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Self-Association of Herpes Simplex Virus Type 1 ICP35 Is via Coiled-Coil Interactions and Promotes Stable Interaction with the Major Capsid Protein

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The ordered copolymerization of viral proteins to form the herpes simplex virus (HSV) capsid occurs within the nucleus of the infected cell and is a complex process involving the products of at least six viral genes. In common with capsid assembly in double-stranded DNA bacteriophages, HSV capsid assembly proceeds via the assembly of an outer capsid shell around an interior scaffold. This capsid intermediate matures through loss of the scaffold and packaging of the viral genomic DNA. The interior of the HSV capsid intermediate contains the viral protease and assembly protein which compose the scaffold. Proteolytic processing of these proteins is essential for and accompanies capsid maturation. The assembly protein (ICP35) is the primary component of the scaffold, and previous studies have demonstrated it to be capable of intermolecular association with itself and with the major capsid protein, VP5. We have defined structural elements within ICP35 which are responsible for intermolecular self-association and for interaction with VP5. Yeast (*Saccharomyces cerevisiae*) two-hybrid assays and far-Western studies with purified recombinant ICP35 mapped a core self-association domain between Ser165 and His219. Site-directed mutations in this domain implicate a putative coiled coil in ICP35 self-association. This coiled-coil motif is highly conserved within the assembly proteins of other alpha herpesviruses. In the two-hybrid assay the core self-association domain was sufficient to mediate stable self-association only in the presence of additional structural elements in either N- or C-terminal flanking regions. These regions also contain conserved sequences which exhibit a high propensity for α helicity and may contribute to self-association by forming additional short coiled coils. Our data supports a model in which ICP35 molecules have an extended conformation and associate in parallel orientation through homomeric coiled-coil interactions. In additional two-hybrid experiments we evaluated ICP35 mutants for association with VP5. We discovered that in addition to the C-terminal 25 amino acids of ICP35, previously shown to be required for VP5 binding, an additional upstream region was required. This region is between Ser165 and His234 and contains the core self-association domain. Site-directed mutations and construction of chimeric molecules in which the self-association domain of ICP35 was replaced by the GCN4 leucine zipper indicated that this region contributes to VP5 binding through mediating self-association of ICP35 and not through direct binding interactions. Our results suggest that self-association of ICP35 strongly promotes stable association with VP5 in vivo and are consistent with capsid formation proceeding via formation of stable subassemblies of ICP35 and VP5 which subsequently assemble into capsid intermediates in the nucleus.

Herpes simplex virus (HSV) capsid assembly occurs in the nucleus of infected cells and is the first step in the production of infectious progeny virions. The products of six viral genes (UL18, UL19, UL26, UL26.5, UL35, and UL38) encoding seven proteins (VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26) participate in capsid morphogenesis (5–8, 13, 25, 29, 30, 31, 35, 36, 39). This highly ordered process proceeds via a pathway somewhat analogous to that of the DNA bacteriophages, in which the outer capsid components assemble around an interior scaffold structure to form capsid intermediates (3, 37, 38). The interior scaffold of these particles is composed principally of the capsid assembly protein and is expelled from the capsid to permit packaging of the genomic DNA and subsequent steps in particle maturation. The virus assembly protein is therefore only transiently associated with the maturing virus particle.

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The HSV capsid assembly protein, termed pre-VP22a or ICP35cd, is a 329-amino-acid phosphoprotein and the product of the UL26.5 gene (2, 19, 33). During capsid maturation the assembly protein undergoes proteolytic maturation by the viral protease at a site 25 amino acids from its C terminus to generate VP22a or ICP35ef (19, 20). The viral protease is encoded by UL26 and is a 635-amino-acid protein which shares its C-terminal 329 amino acids with ICP35 since the UL26.5 gene is transcribed from within that of the protease as a 3′ coterminal transcript and has the same open reading frame (ORF) (19). The protease is synthesized as a precursor protein which undergoes autoproteolytic maturation at two sites, the maturation (M) and release (R) sites. The M site is located 25 amino acids from the C terminus of the protease (Ala610/Ser611) and is also the site of proteolytic maturation of the assembly protein to ICP35ef (7, 11, 20). Cleavage at the R site, which is located in the N-terminal region of the protease (Ala247/Ser248), results in release of the N-terminal 247 amino acids of the precursor which comprise the catalytic domain of the protease, VP24 (20, 43). In mutant viruses which lack the ability to express ICP35cd the C-terminal portion of

the protease which is the product of R site cleavage is capable, albeit at reduced efficiency, of substituting for the assembly protein as the capsid scaffold (22).

The most abundant capsid protein is VP5, the major capsid protein, which along with VP19C, VP23, and VP26 forms the outer shell of the capsid. The remaining capsid proteins (VP22a, VP21, and VP24), the assembly protein, and the cleavage products of the protease occupy the interior of the nascent capsid structure. The virus assembly protein forms the interior core of the assembling capsid and is essential for the appropriate formation of the outer capsid shell. Assembly protein-capsid protein interactions are critical for imparting the correct curvature to the nascent capsid shell and for generating closed capsid structures (9, 23, 40, 41). The assembly protein therefore must interact with itself and other capsid components to perform these functions. The inherent capacity of the assembly protein to self-associate is supported by a number of experimental observations. High-level expression of ICP35cd in insect cells, either alone or in the presence of the protease, results in the formation of scaffold-like structures visible by electron microscopy (18, 32). Moreover, ICP35ef (VP22a) extracted from isolated B capsids can reassociate in vitro to form torus-shaped structures 60 nm in diameter, which closely resemble the interior core of native B capsids (26). ICP35cd has also been shown to interact with the major capsid protein, VP5, in in vitro studies (16, 41) and has been implicated in the localization of other capsid components to the nucleus in cellular transfection assays (18, 28). The precise molecular basis for ICP35 self-association and the influence of ICP35 self-association on its ability to associate with other capsid proteins, such as VP5, during the complex process of capsid morphogenesis remain undefined. We have therefore carried out experiments to characterize in detail the structural requirements for ICP35 self-association, in both in vivo and in vitro assays, and have determined that the interaction of ICP35 with VP5 is positively influenced by its self-association, an observation consistent with the notion that ICP35 and VP5 may assemble into small oligomers, perhaps in the cytoplasm of infected cells, and the notion that these subunits are recruited to the nascent capsid particle in the nucleus.

MATERIALS AND METHODS

Yeast strain and media. *Saccharomyces cerevisiae* strain SFY526 (*MAT***a** *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112* can^r *gal4-542 gal80-538 URA3*::*GAL1-lacZ*) (1) was used for the two-hybrid system assays. Yeast cultures were grown at 30°C in yeast extract-peptone-dextrose broth (BIO101, Inc.). For β-galactosidase assays, yeast transformants were grown on Sabouraud dextrose (SD) plates or in SD culture medium (Difco yeast nitrogen base, appropriate amino acids, 2% glucose).

Plasmid construction and DNA manipulation. Construction and screening of recombinant plasmids was performed with standard procedures. All DNA fragments of the UL26.5 gene (full-length and truncated) were PCR amplified from DNA of plasmid ICP35-pCRII, which contained the ICP35-encoding sequence. The latter was cloned previously from HSV-1 strain KOS1.1 genomic DNA (corresponding to nucleotides 51727 to 52713 of the published sequence; McGeoch et al. [24]). Specific primers bearing an *Eco*RI site for cloning in frame in the two-hybrid system vectors, pGBT9 (GAL4 DNA-binding domain [DBD]) and pGAD424 (GAL4 activation domain [AD]) (Clontech), and bearing a TGA stop codon when used as antisense primers were used for amplification. *Eco*RI-digested PCR products were ligated into pGBT9 and/or pGAD424 and transformed into competent *Escherichia coli* strain DH5α (Gibco-BRL).

Site-directed mutagenesis of ICP35-encoding sequences to introduce the Leu182/Leu186/Met189-to-Ala182/Ala186/Ala189 mutation was performed by PCR amplification of a fragment bounded by unique *Aat*II (5′) and *Eco*RI (3′) restriction sites from plasmid ICP35-pGBT9 template. The mutagenic primer contained the intended mutations and the *Aat*II site found in wild-type DNA. The mutant fragments were reintroduced by ligation into the wild-type plasmid ICP35-pGBT9, from which the corresponding fragment had been removed. For experiments employing VP5-encoding sequences in yeast two-hybrid vectors, the UL19 gene encoding VP5 was isolated as the *Bgl*II N fragment of HSV-1 KOS1.1 genomic DNA and cloned in the *Bam*HI site of pUC19. The 5′ noncoding sequences of UL19 were removed, and an *Eco*RI site was generated immediately upstream of the natural ATG start codon prior to insertion into the vector pGBT9 or the vector pGAD424. This was achieved by PCR amplification of a fragment containing a short 5′ portion of the VP5-encoding sequence and, to generate a DNA fragment containing an *Eco*RI site, containing the ATG start codon and the first 680 base pairs of the gene, with its 3′ end defined by a natural *Hin*dIII site in the HSV-1 sequence. The remaining 3′ portion of the gene was isolated from VP5-pUC19 as a *Hin*dIII-*Xba*I fragment in which the *Xba*I-generated terminus was filled in. Both fragments were then ligated directly into *Eco*RI- and *Sma*I-cleaved pGBT9 and pGAD424.

GST-ICP35 fusion proteins. All glutathione *S*-transferase (GST)-ICP35 fusion proteins were generated by direct isolation of ICP35-encoding DNA fragments by *Eco*RI digestion of the corresponding two-hybrid vector and ligation into the *Eco*RI site in pGEX-4T-1. Expression was performed in *E. coli* DH5α following induction with IPTG (isopropyl-β-D-thiogalactopyranoside).

Yeast transformation. Transformation into SFY526 was performed according to the Clontech Matchmaker Library Protocol (PT1020-1) for small scale transformation. This protocol is based on the method developed by Ito et al. (17), with some modifications. Briefly, 100 ng of each plasmid was cotransformed in the presence of 100 μg of sonicated salmon sperm carrier DNA in lithium acetate. Transformed cells were plated on SD medium deficient in leucine and tryptophan for selection of both plasmids and incubated at 30°C for 3 to 5 days.

β-Galactosidase assays. The protein-protein interactions detected in the yeast two-hybrid assay result in activation of the expression of β-galactosidase from the reporter gene. Measurements of gene activation were performed by colony lift filter assays with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) as the substrate and by liquid assay of yeast cell extracts with CPRG (chlorophenol red-β-D-galactopyranoside) as the substrate. The assays were performed according to the procedures recommended by the manufacturer (Clontech protocol PT1020-1). For the filter-based assay, colonies were subjectively scored positive or negative for interaction based upon intensity of staining after 90 min of incubation with substrate. β-Galactosidase activity in the liquid assay was determined in triplicate, and the mean values were determined. Three fresh colonies for every transformant were first grown for a period of 14 to 20 h in SD medium deficient in leucine and tryptophan at 30°C with shaking and then used to inoculate yeast extract-peptone-dextrose medium. When the cultures reached an optical density at 600 nm (OD_{600}) of 0.6, the cells were harvested, washed, and submitted to two cycles of freezing and thawing. β-Galactosidase activity was then determined spectrophotometrically (at 578 nm) with CPRG as the substrate for color development. Activities were calculated with the following equation: Miller units = $(1{,}000 \times OD_{578})/(\text{elapsed minutes} \times \text{milliliters of culture} \times OD_{600})$.

ICP35 purification. ICP35cd- and ICP35ef-encoding sequences were cloned in plasmid pET11a for protein expression in *E. coli* BL21(DE3)pLysS. The recombinant proteins were purified as follows. The bacterial pellet was resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 25 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 1 mM leupeptin) and sonicated extensively. The supernatant fraction obtained by centrifugation for 30 min at 23,000 × *g* at 4°C was precipitated with 25% saturated ammonium sulfate. Following centrifugation, the protein pellet was resuspended in a buffer containing 20 mM Tris-HCl (pH 8.0), 400 mM NaCl, 0.1 mM EDTA, and 1 mM DTT. Nucleic acids were precipitated with 1% streptomycin sulfate, and the supernatant was dialyzed extensively against a buffer containing 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1.0 mM DTT, and 3 M urea prior to application on a Resource Q column (anion exchange). Bound material was eluted with a linear (0 to 1.0 M) gradient of NaCl in the same buffer. Pooled ICP35-containing fractions were dialyzed against a buffer containing 50 mM MES (morpholineethanesulfonic acid; pH 5.0), 0.1 mM EDTA, 1.0 mM DTT, and 3 M urea and applied to a Resource S column (cation exchange). Bound material was eluted with a linear (0 to 1.0 M) gradient of NaCl in the same buffer. Fractions containing ICP35 were then dialyzed against storage buffer (50 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 100 mM NaCl) and stored at −80°C.

Far-Western analysis. Purified proteins and total *E. coli* lysates were solubilized in Laemmli sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred onto nitrocellulose membranes. The membranes were incubated in phosphate-buffered saline (PBS) containing 20% dried-milk powder for 1 h. After washing three times in PBS containing 1% dried-milk powder, the membranes were then incubated with pure recombinant HSV-1 ICP35 labeled with a nitroiodophenyl (NIP) hapten reagent (4-hydroxyl-5-iodo-3-nitrophenyl acetyl; Promega). The binding reaction was carried out with the ligand (ICP35-NIP) at a concentration of 5 μg/ml for 3 h at room temperature. The membranes were washed four times for 10 min in PBS containing 1% dried-milk powder and specific binding was detected by using an anti-NIP antibody conjugated to horseradish peroxidase (Promega) coupled to the chemiluminescence Super Signal detection system (Pierce). The results were recorded on X-ray films.

TABLE 1. Self-association of ICP35 deletion mutants in yeast two-hybrid assay

Protein	β-Galactosidase activity (Miller units) produced by:[a]		
	Interaction of DBD fusion and AD fusion[b]	DBD fusion background[c]	AD fusion background[c]
ICP35cd(329)	42.6 (+)	8.6 (+)	0.1 (−)
ICP35ef(304C)	18.6 (+)	0.1 (−)	<0.01 (−)
234C	21.6 (+)	0.1 (−)	<0.01 (−)
219C	12.2 (+)	<0.01 (−)	0.1 (−)
N120	5.6 (+)	3.1 (+)	<0.01 (−)
N164	6.5 (+)	0.1 (−)	<0.01 (−)
N53/219C	0.6 (±)	<0.01 (−)	<0.01 (−)

[a] Results from the colorimetric filter assay described in Materials and Methods are indicated in parentheses.

[b] The DBD fusion corresponds to the ICP35 deletion mutant expressed in pGBT9; the AD fusion corresponds to the ICP35 deletion mutant expressed in pGAD424.

[c] Background activities were determined in yeast cells cotransformed with the DBD fusion and pGAD424 (DBD fusion background) or the AD fusion and pGBT9 (AD fusion background).

RESULTS

ICP35 self-association in the yeast two-hybrid system. In order to identify the regions within ICP35 responsible for intermolecular self-interaction we employed the yeast two-hybrid system, which has been used extensively to characterize protein-protein interactions (12). Initial experiments were performed to establish if both the precursor (ICP35cd) and the mature (ICP35ef) forms of the assembly protein would self-interact in this system. The complete coding sequence for ICP35 as a fusion protein with the Gal4 AD and Gal4 DBD was cloned. The encoded fusion proteins, ICP35cd(329)-AD and ICP35cd(329)-DBD, respectively, were tested for their ability to interact when coexpressed in yeast cells containing the Gal4-responsive reporter gene. A high level of β-galactosidase expression, consistent with efficient association between ICP35cd(329)-AD and ICP35cd(329)-DBD, was observed both in colony lift assays and solution-based colorimetric assays of β-galactosidase activity (Table 1). Background levels of reporter activation for each fusion protein were assessed. Yeast strains expressing ICP35cd(329)-AD and the nonfusion Gal4 DBD exhibited negligible background β-galactosidase activation (0.11 units), although significant background in yeast cells expressing ICP35cd(329)-DBD and the nonfusion AD (8.6 units) was measured. These levels of nonspecific reporter activation were low compared to the levels of enzyme activity induced when the Gal4 AD and DBD were both fused to ICP35 sequences. In a similar experiment ICP35ef(304C), which lacks 25 amino acids from the carboxyl terminus of the precursor assembly protein, was tested for self-association (Table 1). As with ICP35cd(329), high levels of reporter activity were observed upon coexpression of ICP35ef(304C)-AD and ICP35ef(304C)-DBD. These experiments confirmed that both the precursor and processed forms of ICP35 are capable of efficient self-association in this assay.

Location of self-association domains in ICP35. We extended these observations in an attempt to locate the domains within ICP35 which contribute to self-association. In these experiments ICP35cd(329)-AD was tested for interaction with various truncated forms of ICP35 fused to the Gal4 DBD (ΔICP35-

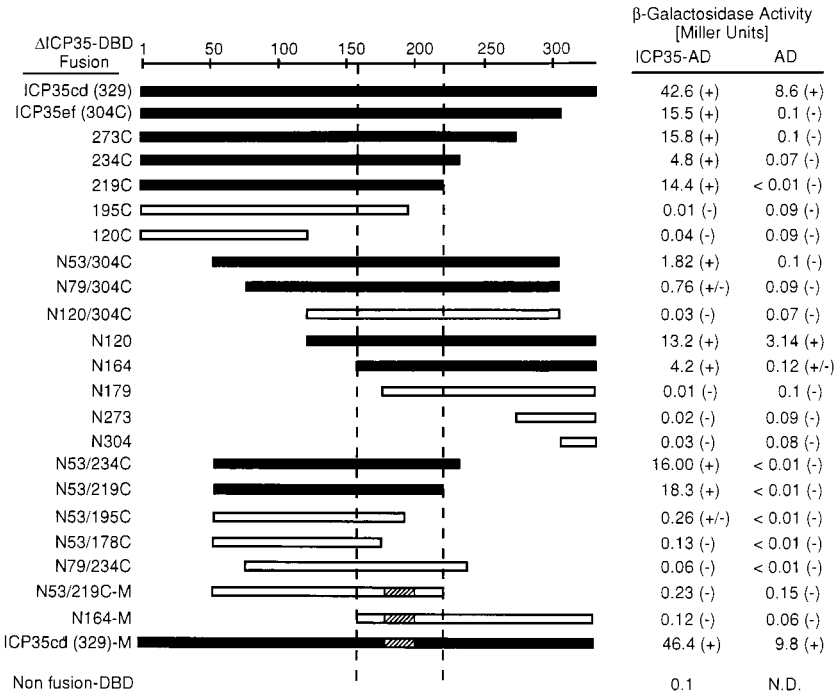

FIG. 1. Two-hybrid analysis of ICP35 self-association. ICP35 deletion and mutant derivatives (ΔICP35-DBD), shown graphically, were expressed as Gal4 DBD fusion proteins and assayed for interaction with ICP35cd expressed as a Gal4 AD fusion protein (ICP35-AD). The levels of reporter activation in yeast cells expressing nonfusion AD with each ICP35-DBD variant are also reported as controls. Reporter gene activation was monitored by colony filter assay with X-Gal (results in parentheses) and by measurement of β-galactosidase activity in crude yeast cell extracts as described in Materials and Methods. The mean β-galactosidase activity, determined from three independent transformants, is presented. ICP35 variants depicted in black were considered positive for interaction with ICP35-AD. Nonshaded constructs were determined to be negative. Hatched boxes indicate the presence of the Leu182/Leu186/Met189-to-Ala182/Ala186/Ala189 mutation (see Fig. 3). Amino acid sequence positions are shown, with broken lines indicating positions 164 and 219. N.D., not done.

DBD). In each case reporter gene activation was compared to background values in yeast strains coexpressing the ΔICP35-DBD fusion and the nonfusion AD or the ICP35cd(329)-AD fusion and the nonfusion DBD. Figure 1 shows the results of these experiments. For ICP35 mutants with progressive resection of the C terminus it was evident that ICP35ef(304C) associated with ICP35cd, consistent with its ability to interact with itself (Table 1), as did 273C, 234C and 219C. Shorter derivatives retaining 195 or fewer amino acids from the N terminus did not support self-association. The N-terminal 219 amino acids of the molecule are therefore sufficient for efficient association with ICP35.

Deletions in the N-terminal region of ICP35 were also evaluated for their effect on self-association. The deletions were evaluated in the context of the ICP35 precursor protein ICP35cd(329) or in ICP35ef(304C), which lacks 25 amino acids from the C terminus. N-terminal deletion of up to 164 amino acids from ICP35cd had little effect on the ability of the mutant to support reporter gene activation, whereas further deletions to positions 179, 273, and 304 destroyed interaction absolutely. This is consistent with a minimal region for self-association of ICP35 located between Ser165 and the C terminus of the molecule. In contrast to these observations, shorter N-terminal deletions of ICP35ef(304C) had a severe impact on interaction. The N-terminal mutant designated N53/304C, lacking 53 amino acids from the N terminus, was active for association, and the mutant N79/304C showed marginal activity in both colony and solution assays of reporter activity. Moreover, in contrast to N120, mutant N120/304C failed to interact with ICP35cd. These results indicated that the N-terminal boundary of a region capable of mediating the interaction of these mutants with ICP35 mapped between Lys120 and the N terminus. Since the analysis of C-terminal truncations of ICP35 (above) indicated that 95 C-terminal amino acids were dispensable for self-association of molecules retaining an intact N terminus, we extended our experiments to test additional N-terminal deletions in ICP35 derivatives lacking these dispensable C-terminal sequences. Mutant N53/234C interacted strongly with ICP35cd, while N79/234C displayed no activity, placing the N-terminal boundary for efficient association between Ser54 and Leu80. Removal of additional C-terminal sequences, as in mutants N53/219C, N53/195C, and N53/178C, defined a minimal interacting region extending from Ser54 to His219.

These experiments therefore identified two overlapping fragments of ICP35 capable of interacting with ICP35cd in two-hybrid experiments. The strongest interaction appears to be mediated by the minimal core region of 166 amino acids between Ser54 and His219. The second minimal region is defined by mutant N164 and is composed of the region extending from Ser165 to the C terminus. The structures responsible for association of this region with ICP35 appear to extend close to the C terminus of the protein, since deletion of 25 amino acids at the C terminus precluded the interaction (compare the results for N120 and N120/304C). We note that this deletion of amino acids in the context of an intact N terminus (equivalent to the result of the proteolytic processing which occurs in infected cells) reduced reporter activity by only twofold (Table 1 and Fig. 1), suggesting that the C-terminal sequences are critical for stable self-interaction only in the absence of a domain proximal to the N terminus. Structural elements in the first 120 amino acids of the protein, between Ser54 and Lys120 and contained within N53/219C, therefore appear capable of compensating for deletions at the C terminus.

These results are readily interpreted in a simple model in which the assembly protein exists in an extended conformation, self-associating via the interactions of three separable domains arranged along the polypeptide chain. The stable self-association of ICP35 detected in the two-hybrid assay appears to require participation of a common central domain, which we have termed the "core self-association domain" (Ser165 to His219 [between the dashed lines in Fig. 1] and additional flanking structural determinants), located either in the N-terminal region (with an N-terminal boundary distal to Ser54) or in the C-terminal region (inactivated by deletion of 25 C-terminal amino acids).

Self-association through homomeric interactions. The above experiments addressed the question of whether fragments of ICP35 expressed as Gal4 DBD fusion proteins could interact with the intact ICP35 protein fused to the Gal4 AD. We extended these studies to determine if the interaction-positive subregions of ICP35 were capable of associating with themselves (Table 1). Identical fragments of ICP35 were fused to both the Gal4 AD and the Gal4 DBD. The ability of 219C and N164 to associate with themselves with an apparent efficiency similar to that with which they interact with the intact ICP35cd fusion protein strongly supports a model in which homomeric association of these regions within the protein is occurring. The minimal domain N53/219C also interacted with itself, albeit yielding a signal somewhat lower than that obtained with ICP35 itself. This is likely a result of poor expression or poor stability of the N53/219C-AD fusion protein, since in contrast to N53/219C-DBD (Fig. 1), it also failed to interact efficiently with ICP35 (data not shown).

Site-directed mutagenesis of a putative coiled coil. Computer-generated alignments of the deduced amino acid sequences of four alpha herpesvirus assembly proteins and secondary structure predictions based upon these sequences (Fig. 2) were inspected. Despite low levels of overall sequence conservation between the assembly proteins, a limited number of short regions exhibiting high levels of conservation are evident. Remarkably, with the exception of one conserved region, the five-amino-acid motif Tyr143 to Glu147, all are revealed to have a high propensity for α helicity. The core self-association domain, Ser165 to His219, common to the two overlapping minimal self-associating fragments of ICP35, contains one of the conserved regions. Moreover the highly conserved sequence of 22 amino acids is predicted to have a high propensity for α helicity and coiled-coil formation (Fig. 2B and 3A). The region exhibits a 4-3 repeat of bulky hydrophobic residues and consequent heptad periodicity, often encountered in coiled coils. We therefore chose to make site-directed mutations at Leu182, Leu186, and Met189 (Fig. 3B) to examine the possibility that this putative coiled coil mediates ICP35 self-association. Each of these hydrophobic residues was replaced with Ala, a relatively conservative mutation which will destabilize potential coiled-coil interactions while not severely attenuating α-helical propensity. These mutations were introduced into ICP35, N53/219C, and N164, and association with ICP35cd (329) was evaluated in the two-hybrid assay (Fig. 1). The mutant forms of N164 and N53/219C, N164-M and N53/219C-M, were negative for interaction with ICP35, suggesting that these residues were critical for the interaction of these minimal domains. On the other hand the three amino acid substitutions had no measurable effect on the ability of the full-length ICP35cd(329)-M to interact with ICP35.

Far-Western analysis of ICP35 self-association. Interpretation of the above genetic analysis, which employed different ICP35 fragments expressed as fusion proteins, can be complicated by potential differences in the level of expression or stability of different fusion proteins and/or differences in their ability to fold correctly upon expression in the yeast cell. We therefore sought to obtain additional biochemical support for

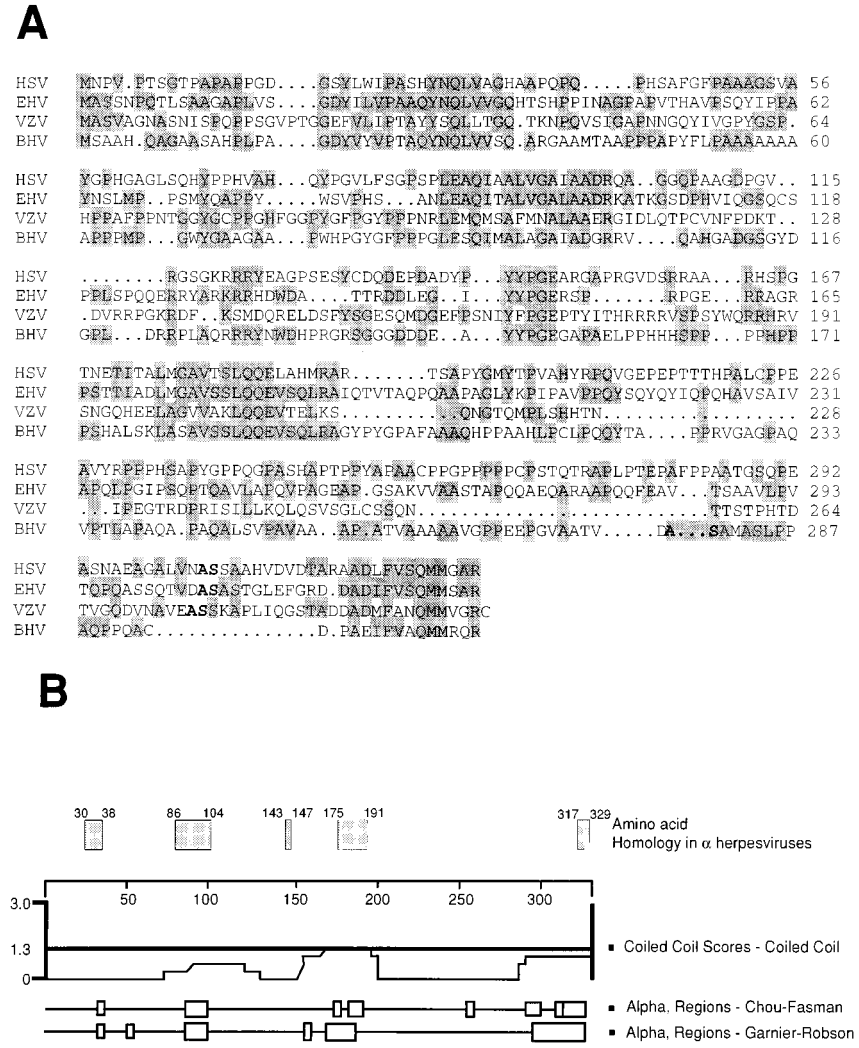

FIG. 2. Alpha herpesvirus assembly protein sequences. (A) Alignment of the deduced amino acid sequences of ICP35 (HSV; UL26.5) and its homologs in equine herpesvirus (EHV; ORF 35.5), varicella-zoster virus (VZV; ORF 33.5) and bovine herpesvirus 1 (BHV; UL26.5 homolog). Amino acid identity to HSV ICP35 is indicated by shading. The Ala-Ser amino acid pair flanking the predicted proteolytic cleavage site is indicated in boldface. (B) Secondary structure predictions for the HSV-1 ICP35 with the indicated algorithms (provided in the Lasergene biocomputing software package; DNASTAR Inc.). Amino acid sequence positions are shown, with the regions of greatest amino acid homology to other alpha herpesvirus assembly proteins indicated by shaded boxes.

our analysis of ICP35 interactions. For this approach we utilized purified recombinant ICP35 as a probe in far-Western assays with both purified recombinant ICP35 and with multiple ICP35 fragments expressed as GST fusion proteins in *E. coli*. Purified ICP35cd and ICP35ef were digested in vitro with the endoprotease Lys-C, which cleaves each protein at a single site between Lys120 and Arg121. The intact proteins and the cleavage products were separated by SDS-PAGE and transferred to nitrocellulose membranes for detection of binding interactions by probing with purified recombinant ICP35 which had been haptenized in vitro with NIP groups. Bound probe was detected on the membrane with a monoclonal antibody to the specific hapten. Figure 4 shows the purified proteins and proteolytic cleavage products (lanes 1 to 4) and far-Western detection of the fragments which bind ICP35 (lanes 5 to 12). For both ICP35cd and ICP35ef the probe binds efficiently to their respective C-terminal fragments (Fig. 4, lanes 6 and 7) but shows little binding to their common N-terminal fragment. Following longer exposure of the blot (Fig. 4, lanes 10 and 11) some binding to the shorter fragment corresponding to residues Met1 to Lys120 could be discerned. Nevertheless, the low level of this signal and subsequent experiments (see below) indicated that this fragment does not contain a major binding site.

In order to evaluate the specificity of these interactions and to determine the feasibility of using the approach to assay ICP35 interaction with multiple mutant proteins in crude or impure form, a preliminary experiment was carried out in which crude cell extracts from *E. coli* expressing either ICP35cd or ICP35ef were probed with ICP35. Both forms of ICP35 were specifically bound by ICP35 in this experiment, illustrating that ICP35 self-association could be detected in the crude lysates by the far-Western approach (Fig. 5). The binding was specific since no significant binding to highly abundant *E. coli* proteins was detected under the binding conditions used. We proceeded to map the domain of ICP35 required for ICP35 self-association in this assay. Crude bacterial cell lysates from cells expressing a series of different GST-ICP35 fusion constructs were probed with purified ICP35. The data from these experiments is shown in Fig. 6, 7, and 8 and summarized in Fig. 9. As

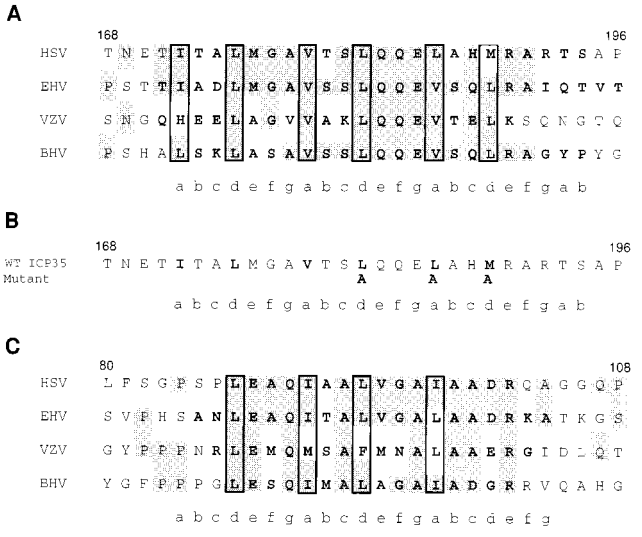

FIG. 3. Putative α-helical regions in assembly protein self-association domains. The alignment of predicted α helices between Ile172 and Ser194 (A) and between Leu87 and Arg102 (C) of HSV-1 ICP35, with homologous sequences in equine herpesvirus (EHV), varicella-zoster virus (VZV) and bovine herpesvirus (BHV), is shown. Amino acids in boldface in each sequence are predicted to be α helix (4, 14, 21). Amino acid positions within the putative helices are designated in standard nomenclature, *a* to *g*. Hydrophobic residues at positions *a* and *d* of the putative helices and constituting the characteristic 4-3 repeat of coiled coils are boxed. Residues which are conserved with respect to HSV-1 ICP35 are shaded. (B) Site-directed mutations introduced into HSV-1 ICP35. WT, wild type.

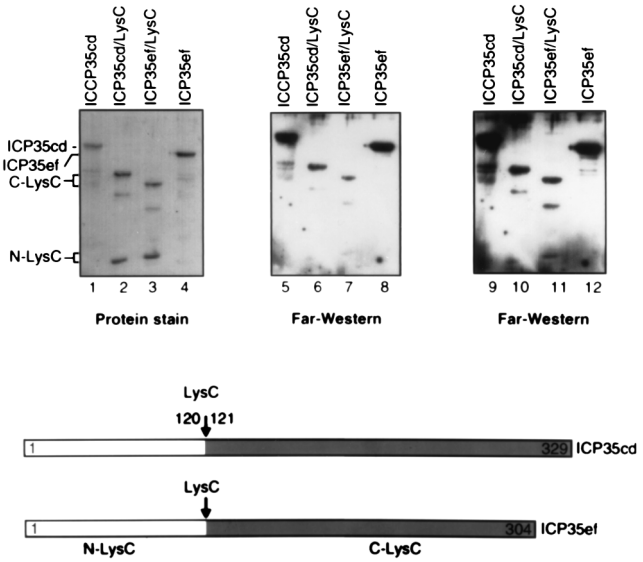

FIG. 4. ICP35 self-interaction by far-Western analysis. Samples (2 μg) containing purified ICP35cd or ICP35ef and their endoproteinase Lys-C digestion products were separated on SDS–15% polyacrylamide gel and transferred onto nitrocellulose membrane. The far-Western procedure was performed as described in Materials and Methods. The digestion with Lys-C was performed according to the manufacturer's recommended procedure. The left panel represents the protein pattern on the nitrocellulose membrane stained with Ponceau S. The middle and the right panels show the results obtained by far-Western analysis with a short (middle) and a long (right) exposure to X-ray film. The location of the unique lysine in ICP35cd and ICP35ef is illustrated in the diagram at the bottom of the figure.

a control, blots containing the fusion proteins were stained with Ponceau S and also probed with an anti-GST monoclonal antibody to confirm the expression of the fusion protein and its migration upon electrophoresis. No significant binding was observed for extracts containing the vector encoding GST (Fig. 6A, lanes 1 and 7; Fig. 6B, lanes 2, 10 and 17; Fig. 7, lanes 2 and 8). Progressive N- and C-terminal deletions (Fig. 6) of ICP35 sequences yielded results consistent with the two-hybrid experiments; the smallest active C-terminal and N-terminal truncations were 219C and N164, respectively. Moreover, the expression of ICP35 fragments containing sequences between these deletion endpoints, N164/234C and N164/219C, mapped a strong self-interacting region (Fig. 7, lanes 5 and 6). Additional fragments, N120/178C (Fig. 7, lane 3) and N179/234C (lane 4), which contain regions overlapping the endpoints of the minimal interacting fragment were negative for association, confirming that a single specific self-association domain could be mapped with this technique to sequences between Ser165 and His219.

Significantly, this region corresponds to the core self-association domain required for self-association of ICP35 fragments in the yeast two-hybrid system. As further confirmation that the same binding interactions we detected in yeast were also responsible for binding in this biochemical assay we introduced the same Leu182/Leu186/Met189 mutations into the core self-association domain and determined their effect on self-association in the far-Western assay. As predicted (Fig. 8) these mutations destroyed the ability of N164/234C and N164/219C to bind the ICP35 probe in this assay. Moreover, N53/219C-M, which contains the additional N-terminal sequences required for association with ICP35 in the two-hybrid assay but is mutated in the core self-association domain coiled-coil motif, also failed to bind ICP35. This is consistent with data from the yeast genetic assay, in which N53/219C-M was inactive.

ICP35 interacts with VP5 in the yeast two-hybrid system. It has been reported that the HSV assembly protein is capable of interacting with the major capsid protein, VP5 (16, 18, 28, 41). In the light of our data bearing on the mapping of self-association domains within ICP35, we wished to determine if ICP35-VP5 interaction could be reproduced in the yeast two-hybrid assay and what regions would be required for such an interac-

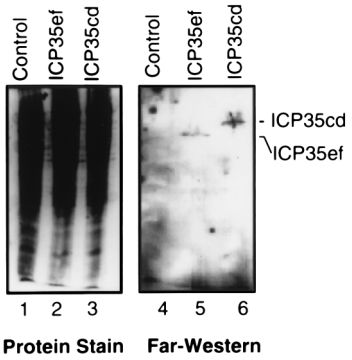

FIG. 5. ICP35 self-interaction in crude bacterial cell extracts as revealed by far-Western analysis. Crude extracts of *E. coli* clones expressing control vector (lanes 1 and 4), ICP35cd (lanes 3 and 6), and ICP35ef (lanes 2 and 5) were separated on SDS–15% polyacrylamide gel, transferred onto nitrocellulose membranes, and incubated with labeled ICP35 probe (see Materials and Methods). The samples were prepared from *E. coli* cultures before (control) and after 3 h of induction (ICP35cd and ICP35ef) with IPTG. The cell pellets were solubilized in Laemmli sample buffer, and the material representing 100 μl of culture was applied to the acrylamide gel. The far-Western analysis was performed according to the procedure described in Materials and Methods. The left panel represents the protein pattern (Ponceau S stain) on the nitrocellulose membrane used for the far-Western analysis. The results of the far-Western analysis are shown in the right panel.

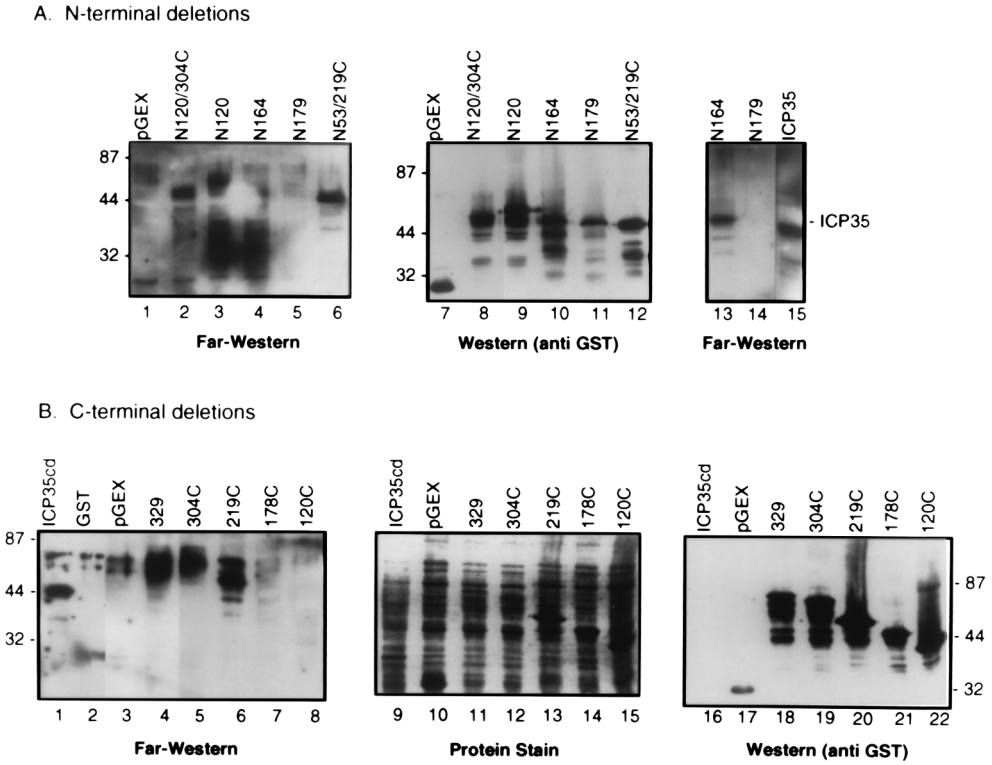

FIG. 6. Far-Western analysis of GST-ICP35 fusion proteins. Crude *E. coli* lysates containing GST-ICP35 fusion proteins in which the ICP35 fragment was an N-terminal (A) or C-terminal (B) deletion derivative were evaluated for their binding to purified ICP35 probe (A, lanes 1 to 6 and 13 to 15; B, lanes 1 to 8) under conditions similar to those described in the legend to Fig. 5. Lanes 13 and 14 in panel A contain identical samples to those in lanes 4 and 5 and 10 and 11 and are included to clarify the phenotype of N164, since a blot artifact prevents clear interpretation of lane 4. The ICP35 deletion derivative is indicated above each lane. Lysates from *E. coli* expressing GST (pGEX; A, lanes 1 and 7; B, lanes 2, 10, and 17) or ICP35cd (B, lanes 1, 9, and 16) were included as controls. The expression of recombinant proteins for each construct was verified by Western analysis with a monoclonal antibody to GST (A, lanes 7 to 12; B, lanes 16 to 22) and protein staining (B, lanes 9 to 15). Molecular weights are shown in thousands.

tion. Figure 10 illustrates the data from studies in which several deletion mutants of ICP35 were evaluated for interaction with VP5. As expected, deletion of the C-terminal 25 amino acids of the ICP35 precursor to generate ICP35ef(304C) or N120/304C completely abolished ICP35-VP5 interaction, suggesting that the 25 amino acids, removed by proteolytic cleavage during HSV capsid assembly, are critical for stable association with the capsid protein. Further resection of the C terminus also generated fragments of ICP35 with no ability to interact with VP5 (273C and 120C). In contrast, progressive deletion of N-terminal sequences (N53, N120, and N164) to position 164 had no effect on interaction with VP5. Deletion to position 179 or beyond (N179, N273, and N304) reduced interaction to background levels. The failure of these fragments to interact with VP5, despite retaining the critical C-terminal 25 amino acids, is consistent with a requirement for additional structural determinants, elsewhere in the ICP35 molecule, for stable interaction with VP5.

Self-association of ICP35 promotes interaction with VP5. The association of ICP35 with VP5 therefore appeared to be dependent upon sequences within the terminal 25 amino acids of the protein and upon structural determinants between Ser165 and Val179. Since a primary ICP35 self-association domain maps between Ser164 and His219 (see above), we speculated that self-association of ICP35 influences VP5 interaction and that this region might participate indirectly in VP5 association by mediating self-association. We therefore tested additional mutants for VP5 association. The mutants Δ234/273 and Δ234/304 (Fig. 10A) are each capable of efficient self-association (data not shown) but have deletions proximal to the VP5-binding C-terminal 25 amino acids. These mutants efficiently interacted with VP5, suggesting the upstream region retained in N164 and required for VP5 interaction maps be-

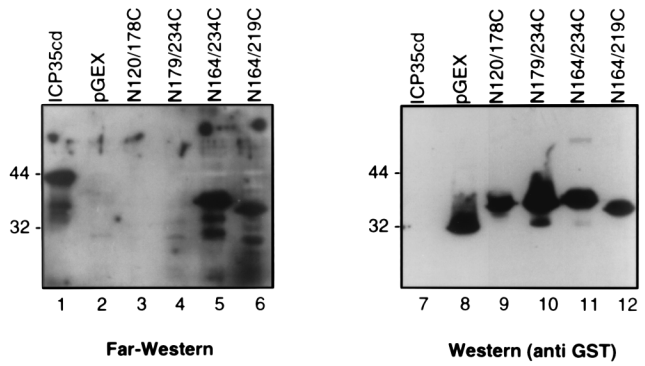

FIG. 7. Far-Western analysis of GST-ICP35-internal-fragment fusion proteins. Fusion proteins containing the designated internal fragments of ICP35 fused to GST were analyzed to confirm the boundaries of the internal region necessary for ICP35 binding. Crude bacterial extracts were analyzed by far-Western (lanes 1 to 6) analysis under conditions similar to those described in the legend to Fig. 5. Expression of the recombinant proteins was confirmed by Western blotting with a monoclonal antibody to GST (lanes 7 to 12). Purified ICP35cd was used as a positive control for ICP35 binding (lanes 1 and 7), and lysates from *E. coli* containing the pGEX vector were used as a control for GST expression (lanes 2 and 8). Molecular weights (in thousands) are indicated on the left.

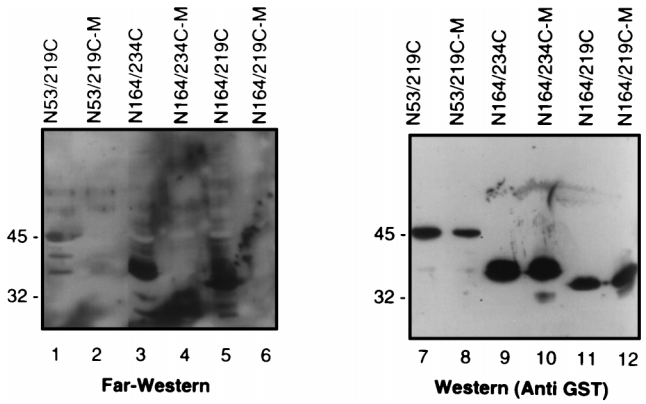

FIG. 8. Far-Western analysis of site-directed mutants in the core self-association domain. Crude bacterial lysates containing the wild-type (lanes 1, 3, 5, 7, 9, and 11) or mutant (lanes 2, 4, 6, 8, 10, and 12) forms of GST-N53/219C (lanes 1, 2, 7, and 8), GST-N164/234C (lanes 3, 4, 9, and 10), and GST-N164/219C (lanes 5, 6, 11, and 12) were analyzed for binding to ICP35 by far-Western analysis (lanes 1 to 6) under conditions similar to those described in the legend to Fig. 5. Western analysis (lanes 7 to 12) was used to evaluate the expression of recombinant GST fusion proteins and to confirm that the mutants were expressed at levels similar to those for the constructs without mutations. Each mutant construct contained Ala substitution mutations within the ICP35 self-association domain at Leu182, Leu186, and Met189 (ICP35 amino acid sequence numbering).

tween His234 and Ser165. This is also consistent with a role for the core self-association domain in VP5 association. We therefore tested ICP35 variants with site-directed mutations in the putative coiled coil of the core self-association domain. The self-association deficient mutant N164-M (Fig. 10B), containing the mutations in the coiled-coil motif, failed to interact with VP5 at significant levels. Control experiments confirmed that the N164, N179, and N164-M fusion proteins are expressed at similar steady-state levels in yeast cells, suggesting that differences in their VP5-binding phenotypes are not simple consequences of differential stability or expression of these proteins. Differences in their structural features must therefore be responsible for influencing their protein-protein interaction phenotype.

These data were consistent with a role for the ICP35 core self-association domain (Ser165 to His219) in VP5 binding but do not distinguish between the possibility that this protein domain has direct binding contacts with VP5 and the possibility that this protein merely affects binding of VP5 indirectly by mediating ICP35 self-association. This was addressed by two approaches. In the first, we assayed ICP35cd(329)-M for VP5 association and compared the result with N164-M (Fig. 10B). This mutant contains the amino acid substitutions in the putative coiled coil of the principal self-association domain (Fig. 3B) but retains self-association capacity in yeast cells, most likely as a consequence of the contributions of the N- and C-terminal domains of the protein to binding. As expected this mutant was highly active for VP5 binding, suggesting that the same mutations in N164 were having their effect through disruption of self-association and not through interruption of a specific binding contact with VP5. A short 56-amino-acid C-terminal segment of ICP35 (N274) was negative for VP5 interaction, despite containing the essential C-terminal 25-amino-acid VP5 interaction site, but strongly interacted when linked to a self-association competent fragment of ICP35 (Met1 to His234; 234C in Fig. 1). We therefore assayed the ability of a heterologous and well-characterized dimerization domain, the leucine zipper of the yeast GCN4 transactivator protein (15), to confer VP5 binding ability to this C-terminal fragment of the assembly protein. The resulting fusion protein (N274-GCN4LZ) has a 31-amino-acid coiled-coil segment from GCN4 linked to the terminal 55 amino acids of ICP35. This fusion protein efficiently interacted with VP5, while the isolated leucine zipper (GCN4LZ) exhibited no interaction (Fig. 10B). These results suggest that dimerization of the ICP35 C-terminal VP5-binding domain promotes interaction with VP5.

DISCUSSION

The capacity of ICP35 to associate into the macromolecular structure composing the capsid scaffold is central to its function. The structural basis for the protein-protein interactions which stabilize these macromolecular assemblies is unknown. In this study we mapped the structural domains responsible for self-interaction of the HSV-1 assembly protein. Expression of ICP35 subdomains in the yeast two-hybrid system permitted us to define two distinct but overlapping regions of the protein, Ser54 to His219 and Ser165 to Arg329, which self-associate in yeast. Each contains a common region, which we have termed the core self-association domain, located centrally in the protein between Ser165 and His219. In the simplest model resolving the data, ICP35 exists as an extended polypeptide chain throughout which multiple self-interacting domains are disposed. The results also suggest that the participating polypeptide chains associate in a parallel orientation, probably through homomeric molecular interactions. Self-association of these parallel polypeptide chains with sufficient affinity to be positive for interaction in the yeast two-hybrid assay requires participation of the core domain and at least one of two flanking domains. Amino acid sequences of ICP35 proximal to the N terminus relative to the core domain appear to contribute more strongly to self-interaction than distal sequences, since the self-interaction of mutants in which the C-terminal flanking sequences are removed is considerably stronger than the self-interaction of mutants in which the N terminus is removed (compare 219C with N164; Fig. 1). Of the three regions, Ser54 to His164, Ser165 to His219, and Pro220 to Arg329, which

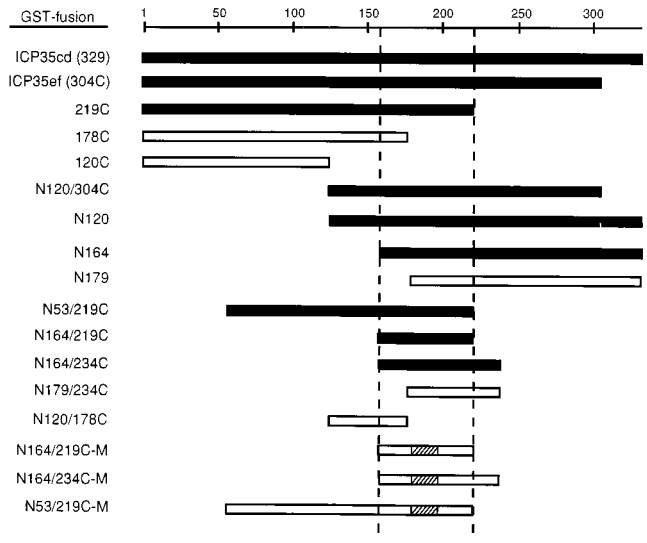

FIG. 9. Self-association domain of ICP35 mapped by far-Western analysis. A summary of the far-Western mapping data presented in Figs. 6 to 8 is shown. Constructs shaded black are positive for interaction with ICP35. Those which are unshaded did not interact. Hatched boxes indicate the presence of the Leu182/Leu186/Met189-to-Ala182/Ala186/Ala189 mutation (see Fig. 3). Amino acid sequence positions are shown, with broken lines indicating positions 164 and 219.

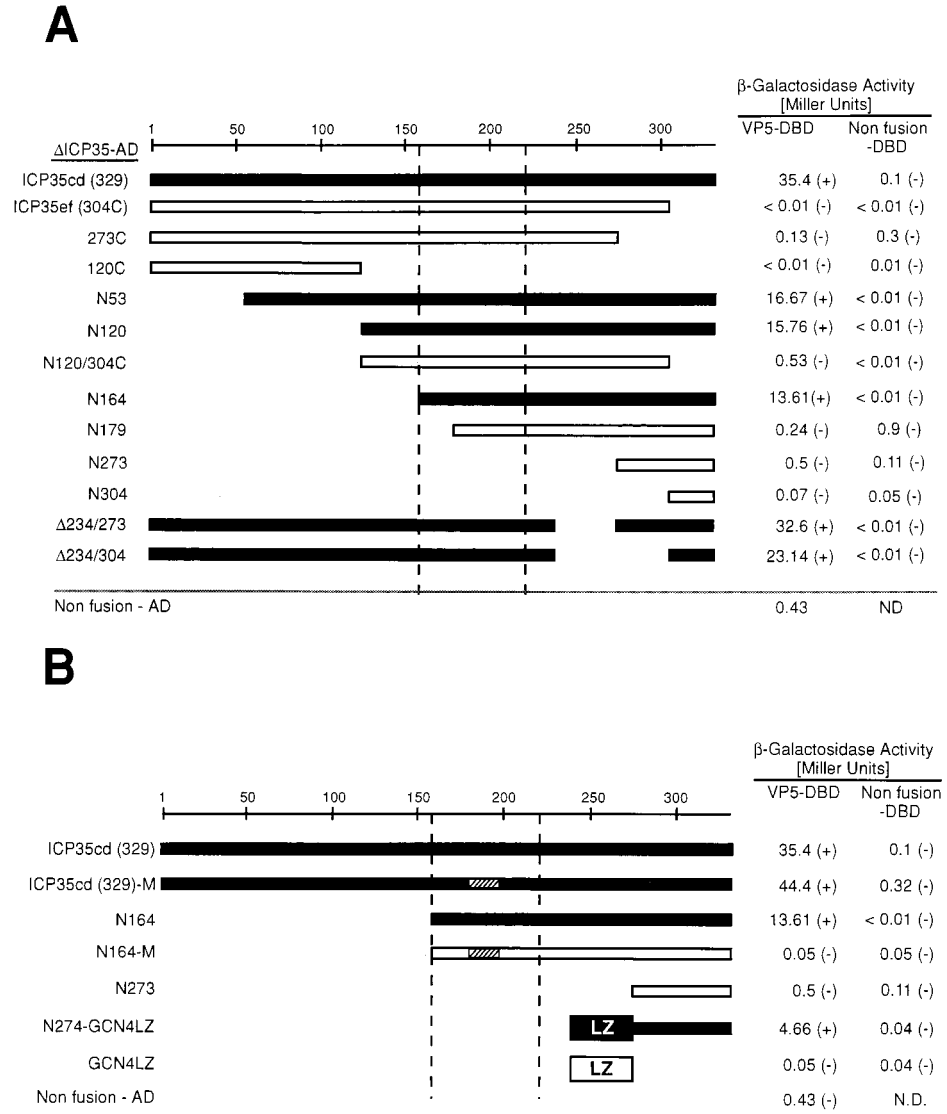

FIG. 10. Yeast two-hybrid analysis of HSV-1 ICP35 interaction with VP5. (A) The indicated ICP35 deletion and mutant derivatives (ΔICP35-AD) were expressed as Gal4 AD fusion proteins and assayed for interaction with HSV-1 VP5 expressed as a Gal4 DBD fusion protein (VP5-DBD). The levels of reporter activation in yeast cells expressing the nonfusion Gal4 DBD (nonfusion DBD) with each ICP35-AD variant are also reported as the controls. Reporter gene activation was monitored by colony filter assay with X-Gal (results in parentheses) and by measurement of β-galactosidase activity in crude yeast cell extracts, as described in Materials and Methods. The mean β-galactosidase activities determined from three independent transformants are presented. ICP35 variants depicted in black were considered positive for interaction with VP5-DBD. Nonshaded constructs were determined to be negative. No ΔICP35-AD derivative scored positive for interaction with nonfusion DBD in the subjective colony filter β-galactosidase assay. Hatched boxes indicate the presence of the Leu182/Leu186/Met189-to-Ala182/Ala186/Ala189 mutation (B) (see Fig. 3). The GCN4 protein leucine zipper (LZ) motif (GCN4 amino acids 250 to 281) is indicated (B). Amino acid sequence positions are shown, with broken lines indicating positions 164 and 219. N.D., not done.

contain structural determinants contributing to self-association, none were independently capable of stable self-association in this assay. Mutants deleted of one or another flanking domain remained competent for interaction, as did a mutant containing discrete mutations within the core domain but retaining both intact flanking domains. This suggests that these domains may be partially redundant in function and that stable interaction may occur when any combination of two self-associating domains is present.

Complementary far-Western experiments identified the central core domain, Ser165 to His219, as the principal self-association domain of ICP35. Indeed this 55-amino-acid segment of the protein was both necessary and sufficient for self-association in far-Western assays. In contrast with results from two-hybrid assays, no contribution of regions outside this core domain could be discerned. This may be due to the greater sensitivity of the far-Western assay compared to the yeast two-hybrid system, enabling it to detect weaker binding interactions. We also consider it possible that regions distal or proximal to the central core domain do not mediate direct binding interactions but contribute to increased stability or expression of particular fusion proteins in yeast or play a role in conformational stabilization of the core domain. Another possibility is that the in vitro assay only partially reflects the in vivo interaction of ICP35 molecules. It is known that ICP35 is phosphorylated in HSV-infected cells (2), and it is feasible that such modification could modulate the affinity of ICP35 self-interaction. The phosphorylation of ICP35 in yeast cells could

thus explain why the core domain is not sufficient for stable self-interaction in the two-hybrid assay. This possibility is particularly intriguing since parallel homomeric association of the polypeptide chains might well be expected to juxtapose negatively charged phosphoamino acids, perhaps reducing the stability of the complex. The potential effects of in vivo protein modification are not addressed in our far-Western experiments, which utilized recombinant proteins expressed in *E. coli.*

Parallel-oriented coiled coils are proposed to be the underlying structural basis for ICP35 self-association. Coiled coils are composed of bundles of α helices wound into a superhelix and are characterized by the regular packing of amino acid side chains within the interface. The nature of the structure requires a heptad repeat of the chemical nature of the amino acid side chains, most notably for the residues at positions *a* and *d* in the helix (Fig. 3). Hydrophobic amino acids in these positions constitute the helix interface and are evident as a 4-3 repeat in the primary amino acid sequence. A short coiled coil spanning the residues Ile172 to Ser194 is found in the core self-association domain of ICP35 and is responsible for mediating the self-interaction of this region. This motif is highly conserved between the assembly proteins of the alpha herpesviruses, which otherwise exhibit limited sequence conservation. Mutation of a subset of amino acids predicted to participate in the interface of the coiled-coil region destroyed the capacity for self-association. In considering the requirement of the participation of flanking regions of the assembly protein for self-association in two-hybrid assays, we examined sequence alignments and secondary structure predictions for the assembly proteins. It is possible that the additional putative α-helical regions which correspond to highly conserved motifs within the assembly proteins contribute to the stability of association through additional coiled-coil interactions (Fig. 2B and 3C). For example, a short conserved region having a propensity for coiled coil and containing a short 4-3 hydrophobic repeat is located between Leu87 and Arg102. Similarly the C terminus of the protein contains a highly conserved segment (amino acids 317 to 329) which has a high propensity for α helicity. This region lacks the 4-3 repeat of bulky hydrophobes commonly observed in well-characterized coiled coils but possesses the potential for forming an extensive amphipathic helix. This region is responsible for interaction of the assembly protein with VP5 and has been reported to exist in an α-helical conformation (16). The capacity of this α helix to self-associate through its hydrophobic surfaces is, however, uncertain. It is worth noting that each putative coiled coil we have identified in ICP35 is shorter than many structurally characterized coiled coils. The stable self-association of the ICP35 molecule may thus be attributable to the aggregate affinity of multiple weak homomeric coiled-coil interactions.

Our results have extended those of Desai et al. (9) who, in similar experiments, demonstrated self-interaction of ICP35 and N-terminal fragments as short as 198 amino acids. These fragments would be expected to retain the intact coiled-coil motif we have defined in the core self-association domain. Interestingly, the cytomegalovirus assembly protein contains an extended region with significant propensity for coiled-coil formation, although it is not positionally conserved with respect to such regions in HSV. We note, however, that experiments to map self-association domains within that protein have located a short domain, Ala31 to Tyr123, closer to the N terminus of the protein (14a). In contrast to the region mapped for HSV, this region contains no sequence with an obvious propensity for α helicity or coiled-coil formation (unpublished data). The significance of these differences and the potential role of coiled coils in the function of the cytomegalovirus assembly protein remain to be established.

We also examined the association of ICP35 with VP5 in the two-hybrid system. Our experiments confirmed that the 25 C-terminal amino acids of the ICP35 precursor are required for this interaction (16, 41). Moreover, our results indicated that an additional upstream region of ICP35 was required for the interaction and mapped to the core self-association domain between Ser165 and His219. In addition, self-association deficient variants of ICP35 containing site-directed mutations in the putative self-interacting coiled-coil region failed to interact with VP5 despite retaining the C-terminal 25 amino acids distal to the protease cleavage site. The same mutations, which in the context of the full-length ICP35 molecule failed to perturb self-association to a significant degree in the two-hybrid system, likewise had no effect on VP5 binding. This lack of effect rules out a role for direct contact of the putative coiled-coil sequences with VP5 in stabilizing the ICP35-VP5 complex. Furthermore, the substitution of a heterologous dimerization domain, the coiled coil of the yeast GCN4 protein, for the self-associating region of ICP35 rescued VP5 interaction of a short 56-amino-acid C-terminal segment of ICP35. These data strongly support the hypothesis that self-association of the assembly protein is required for interaction with VP5. The apparent prerequirement for self-association of ICP35 for binding to VP5 is contrary to the findings reported by Hong et al. (16). Those investigators demonstrated that a peptide mimicking the C terminus of ICP35 could compete for interaction of VP5 with a GST-ICP35 fusion protein, although data concerning the monomeric status of the peptides used were not presented. Nevertheless, we have observed that with a stronger promoter driving higher levels of expression of the oligomerization-deficient mutants of ICP35 (N179 and N164-M) in the yeast two-hybrid system, significant levels of interaction can be observed over background (unpublished data). ICP35 derivatives which retained the capacity to self-associate still retained differentially higher levels of VP5 interaction in these assays. It is therefore likely that efficient self-association of ICP35 promotes complex formation with VP5 molecules in vivo.

ICP35 forms the core around which the exterior capsid components assemble. It has been previously shown to interact directly with VP5 in the shell via its C-terminal sequences (16, 41). The participation of the assembly protein in capsid assembly is essential for the formation of closed capsids and appears to influence the curvature of the outer capsid shell (9, 23, 41). Data from in vitro reconstitution of capsids is consistent with assembly proceeding via the stoichiometric addition of assembly protein and VP5 to the nascent capsid structure, and it is envisaged that small subassemblies of VP5 and ICP35 are recruited to the growing structure, similar to the mechanism proposed for *Salmonella* bacteriophage P22 capsid assembly (27, 33, 34). The assembly protein exists as a highly elongated molecule, anchored at its C terminus to VP5 and projecting into the interior of the nascent capsid as an extended molecule capable of lateral interactions with neighboring assembly protein molecules (42). The putative coiled coils within ICP35 that we have proposed appear to mediate self-association of ICP35 and to promote interaction with VP5. It is therefore likely that these interactions mediate the assembly of small VP5-ICP35 oligomers which may serve as the subunits for capsid assembly in the nucleus. It is tempting to speculate that lateral interactions between bundles of helices participating in coiled coils could be the basis for scaffold assembly in the nascent capsid, perhaps forming a protein micelle with a preferred radius of curvature, as proposed by Trus and colleagues (42) from their electron microscopy studies of HSV capsid intermediates.

Their interpretation of the radial density of the interior scaffold is consistent with self-association of ICP35 occurring through lateral interaction of three distinct domains. It is possible that the three regions influencing self-association that we have mapped correspond to the regions of the assembly protein exhibiting greatest radial density.

Although the role of ICP35 in capsid assembly is now generally well established, a number of more specific questions remain. One of these pertains to the mechanism by which the assembly protein is expelled from the capsid upon DNA packaging. It is difficult to envisage that the scaffold can escape from the capsid intermediate without at least partial disaggregation of its higher-order structure. The proteolytic cleavage at the C terminus (M site) of the assembly protein (and protease), which is essential for capsid maturation, clearly has the capacity to release ICP35 from a binding interaction with the capsid shell. It remains to be determined whether this can sufficiently destabilize the macromolecular complex to mediate dissolution of the scaffold or whether additional factors are involved. The removal of 25 amino acids from the C terminus may reduce the affinity with which ICP35 molecules associate. Our data (Table 1) are consistent with those of Desai and Person (10) who described a twofold reduction in interaction in the yeast two-hybrid assay. It is therefore possible that in vivo the characterized cleavages of the assembly protein play an important role in modulating stability of the capsid scaffold by releasing it from VP5 interaction and destabilizing self-interaction affinity. It is possible that the scaffold is critically dependent on the aggregate of simultaneous interactions between ICP35 molecules and between ICP35 and VP5 molecules for its integrity. This would provide for its rapid destabilization and removal upon, or prior to, DNA packaging. In addition, as discussed above, ICP35 is a phosphoprotein, and specific phosphate additions in the infected cell might have significant effect upon the consequences of proteolytic modification on scaffold stability. It will be interesting to identify the position of phosphorylation of ICP35 in relation to those sequences that we have designated as important for self-association.

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