enhanced cleavage was more pronounced at the N-terminal site, while the C-terminal cleavage product of HSV-1 protease, Prb, was barely detectable during time courses of coexpression. In HSV-1 the cleavage sites are between residues 247 and 248, and at the C-terminus, between residues 610 and 611. The cleavage generates two polypeptides, an amino-terminal polypeptide, designated Prn, and a carboxyl-terminal polypeptide, corresponding to ICP35a. Probably identical to VP24, Prn is encoded by the first 247 codons of UL26 and functions as a protease [2].

Person *et al* suggested that VP21, which is probably identical to ICP35a (Na), starts at codon 248 and probably extends to the end of the ORF (codon 635), and ICP35b (Nb) arises by carboxyl-terminal cleavage of ICP35a [2]. However, Weinheimer *et al* proposed that VP21 is Nb, based on their results [14]. The doublet-band formation characteristics of Pra and Prb, ICP35a and b, ICP35c and d, and ICP35e and f, but not of Prn is a function of the amino acid sequences shared by UL26 and UL26.5.

In order to specify the bands which appeared under different conditions, bands of the VP22a family were named according to their increasing electrophoretic mobility. In this study, the VP22a virion-form bands were designated as VP22a-V5 to VP22a-V10. The VP22a infected-cell-form bands were designated as VP22a-IC1 to VP22a-IC13. The molecular weights for the first four bands from VP22a infected-cells forms (IC1-IC4) and the other close-spaced bands were within the ranges from 82 to 68 kDa and 52 to 36 kDa. The major bands within the infected-cell form, VP22a-IC7

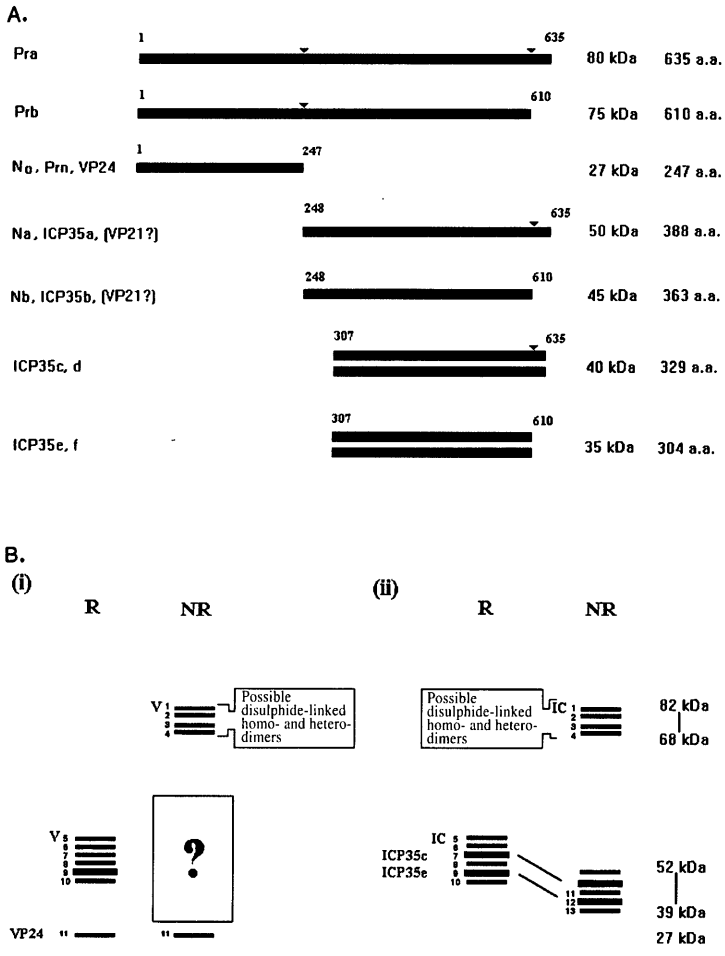

Fig. 5. Schematic representation of the UL26 and UL26.5 products shows in this study. **A.**, Diagram of cleavage products of HSV-1 protease. HSV-1 protease cleavage sites are indicated with arrowheads. Numbering of amino acid is relative to full-length protease, Pra. **B.**, Diagram and nomenclature of the bands are recognized by monoclonal antibody 406 shown in this study. This model is proposed in order to explain the multiple species of VP22a virion (i) and infected-cell (ii) forms under reducing (R) and nonreducing (NR) conditions

and IC9, may correspond to ICP35c and e, respectively. The shift assays presented in Figs. 3A and B suggest that polypeptide V9 contains intramolecular disulfide bonding, and nonreduced polypeptide V9 has a similar molecular weight to polypeptide V10, at a 1/10 to 1/50 dilution of reducing agents (lanes 7-10), but no bands can be detected with further dilution (1/100-1/1000; lanes 2-6 and 11-13). For the VP22a infected-cell form, polypeptides IC7 and IC9 were shown to migrate faster than the equivalent molecular-weight polypeptides IC8 and IC10, respectively, at a 1/10 to 1/50 reducing-agent dilution. Eventually, IC7 and IC9 moved with the same molecular weight as IC10 and IC12, respectively with high dilutions (1/100-1/1000) of reducing agents and nonreducing conditions. Based on this evidence, we suggest that polypeptides IC7 and IC9 both contain intramolecular disulfide bonding since reduced polypeptides IC7 and IC9 have the same molecular weight as nonreduced polypeptides IC10 and IC12. The intermediate status of reduction was also manifested using this assay. The figures generated by this study also reveal visible spots between adjacent slab tracks due to the lateral diffusion of reducing agents from one track to another during the course of the electrophoresis.

The polypeptides IC1-IC4 examined in this study were more abundant under nonreducing conditions and

may be nonreduced species with molecular weights similar to the UL26-gene products, Pra and Prb. If we accept this premise, the polypeptides IC1-IC4 may be disulfide bond-linked homo- or heterodimers of ICP35, which exist only under nonreducing conditions. The polypeptide V11 may be identical to VP24 since it revealed a similar molecular weight (27 kDa) and a single band. The polypeptides IC11, IC12 and IC13 were detected only under nonreducing conditions, suggesting that they are nonreduced polypeptides (Figs. 2B,4). For other polypeptides studied, the information obtained was inadequate in terms of drawing rigorous conclusions, that means it was not feasible to distinguish which band corresponds to VP21 and designate every band on an SDS-PAGE gel.

VP22a is often referred to as an " assembly protein" and analogues have been identified in other herpes viruses, including PRV (35 kDa), EHV-1 (45 kDa), and HVS (31-29 kDa) [10,15-17]. In HCMV, B capsids contain a 36 kDa protein which has been designated as the " assembly protein", based on its similarities to analogues in the Colburn CMV strain (37 kDa) and HSV (i.e. VP22a). The assembly protein for HCMV is encoded by the C-terminal portion of the UL80 ORF which is homologous to the HSV-1 UL26 ORF [18, 19]. The B-capsid assembly protein was found to be abundant in noninfectious enveloped particles [20]. The 37 kDa assembly protein of HCMV B capsids was shown to have a 40 kDa precursor. Pulse-chase radiolabeling experiments revealed that conversion of the precursor to the product was slow, requiring over 6 h for completion, and correlated with movement from the cytoplasmic to the nuclear fraction of NP-40-disrupted cells. This 40 kDa protein, synthesized *in vitro*, forms disulfide-linked homodimers, but the 37 kDa assembly protein does not [21].

Since the data generated by this study is contradictory with the findings by Gibson and Roizman in 1972 [6], it is suggesting that VP22a may not be the intermediate scaffold protein but may be existing in two different forms (within purified virus and virus-infected cells which contain both inter- and intramolecular disulfide bondings), the functional role of this protein and these disulfide bonds in nucleocapsid assembly. The significance of the VP22a disulfide bonding is still unknown, although it is generally accepted that disulfide bridges increase protein stability.

To summarize, we suggest VP22a is present within purified HSV-1, but in a different form from that obtained from virus-infected cells. Both forms of VP22a may contain intramolecular, and probably intermolecular, disulfide bonding. During capsid assembly processes the VP22a infected-cell form may be modified to the virion form prior to full capsid formation. TheVP22a-virion form may be present outside the surface of full capsids and the VP22a infected-cell form functions inside empty capsids. The doublets of VP22a-V7 and 8, VP22a-V9 and 10, and IC7 and 8 (ICP35c and d), IC9 and 10 (ICP35e and f) may also arise from some kind of modification. The VP22a protein may not be a " scaffold protein", rather undergoing conformational alterations and thus not detectable on previous attempts. If there is a " scaffold protein", we suggest that it may be VP21 since its role is uncertain and the amount of VP21 is less than VP22a in samples tested. Interestingly, these speculations offer an explanation for the incongruent findings generated by this study. Recently, Thomsen *et al* have suggested that VP21 may serve as a scaffold protein [22] and either VP21 performs the scaffolding function in a different way to VP22a, or, VP21 forms a shell that is not readily apparent using electron microscopy since there is no capsid inner core and only an outer (icosahedral) shell present in all 26.5-infected cells (insect cells infected with recombinant baculoviruses expressing UL18, UL19, UL26, UL35 and UL38 genes). In this study, multiple species of both forms of VP22a were revealed by monoclonal antibody, but it may not be possible to elucidate the actual numbers for each VP22a-band form using SDS-PAGE techniques.

Acknowledgment

This study was supported by a grant (No.CSMC-84-OM-B-005) from Chung Shan Medical and Dental College, Taichung, Taiwan, ROC.

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