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The Putative Leucine Zipper of the U_L6-Encoded Portal Protein of Herpes Simplex Virus 1 Is Necessary for Interaction with pU_L15 and pU_L28 and Their Association with Capsids[▽]

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Herpes simplex virus (HSV) type 1 capsids contain a single portal vertex that is composed of 12 copies of the U_L6 gene product (pU_L6), which forms a pore through which DNA is inserted during packaging. This unique vertex is also believed to comprise the site with which a molecular motor, termed the terminase, associates during the DNA packaging reaction. In HSV, the terminase likely comprises the U_L15, U_L28, and U_L33 proteins (pU_L15, pU_L28, and pU_L33, respectively). The current study was undertaken to identify portal domains required for interaction with the terminase. Both the amino and carboxyl termini, as well as amino acids 422 to 443 of pU_L6 forming a putative leucine zipper motif, were critical for coimmunoprecipitation with pU_L15 in the absence of other viral proteins. Amino acids 422 to 443 were also necessary for interaction with pU_L28 in the absence of other viral proteins. By using an engineered recombinant virus, it was further determined that although amino acids 422 to 443 were dispensable for interaction with scaffold protein and incorporation of portal protein into capsids, they were necessary for coimmunoprecipitation of pU_L6 and pU_L15 from infected cell lysates, association of optimal levels of pU_L15, pU_L28, and pU_L33 with capsids, and DNA cleavage and packaging. These data identify a portal protein domain critical for terminase association with the capsid and suggest that both the pU_L15- and pU_L28-bearing terminase subunits mediate docking of the terminase with the portal vertex.

The DNA replication machinery of herpes simplex virus (HSV) produces concatameric viral DNA in the nuclei of infected cells that is cleaved and packaged into preformed capsids (reviewed in references 2 and 4). These icosahedral capsids contain a unique pore, termed the portal vertex, through which viral DNA is inserted (15). The portal vertex comprises 12 copies of the U_L6 gene (23). The portal also likely functions as the docking site of the terminase enzyme (24, 27, 30), which is responsible for cleavage of viral DNA and is a key part of the molecular motor that drives DNA through the portal during the packaging reaction (2, 19). In HSV, the terminase likely comprises the U_L15, U_L28, and U_L33 gene products (5, 10, 26). The U_L15 protein (pU_L15) is believed to provide the energy for the packaging reaction through hydrolysis of ATP because it contains a highly conserved ATP binding motif that is essential for DNA packaging (7, 29); pU_L28 likely provides sequence-specific DNA binding activity (1), and pU_L33 enhances the pU_L15/pU_L28 interaction, primarily through its interactions with U_L28 (26).

Incorporation of the portal into the capsid is mediated by its interaction with amino acids 143 to 151 of ICP35, the major component of the internal shell of the two-shelled capsid (9, 18, 28). In the absence of this scaffold protein sequence, the portal protein fails to interact efficiently with ICP35 in vitro and is not incorporated into the capsids.

Like those of all herpesviruses, the HSV type 1 (HSV-1) portal protein contains a potential leucine zipper between amino acids 422 and 443, in which three invariant leucines are separated by 6 amino acids, thus potentially placing them on one side of an α-helix (data not shown). Because such motifs have been implicated in a number of protein-protein interactions, and protein interactions represent critical functions of the portal vertex, this motif has garnered experimental interest. Specifically, deletion of codons 409 to 473, or changing leucines at positions 429 and 436 to glutamic acid, reduced incorporation of the portal into capsids and precluded normal formation of portal rings in vitro (14).

One goal of the current work was to determine how the terminase docks with the capsid. The portal protein pU_L6 coimmunoprecipitates with both pU_L15 and pU_L28, suggesting these proteins interact in vivo (27). This hypothesis is also supported by observations that pU_L6 (i) coimmunoprecipitates with either pU_L15 or pU_L28 overexpressed in insect cells and (ii) can alter the localization of pU_L28 and pU_L15 in mammalian cells when these proteins are coexpressed with pU_L6 in the absence of other viral proteins (24).

Because our previous observations (27) indicated that the coimmunoprecipitation of pU_L15 and pU_L6 from infected cells is more robust than that of pU_L28 and pU_L6, we focused primarily on the pU_L6/pU_L15 interaction in the current study. The data indicated that although codons 422 to 443 of U_L6 were dispensable for interaction with scaffold protein and incorporation of the portal into the capsid, they were critical for (i) DNA cleavage and packaging, (ii) interaction between pU_L15 and pU_L6 in lysates of both uninfected and infected cells, (iii) coimmunoprecipitation with transiently expressed pU_L6 and pU_L28, and (iv) association of normal levels of

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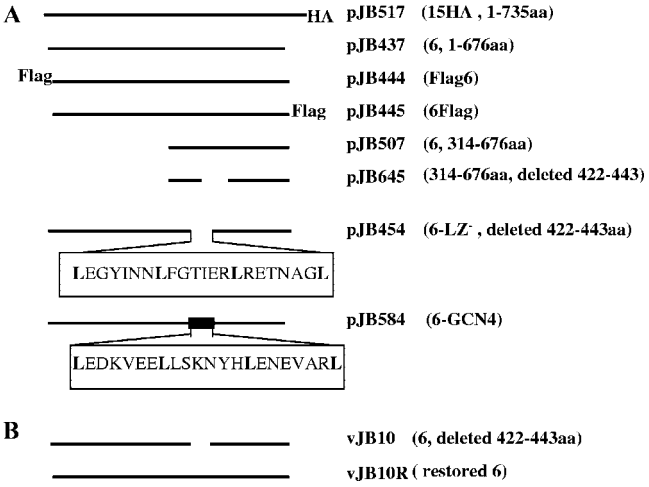

FIG. 1. (A) Schematic diagram of the full-length pU_L15 (top line), pU_L6 (second line from top), and U_L6 mutant (other lines) proteins. The designation of the plasmid and genotype are indicated on the right. Numbers in the rightmost column followed by aa indicate the amino acids present in the corresponding construct. (B) Schematic diagram of the U_L6 gene of the deletion mutant virus vJB10 or its corresponding genetically restored virus vJB10R.

pU_L15, pU_L28, and pU_L33 with the capsid. These data suggest that docking of the terminase with the capsid involves interactions between the portal protein and both the U_L15-encoded and pU_L28-encoded terminase subunits and shed new light on the importance of the putative leucine zipper of the portal in HSV DNA packaging.

MATERIALS AND METHODS

Viruses and cells. CV1, Vero, and rabbit skin cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 100 U penicillin per ml, and 100 μg of streptomycin per ml. The Flp-In-CV1 cell line was purchased from Invitrogen and was grown in DMEM supplemented with 10% newborn bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml Zeocin. The CV6 and CV6M cell lines described in this paper were cultured in DMEM supplemented with 10% newborn bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 200 μg/ml of hygromycin B. HSV-1 strain F [HSV-1(F)] and U_L6 null virus derived from HSV-1 strain 17 were described previously (8, 17). Recombinant virus vJB10 and its restored virus vJB10R are described in this paper.

Plasmids. The full-length U_L15 gene with a hemagglutinin (HA) tag inserted in-frame at the C terminus was amplified by PCR and cloned into the HindIII and EcoRV sites located in the multiple cloning site of expression vector pCDNA3. The resulting plasmid was designated pJB517. Plasmid pJB437 containing the entire U_L6 coding sequence was described previously (27). U_L6 genes fused at the 5′ or 3′ end to DNA encoding a Flag epitopic tag were amplified by PCR and cloned into the BamHI and EcoRI sites located in the multiple cloning site of expression vector pcDNA3. The resulting constructs were designated pJB444 and pJB445, respectively. To delete codons 422 to 443 from U_L6, encoding a putative leucine zipper, two-step PCR was performed. The resultant PCR amplicons were cloned into pcDNA3 at the BamHI and EcoRI sites, and the resulting plasmid was designated pJB454. Similarly, amino-terminally truncated U_L6 (as shown in the diagram of Fig. 1) was amplified by PCR, using pJB437 or pJB454 as a template. PCR products were cloned into the BamHI and EcoRI sites of pCDNA3, and the resulting plasmids were designated pJB507 and pJB645, respectively. To construct pJB456 and pJB557, plasmids pJB347 and pJB454 were digested with BamHI and NotI, and the inserts were purified and cloned into pcDNA5/FRT at the BamHI and NotI sites. All plasmid constructs were confirmed by immunoblotting after transient expression in mammalian cells and DNA sequencing by the Cornell University DNA sequencing and genotyping core facility (data not shown). To replace the sequence for the putative leucine zipper of pU_L6 with the amino acid sequence of the GCN4 leucine zipper (LEDKVEELLSKNYHLENEVARL), two-step PCR was performed, the final PCR amplicons were cloned into pCDNA3 at the BamHI and EcoRI sites, and the resulting plasmid was designated pJB584.

Plasmid pJB112, containing the U_L28 coding sequence cloned into pCDNA3, was described previously (22). Plasmid pCAGGS-nlsCre, expressing Cre recombinase, was a gift from Michael Kotlikoff, Cornell University. Plasmids pBAD-I-SceI, containing the gene encoding the *Saccharomyces cerevisiae* I-SceI endonuclease, and pEPkan-S, containing *aphAI* (encoding kanamycin resistance) adjacent to an I-SceI restriction site, were gifts from Nikolaus Osterrieder, Cornell University.

Construction of complementing cell lines. CV6 and CV6M cell lines were made by using the Flp-In-CV1 system (Invitrogen) according to the manufacturer's protocol and as described previously (28). Briefly, either pJB456 or pJB557 was cotransfected with plasmid pOG44, encoding Flp recombinase under the constitutive cytomegalovirus promoter/enhancer, into an engineered cell line (Flp-In-CV1). Correct insertion of the shuttle vector caused simultaneous loss of Zeocin resistance and gain of hygromycin resistance. After recombination, cells resistant to hygromycin were selected by growth in DMEM supplemented with 10% newborn bovine serum and 200 μg/ml hygromycin B.

Construction of recombinant viruses. Production and characterization of a bacterial artificial chromosome (BAC) containing the entire HSV-1(F) genome was described previously (20). Recombinant viruses were constructed by en passant mutagenesis, a two-step Red-mediated recombinant system described by Tischer et al. (22). The details of the procedure and their use in construction of recombinant HSV-1 genomes were also described previously (27). The primers for the production of a PCR amplicon for eventual deletion of codons 422 to 443 from the U_L6 gene in the HSV-1(F)-containing BAC were as follows: forward, TTCCGCACGGCCGTGGTTAACAACATCAACGGCGTGGCGACCCAAT TGCAGGAGTAGGGATAACAGGGTAATCGATTT; reverse, CCGGAGCT CGCGGTCGCGCTCCTGCAATTGGGTCGCCACGCCGTTGATGTTGTT GCCAGTGTTACAACCAATTAACC.

The expected mutation in the BAC DNA was confirmed by DNA sequencing, and the resulting recombinant BAC was designated bJB10. Purified bJB10 BAC DNA was cotransfected with a Cre expression plasmid (see above) into CV6 cells expressing pU_L6. The presence of viable recombinant virus was confirmed by plaque formation on complementing CV6 cells, and the resulting virus was subjected to two further rounds of plaque purification. The genotype of the recombinant virus, designated vJB10, was confirmed by PCR and DNA sequencing, whereas the viral phenotype was characterized as described below in Results. To repair the mutated U_L6 gene, rabbit skin cells were cotransfected with vJB10 viral DNA and linearized pRB132, which contains the gene fragment from bases 11820 to 21655 of the HSV-1 genome, according to the numbering scheme of McGeoch et al. (13). The virus arising from homologous recombination was able to form plaques on rabbit skin cells and was designated vJB10R. The genotype of vJB10R was confirmed by DNA sequencing, immunoblotting, and Southern blot analyses (data not shown).

Immunoprecipitation and immunoblotting. These procedures were performed essentially as described previously (23, 24). Briefly, CV1 cells were either transfected with expression plasmids containing U_L15, U_L28, and U_L6 or its derivatives or infected with wild-type or recombinant viruses. At 24 h after transfection or 18 h after infection, the cells were washed with cold phosphate-buffered saline (PBS) and lysed in cold radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, and 1× protease inhibitor cocktail (Roche). Rabbit anti-HA polyclonal antibody (HA-probe, Y-11, SC-805 diluted 1:200; Santa Cruz Biotechnology) was used for pU_L15 immunoprecipitation, whereas pU_L6-specific rabbit polyclonal serum (diluted 1:100) was used to immunoprecipitate pU_L6. Anti-ICP35 monoclonal antibodies (MCA 406; AbD Serotec) were used for immunoprecipitation at a dilution of 1:200. pU_L28-specific rabbit polyclonal antisera were diluted 1:100 to immunoprecipitate pU_L28. Immune complexes, RIPA buffer-solubilized clarified lysates, total lysates solubilized in 1% sodium dodecyl sulfate (SDS) and beta-mercaptoethanol, and in some experiments, SDS-denatured purified B capsids were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes for immunoblotting.

Immunoblots were probed with anti-HA antibodies diluted 1:1,000, anti-pU_L15C diluted 1:1,000, anti-pU_L28 diluted 1:1,000, anti-pU_L33 diluted 1:400, anti-ICP35 (MCA 406; AbD Serotec) diluted 1:2,000, anti-VP5 (HA018-100; Virusys Corporation) diluted 1:1,000, and/or anti-pU_L6 polyclonal antiserum diluted 1:1,000. The bound immunoglobulins were revealed by reaction with horseradish peroxidase-conjugated anti-immunoglobulins and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). Where applicable,

TABLE 1. Virus replication assay

Virus	Genotype of U_L6	Cell type infected/cell type used for plaque assay	Titer (PFU/ml)[a]
vJB10	Deletion of codons 422 to 443	CV1/CV6	$<10^2$
vJB10	Deletion of codons 422 to 443	CV6/CV6	$1.2 \times 10^7 \pm 0.5 \times 10^7$
vJB10	Deletion of codons 422 to 443	CV6M/CV6	$<10^2$
vJB10R	Deleted codons restored	CV1/Vero	$2.0 \times 10^7 \pm 0.76 \times 10^7$
HSV-1(F)	Wild type	CV1/Vero	$2.5 \times 10^7 \pm 0.2 \times 10^7$
HSV-1(F)	Wild type	CV6/Vero	$2.6 \times 10^7 \pm 0.5 \times 10^7$
HSV-1(F)	Wild type	CV6M/Vero	$6.5 \times 10^4 \pm 0.8 \times 10^4$

[a] Virus titers were determined as described in Materials and Methods. The data represent means ± standard deviations for three independent experiments.

the image intensities of specific bands on the immunoblots were quantified with an LAS-3000 mini Fujifilm imaging system (Fuji Photo Film Co., Ltd.).

Capsid purification. CV1 cell monolayers from two 850-cm^2 roller bottles were infected with either HSV-1(F) or mutant viruses at a multiplicity of infection (MOI) of 5 PFU/cell. The cells were harvested 20 hours later and washed with cold PBS. Cell pellets were suspended in 25 ml of lysis buffer (1 mM dithiothreitol, 1 mM EDTA, 20 mM Tris [pH 7.6], 500 mM NaCl, 1% Triton X-100, and protease inhibitor), sonicated briefly, and precleared by spinning at $10,000 \times g$ for 15 min. The precleared lysates were pelleted through a 5-ml 35% sucrose cushion in TNE buffer (20 mM Tris-HCl [pH 7.6], 500 mM NaCl, 1 mM EDTA), in an SW28 ultracentrifuge tube at 24,000 rpm for 1 h. The pellets were resuspended in TNE buffer and applied to 20% to 50% sucrose gradients in SW41 ultracentrifuge tubes followed by centrifugation at 24,500 rpm for 1 h. After centrifugation, the light-refracting B capsid band was removed with a Pasteur pipette. Purity of the capsid preparations was evaluated by transmission electron microscopy and negative staining (data not shown).

Southern blotting. Approximately 2×10^6 CV1 cells were infected with HSV-1(F), vJB10, or vJB10R. At 18 h postinfection, viral DNA was extracted, digested with BamHI, separated on 0.8% agarose gels, and transferred to nylon membranes as described previously (24). The bound DNA was UV cross-linked to the membrane and hybridized with a denatured [^{32}P]dCTP-labeled BamHI P fragment of HSV-1(F) DNA at 42°C for 24 h. The membrane was washed twice with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) at 42°C for 15 min each time and once with 0.1× SSC–0.1% SDS for 1 h at 64°C and then fluorographed by exposure to X-ray film at −80°C in the presence of intensifying screens.

Virus replication assay. Approximately 2×10^6 cells in 25-cm^2 flasks were infected with the viruses indicated in Table 1 at an MOI of 0.1 PFU/cell. After adsorption for 2 hours at 37°C with shaking, the inoculates were removed, and the cells were washed with CBS buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3.0]) to remove residual infectivity. The cells were then washed with PBS once and overlaid with 5 ml of DMEM supplemented with 5% newborn calf serum. Twenty-four hours after infection, virus was harvested by three cycles of freezing and thawing, and the infectious yields were determined by plaque assay on the cell monolayers indicated in Table 1.

RESULTS

To identify domains of pU_L6 necessary for interaction with pU_L15, we cloned pU_L15 in-frame with an HA tag at its C terminus and wild-type U_L6 into plasmids such that their expression was under the control of the human cytomegalovirus early promoter/enhancer. We chose this site in pU_L15 to insert the HA tag because other insertions (cytomegalovirus glycoprotein B or Flag epitopes) at the C terminus of pU_L15 have been tolerated with little effect on virus replication (3). The engineered plasmids were transfected into CV1 cells. Lysates of the transfected cells were then subjected to immunoprecipitation with HA-specific or pU_L6-specific antibodies, and the presence of pU_L6 and pU_L15-HA in immunoprecipitated material was assessed by immunoblotting.

As shown in Fig. 2, lane 2, pU_L15 and pU_L6 were expressed to readily detectable levels in lysates of the transfected cells, but not in mock-transfected cells. Moreover, the HA-specific antibody readily immunoprecipitated HA-tagged pU_L15, and the pU_L6-specific antibody successfully immunoprecipitated pU_L6. Most importantly for the purposes of this report, material immunoprecipitated with the HA-specific antibody also contained pU_L6, and reaction with the pU_L6 antibody caused coimmunoprecipitation of pU_L15. We conclude that transiently expressed pU_L15 and pU_L6 interact as assessed by immunoprecipitation.

To identify domains of pU_L6 involved in interactions with the terminase subunit pU_L15, a series of transient expression constructs were made; their relevant sequences are indicated in Fig. 1A. Expression plasmids bearing these constructs were contransfected with plasmids expressing full-length pU_L15-HA into CV1 cells, and lysates of the cells were immunoprecipitated with the HA- and pU_L6-specific antibodies. As shown in Fig. 2, lanes 3 and 4, placement of a Flag epitopic tag at either the C terminus or the N terminus of pU_L6 did not interfere with the expression or immunoprecipitation of pU_L6 with its cognate antibody, but it completely precluded coimmunoprecipitation with pU_L15. These data suggest that both the N and C termini of pU_L6 are important for the interaction with pU_L15. To confirm that the N and C termini of pU_L6 were necessary for the coimmunoprecipitation with pU_L15, a plas-

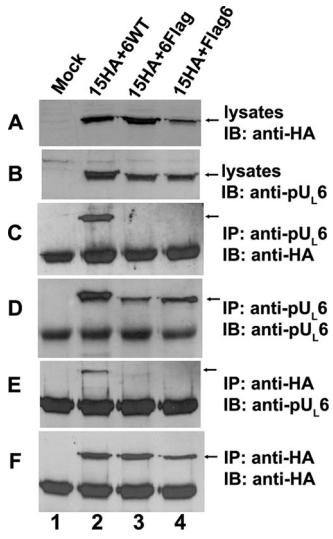

FIG. 2. Insertions of Flag epitopic tags at the N or C terminus of pU_L6 preclude interaction with transiently expressed pU_L15. CV1 cells (2×10^6) were cotransfected with plasmids encoding pU_L15-HA and wild-type U_L6 (lane 2) or Flag-tagged pU_L6 (lanes 3 and 4) or were mock transfected (lane 1). Twenty-four hours after transfection, reciprocal coimmunoprecipitation with either anti-pU_L6 or anti-HA antibodies was performed. Cell lysates and immunoprecipitated proteins were electrophoretically separated in denaturing 12% polyacrylamide gels and transferred onto a nitrocellulose membrane. The transferred proteins were probed with anti-pU_L6 (B, D, and E) or anti-HA (A, C, and F) antibodies. Bound immunoglobulins were revealed by enhanced chemiluminescence. IP, immunoprecipitation with the indicated antibody; IB, immunoblotting with the indicated antibody. The arrows indicate the positions of proteins of interest.

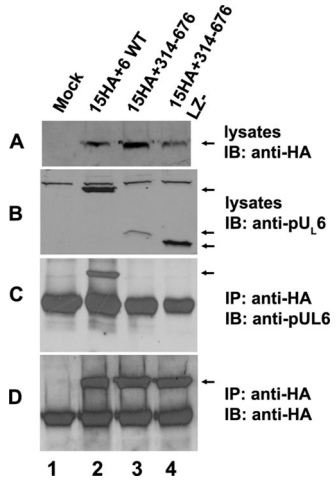

FIG. 3. Only the full-length pU_L6 interacts with pU_L15 in transiently transfected cells. CV1 cells (2×10^6) were mock transfected (lane 1) or cotransfected with plasmids encoding pU_L15-HA and full-length pU_L6 (lane 2), N-terminally truncated pU_L6 (lane 3), or N-terminally truncated pU_L6 lacking the putative leucine zipper (lane 4). Twenty-four hours after transfection, coimmunoprecipitation was performed with anti-pU_L6 antibodies. Cell lysates and immunoprecipitated material were separated on 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The transferred proteins were probed with anti-pU_L6 (B and C) or anti-HA (A and D) antibodies. Bound immunoglobulins were revealed by reaction with appropriately conjugated anti-immunoglobulins followed by enhanced chemiluminescence. IP, immunoprecipitation with the indicated antibody; IB, immunoblotting with the indicated antibody. The arrows indicate the positions of proteins of interest.

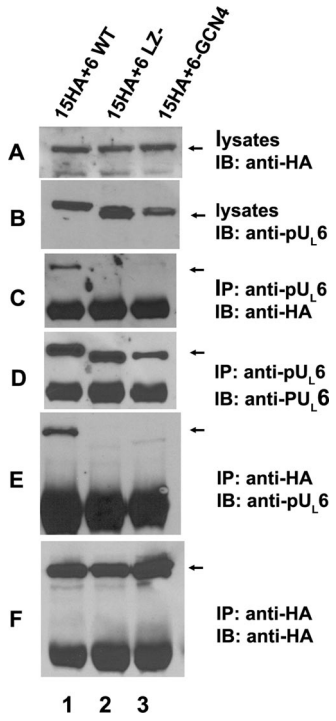

FIG. 4. The putative leucine zipper of pU_L6 of HSV-1(F) is required for pU_L6 and pU_L15 interaction in the absence of other viral proteins. CV1 cells were cotransfected with plasmids encoding full-length pU_L15 with a C-terminal HA tag and wild-type pU_L6 (lane 1), pU_L6 lacking amino acids 422 to 443 (lane 2), or a pU_L6-GCN4 chimera (lane 3) in which codons 422 to 443 of U_L6 were replaced with the sequence encoding the GCN4 leucine zipper. Twenty-four hours after transfection, reciprocal coimmunoprecipitations were performed with anti-pU_L6 or anti-HA antibodies. Cell lysates and immunoprecipitated proteins were separated on denaturing 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The transferred proteins were probed with anti-pU_L6 (B, D, and E) or anti-HA (A, C, and F) antibodies. Bound immunoglobulins were detected as indicated in the legend to Fig. 3. IP, immunoprecipitation; IB, immunoblotting. The arrows indicate the positions of proteins of interest.

mid construct encoding pU_L6 amino acids 314 to 676 (Fig. 3) and a construct encoding only the first 321 amino acids of pU_L6 (not shown) were coexpressed with pU_L15 and were subjected to immunoprecipitation with HA antibodies. As shown in Fig. 3, the C-terminal amino acids 314 to 676 of pU_L6 were expressed and were recognized by the pU_L6 antibody, but they did not coimmunoprecipitate with pU_L15-HA. Similar negative results were obtained upon expression of the first 321 codons encoding the sequence of pU_L6 followed by attempted coimmunoprecipitation of pU_L15-HA with the HA antibody (not shown). We conclude that both N and C termini are necessary for interaction with pU_L15 in this transient immunoprecipitation assay.

To investigate the role of the putative leucine zipper in coimmunoprecipitation of transiently expressed pU_L6 and pU_L15, codons 422 to 443 of U_L6 were deleted, the truncated protein was coexpressed with pU_L15-HA, and the lysates were subjected to immunoprecipitation with pU_L6- and HA-specific antibodies. As shown in Fig. 4, lane 2, the absence of U_L6 codons 422 to 443 precluded coimmunoprecipitation of the truncated pU_L6 and full-length pU_L15, despite the presence of ample levels of soluble pU_L6 and pU_L15 proteins in the starting lysates, and the successful coimmunoprecipitation of both proteins with their cognate antibodies. These data indicate that the putative leucine zipper in pU_L6 is required for the interaction with pU_L15. To determine if a known leucine zipper could complement the function of codons 422 to 443, these codons were replaced with DNA encoding the leucine zipper of GCN4 (16) and the protein was coexpressed with pU_L15 and subjected to the coimmunoprecipitation reaction. As shown in Fig. 4, lane 3, the GCN4 motif was not sufficient to restore the ability of truncated pU_L6 to interact with pU_L15. Thus, either the GCN4 motif is unable to form a leucine zipper when expressed in the context of pU_L6, or codons 422 to 443 mediate the interaction with pU_L15 through means other than as a leucine zipper.

To determine whether pU_L6 and pU_L28 can interact in the absence of other viral proteins in mammalian cells and whether codons 422 to 443 affected this interaction, CV1 cells were transfected with plasmids encoding full-length pU_L28 and either wild-type pU_L6, pU_L6 lacking sequences corresponding to codons 422 to 443, or pU_L6 in which the sequences corresponding to codons 422 to 443 was replaced by the GCN4 leucine zipper motif (16). Lysates of the cells were then reacted with pU_L28-specific antibody, and the presence or absence of pU_L6 and pU_L28 in immunoprecipitated material was determined by immunoblotting. As shown in Fig. 5, lane 1, wild-type pU_L6 was readily coimmunoprecipitated with pU_L28-specific antibody. In contrast, the absence of the leucine zipper of

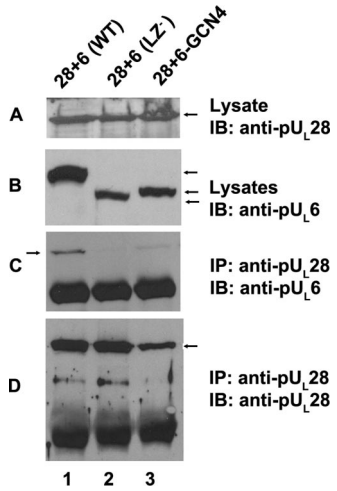

FIG. 5. The putative leucine zipper of HSV-1(F) pU_L6 is required for interaction with pU_L28 in the absence of other viral proteins. Cells were transfected with plasmids encoding full-length pU_L28 and wild-type pU_L6 (lane 1), pU_L6 lacking codons 422 to 443 (lane 2), or pU_L6 in which codons 422 to 443 were replaced by a leucine zipper of GCN4 (lane 3). The cells were lysed 24 h later and either immunoblotted with antibodies directed against pU_L6 or pU_L28 (A and B) or subjected to immunoprecipitation with antibody directed against pU_L28. The presence of pU_L6 or pU_L28 in immunoprecipitated material was determined by immunoblotting (C and D, respectively). IP, antibody used for immunoprecipitation in the corresponding panel; IB, antibody used for immunoblotting in the corresponding panel. The arrows indicate the positions of proteins of interest.

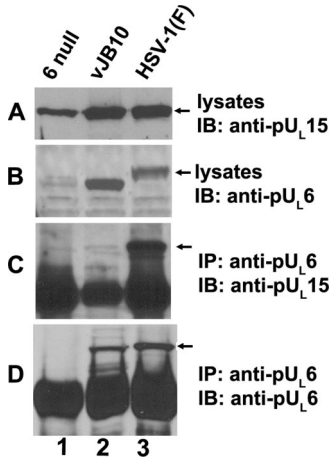

FIG. 6. The putative leucine zipper of HSV-1(F) pU_L6 is required for the interaction between pU_L6 and pU_L15 in virus-infected cells. CV1 (2×10^6) cells were infected with a U_L6 null mutant, leucine zipper deletion virus (vJB10), or HSV-1(F) at an MOI of 5 PFU per cell. Eighteen hours after infection, cells were lysed in RIPA buffer with 1 M NaCl, and immunoprecipitation was performed with anti-pU_L6 antibody. Cell lysates and immunoprecipitated proteins were electrophoretically separated on four separate polyacrylamide gels and immunoblotted with anti-pU_L15C or pU_L6-specific antibodies. Bound immunoglobulins were revealed as indicated in the legend to Fig. 3. The arrows indicate the positions of proteins of interest. Note that the gels were run for different periods of time, accounting for the different migrations of bands in the different panels. IP, antibody used for immunoprecipitation in the corresponding panel; IB, antibody used for immunoblotting in the corresponding panel.

pU_L6 precluded this coimmunoprecipitation whether or not it was replaced with the GCN4 leucine zipper motif.

To determine the role of codons 422 to 443 of pU_L6 in infected cells, a recombinant virus lacking these codons was constructed as detailed in Materials and Methods. The virus was designated vJB10. Initial experiments indicated that vJB10 was unable to propagate on CV1 cells, so viral stocks were generated on CV1 cells engineered to express U_L6. To confirm the conclusion that the deletion within U_L6 was lethal for viral replication, CV1 cells, CV6 cells (CV1 cells expressing pU_L6), and CV6M cells (CV1 cells expressing U_L6 lacking codons 422 to 443) were infected with wild-type HSV-1(F) and vJB10. After 24 h, the cells were frozen and thawed three times, and the titer of infectious virus was determined on CV6 or Vero cells as indicated in Table 1. The results indicated that the U_L6 deletion virus was unable to produce substantial amounts of infectious virus upon infection of either CV1 or CV6M cells. In contrast, the virus was able to replicate to levels approaching those produced by HSV-1(F) in CV6 cells. Interestingly, CV6M cells were refractory to replication of HSV-1(F) such that titers were reduced approximately 380-fold compared to those of infectious virus obtained from CV1 or CV6 cells. These data suggest that the mutant pU_L6 expressed in CV6M cells served in a dominant negative fashion to inhibit wild-type virus replication.

To determine the role of pU_L6 amino acids 422 to 443 on the pU_L6/pU_L15 interaction in infected cells, CV1 cells were infected with HSV-1(F) or vJB10, and infected cell lysates were immunoprecipitated with pU_L6- or pU_L15-specific antisera. The presence of either pU_L6 or pU_L15 in immunoprecipitated material was then assessed by immunoblotting. As shown in Fig. 6, the absence of codons 422 to 443 did not preclude expression or solubility of pU_L6 but abrogated coimmunoprecipitation with pU_L15. This was despite the fact that wild-type pU_L6 and pU_L15 coexpressed in HSV-1(F)-infected cells readily coimmunoprecipitated. We therefore conclude that U_L6 codons 422 to 443 are required for interaction with pU_L15 in infected cell lysates.

To determine the effect of the deletion of codons 422 to 443 on viral DNA cleavage, cells were infected with HSV-1(F), vJB10, or vJB10R, a virus derived from vJB10 but containing a U_L6 gene restored to wild-type sequences. DNA was purified from the infected cells, digested with BamHI, electrophoretically separated on an agarose gel, transferred to a nylon membrane, denatured, and reacted with a radiolabeled probe bearing end-specific sequences. As noted previously (3), this probe hybridizes to the BamHI junction S-P fragment and the S-terminal BamHI P fragment in BamHI-digested HSV-1(F) DNA; this was also the case in digested vJB10R DNA. In contrast, only the S-P fragment was recognized in digested vJB10 DNA (Fig. 7). Probing the Southern blot with an L-terminal probe produced similar results (data not shown), indicating that neither DNA cleavage event occurs in cells infected with vJB10. As might be expected in the absence of DNA cleavage, we also noted that only capsids lacking DNA were detected in cells infected with the virus lacking U_L6 codons 422 to 443 (not shown). We conclude that U_L6 codons 422 to 443 are necessary for cleavage of concatameric viral DNA.

To determine whether the deletion of codons 422 to 443

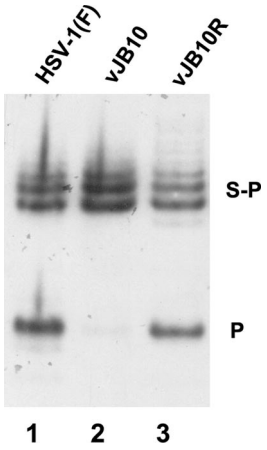

FIG. 7. The virus with mutant U_L6 lacking the putative leucine zipper is defective in viral DNA cleavage. Approximately 2×10^6 CV1 cells were infected with 5 PFU per cell of HSV-1(F), vJB10, or vJB10R, which was derived from vJB10 but contains a genetically restored U_L6 gene. Viral DNA was extracted about 15 hours after infection, digested with BamHI, transferred to a nylon membrane (0.45 μM), and hybridized with a radiolabeled BamHI P fragment of HSV-1(F) DNA. Bound probe DNA was detected by fluorography.

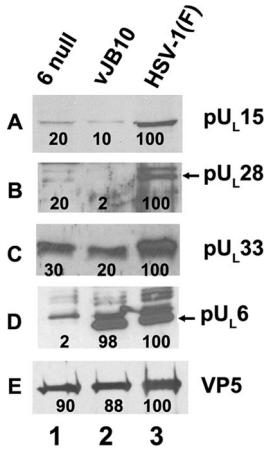

FIG. 9. Immunoblot of B capsids probed with pU_L15-, pU_L6-, pU_L33-, pU_L28, or VP5-specific antibodies. Approximately 4×10^8 CV1 cells were infected with viruses U_L6 null, vJB10, or HSV-1(F) at an MOI of 5 PFU per cell. Twenty hours after infection, capsids were purified, and B capsids were denatured, separated by SDS-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane, followed by immunoblotting with anti-pU_L15C, anti-pU_L6, or anti-VP5 antibodies. Bound immunoglobulin was revealed by enhanced chemiluminescence as described in the legend of Fig. 3. The intensity of each band was determined using an LAS-3000 mini Fujifilm imaging system. The intensity of the band on each panel is reported as a percentage of the signal in the lane containing HSV-1(F) proteins and is shown below each band.

affected interaction with scaffold proteins, cells were mock infected or infected with the U_L6 null virus, vJB10, or HSV-1(F), lysed, and subjected to immunoprecipitation with anti-ICP35 antibody. As shown in Fig. 8, ICP35 was efficiently immunoprecipitated with its cognate antibody from all three infected cell lysates. Immunoblotting of immunoprecipitated material with the pU_L6-specific antibody revealed that pU_L6 was coimmunoprecipitated with ICP35 from lysates of cells infected with HSV-1(F) and vJB10. We therefore conclude that the defect in DNA cleavage induced by the absence of U_L6 codons 422 to 443 was not attributable to a failure of pU_L6 to interact with scaffold protein.

To determine the effect of codons 422 to 443 on the association of portal protein and pU_L15 with capsids, B capsids were purified from cells infected with HSV-1(F), the U_L6 null virus, or vJB10. The capsids were then denatured, and associated proteins were electrophoretically separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and subjected to immunoblotting with pU_L6-, pU_L15-, or VP5-specific antibodies, the latter serving as a loading control. The relative levels of the chemiluminescent signals on the immunoblots were then quantified by densitometry, and the level of signal was normalized to the levels obtained in the lanes containing HSV-1(F) capsid proteins. The results are shown in Fig. 9. Given that other terminases have been shown to interact with both the major capsid protein and portal protein (19), it was not unexpected that some terminase proteins were detected in capsids lacking portals (i.e., those from cells infected with the U_L6 null mutant). The deletion in vJB10 did not reduce the incorporation of pU_L6 into the capsid discernibly, whereas the level of pU_L15 incorporated into vJB10 capsids was reduced by approximately 90%. This level was similar to the levels detected in pU_L6 null capsids. We therefore conclude that the deletion of codons 422 to 443 precludes the association of pU_L15 with the portal vertex.

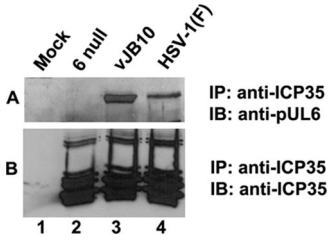

FIG. 8. Deletion of the putative leucine zipper of pU_L6 does not block interaction with the scaffold protein ICP35. CV1 cells were infected with 5 PFU per cell of the U_L6 null mutant, leucine zipper deletion virus (vJB10), or HSV-1(F). Eighteen hours after infection, the cells were lysed in RIPA buffer and reacted with ICP35-specific monoclonal antibody. Immunoprecipitated proteins were immunoblotted with anti-pU_L6 (A) or anti-ICP35 (B) antibodies. Bound immunoglobulins were revealed by reaction with appropriately conjugated immunoglobulins followed by enhanced chemiluminescence.

To determine whether capsid association of other putative terminase subunits requires U_L6 codons 422 to 443, immunoblots of capsids purified from cells infected with HSV-1(F), vJB10, or the U_L6 null virus were also probed with antibodies directed against pU_L28 and pU_L33. The results as shown in Fig. 9 indicated that levels of pU_L28 and pU_L33 were reduced to various extents in vJB10 mutant capsids compared to those in wild-type capsids.

DISCUSSION

The data presented herein indicate that codons 422 to 443 of U_L6 are critical for (i) interaction with pU_L15 and pU_L28, as assessed by immunoprecipitation, and (ii) the association of all three putative terminase subunits with portal-bearing capsids.

This is the first genetic evidence of a domain of pU_L6 critical to interaction with putative terminase subunits in infected cells and capsids.

The observations that both pU_L15 and pU_L28 can interact with pU_L6 independently suggest that docking of the terminase with the portal involves interactions between the two larger subunits of the terminase and the portal. This conclusion is consistent with the data of others showing that pU_L28 and pU_L15 can interact with pU_L6 independently (24). Parenthetically, we did not detect an interaction between pU_L6 and pU_L33 in the absence of other viral proteins as assessed by immunoprecipitation (data not shown).

In the absence of structural data, it is unclear that a leucine zipper is actually encoded by codons 422 to 443 of U_L6. Possibly the best arguments for such a motif are the conservation of aligned leucines in a number of herpesvirus portals and the observation that substitution of leucines 429 and 436 with glutamic acids precludes normal portal ring formation and incorporation of the portal into capsids (14). Nevertheless, replacement of the putative leucine zipper with a sequence known to form such motifs in other contexts was insufficient to restore pU_L15 or pU_L28 binding (Fig. 4 and 5).

Although the structure of pU_L6 is unknown, alignment of herpesvirus portal proteins with the solved structures of bacteriophage ϕ29 and SPP1 portals potentially reveals a structurally conserved region consisting of several α-helices (12). The most C-terminal of these helices (helix 6) is predicted at amino acids 406 to 454 of pU_L6 by using PSIPRED version 2.6 (6, 11). Helix 6 is a long, kinked α-helix that emanates roughly perpendicularly to the DNA channel, forming an external ridge or wing in the SPP1 portal (12). In the bacteriophage systems studied, this helix is proposed to interact with other portal subunits through a relatively sparse set of hydrogen bonds rather than through a leucine zipper.

The mutations described here and in previous studies are predicted to affect the stability, charge, register, and/or length of helix 6 as follows: (i) deletion of codons 409 to 473 should completely remove the helix, (ii) deletion of codons 422 to 443 should decrease its length by 7 amino acids and truncate and displace a hydrophobic patch by about 140 degrees relative to that of the wild type motif, (iii) replacement of codons 422 to 443 with the GCN4 leucine zipper should maintain the position of the leucines and hydrophobic patch and lengthen the helix by 9 amino acids, and (iv) changing leucines 429 and 436 to glutamic acids should disperse hydrophobic residues, precluding a prominent hydrophobic region.

Normal levels of portal proteins were incorporated into capsids purified from cells infected with vJB10 (Fig. 9) but were less efficiently incorporated into capsids when (i) codons 409 to 473 were deleted or (ii) leucines 429 and 436 were changed to glutamic acids (14). Given that vJB10 preserves a remnant (albeit displaced) of the hydrophobic region of helix 6, the data suggest that hydrophobic interactions are involved in intersubunit interactions within the HSV-1 portal. On the other hand, the failure of GCN4 to restore terminase binding of the deletion in helix 6 argues against the possibility that subunit interactions involve interdigitation of leucines as in a classic leucine zipper.

As might be expected given the structure of other portals, attempts to demonstrate an interaction between pU_L6 amino acids 422 to 443 with either pU_L6, pU_L28, or pU_L15 have not been successful (not shown). Taken together, the data support the idea that this region plays an indirect role in portal/terminase interactions, probably by ensuring proper portal morphology.

Our data also suggest that both amino and carboxyl termini of pU_L6 are necessary for interaction with pU_L15. Specifically, insertion of epitopic tags into the N or C terminus of pU_L6 did not preclude protein solubility, expression of the recombinant proteins, or interaction with ICP35 (not shown), but precluded coimmunoprecipitation with pU_L15 in transient expression assays. Moreover, deletion of either the N terminus or C terminus of pU_L6 precluded coimmunoprecipitation with pU_L15. Little is known about the orientation of pU_L6 in the capsid other than the fact that epitopes within the C-terminal 298 amino acids of pU_L6 are located at the external surface (15, 21, 25). Moreover, the termini are not homologous to bacteriophage portal proteins and so do not provide clues as to function. Thus, it is uncertain if the pU_L6 termini interact directly with pU_L15 or act to ensure proper conformation of pU_L6 and, thereby, promote interaction with pU_L15 indirectly.

The large number of U_L6 mutations that can preclude the interaction with pU_L15 is striking to us, and we speculate that terminase docking is exquisitely dependent on proper conformation of the portal in the capsid. This is supported by the observation that expression of the defective portal protein acts as a dominant negative inhibitor of wild-type virus (Table 1), presumably because optimal portal conformation cannot tolerate defective subunits. Because the terminase with bound DNA may have a choice of a number of potential capsids with which to dock, this structural constraint to at least the pU_L15/pU_L6 interaction may represent an important assembly checkpoint to ensure efficient viral DNA cleavage and packaging.

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