A Mutation in the Human Herpes Simplex Virus Type 1 UL52 Zinc Finger Motif Results in Defective Primase Activity but Can Recruit Viral Polymerase and Support Viral Replication Efficiently[▽]

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Herpes simplex virus type 1 (HSV-1) encodes a heterotrimeric helicase/primase complex consisting of UL5, UL8, and UL52. UL5 contains conserved helicase motifs, while UL52 contains conserved primase motifs, including a zinc finger motif. Although HSV-1 and HSV-2 UL52s contain a leucine residue at position 986, most other herpesvirus primase homologues contain a phenylalanine at this position. We constructed an HSV-1 UL52 L986F mutation and found that it can complement a UL52 null virus more efficiently than the wild type (WT). We thus predicted that the UL5/8/52 complex containing the L986F mutation might possess increased primase activity; however, it exhibited only 25% of the WT level of primase activity. Interestingly, the mutant complex displayed elevated levels of DNA binding and single-stranded DNA-dependent ATPase and helicase activities. This result confirms a complex interdependence between the helicase and primase subunits. We previously showed that primase-defective mutants failed to recruit the polymerase catalytic subunit UL30 to prereplicative sites, suggesting that an active primase, or primer synthesis, is required for polymerase recruitment. Although L986F exhibits decreased primase activity, it can support efficient replication and recruit UL30 efficiently to replication compartments, indicating that a partially active primase is capable of recruiting polymerase. Extraction with detergents prior to fixation can extract nucleosolic proteins but not proteins bound to chromatin or the nuclear matrix. We showed that UL30 was extracted from replication compartments while UL42 remained bound, suggesting that UL30 may be tethered to the replication fork by protein-protein interactions.

DNA replication is a universal process, many aspects of which are shared between prokaryotes, eukaryotes, and their viruses. In fact viruses have provided important models for the study of eukaryotic replication in part because they are amenable to biochemical, genetic, and other manipulations. Herpes simplex virus type 1 (HSV-1) contains a 152-kb double-stranded DNA genome and encodes seven proteins that are essential for its replication, including the origin binding protein (UL9), the single-stranded DNA (ssDNA) binding protein (UL29/ICP8), the heterotrimeric helicase/primase complex (UL5, UL8, and UL52), and the DNA polymerase (UL30 and its accessory factor UL42) (6, 11, 37, 40). HSV is a major human pathogen that can be life threatening even in immunocompetent adults. Most of the current anti-HSV treatments, such as the polymerase inhibitor acyclovir, are targeted to the viral polymerase (13). The emergence of drug resistance mutations from the polymerase inhibitors, however, requires that we continue seeking new targets. In fact, the helicase/primase has been exploited as an antiviral target, and two classes of highly potent helicase/primase inhibitors have been reported (14, 31).

The three-component HSV-1 helicase/primase complex exhibits several enzymatic activities, including 5'-to-3' helicase, primase, ssDNA-dependent nucleoside triphosphatase (NTPase),

and DNA binding activities (11, 40). A subcomplex of UL5 and 52 displays all four biochemical activities (17). The UL5 gene contains seven motifs that are conserved in members of helicase superfamily I (24, 25). Mutations in these motifs abolish helicase but not primase activity, indicating that UL5 is most likely the helicase subunit (26, 56). UL52, on the other hand, contains motifs conserved in other primases, including an internal catalytic DXD motif and a C-terminal zinc finger motif. Mutations in the DXD motif abolish the primase activity but not the helicase activity, suggesting that UL52 is likely the primase subunit (18, 32). Interestingly, however, several lines of evidence suggest a complex interdependence between the UL5 and UL52 subunits. Both proteins must be expressed together in order to obtain active protein, suggesting that protein-protein interactions between UL5 and UL52 are extensive (16, 17). It appears that cotranslation may be required for proper folding. In addition, mutations in the putative DNA binding domain of UL52 exhibit profound effects on the helicase and ATPase activities of the subcomplex (4). Although not essential for the enzymatic activities of the subcomplex, the UL8 subunit greatly stimulates its activities (19, 20, 28, 47, 50, 51). UL8 also interacts with other replication proteins such as UL9, ICP8, and UL30 (9, 19, 28, 41, 42, 44, 50), suggesting a possible regulatory role for UL8 at the replication fork.

The UL52 subunit contains a zinc finger motif of the Cys-His-Cys-Cys variety at its C terminus (Fig. 1), which is highly conserved in prokaryotic, eukaryotic, and viral primases (29, 45). Zinc finger motifs can be involved in DNA binding, protein-protein interactions, and protein-lipid interactions, as well as in maintaining zinc homeostasis and protein structural in-

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FIG. 1. Sequence alignment of the herpesvirus primase zinc finger region homologues. The sequence alignment was performed using PILEUP, a component of the GCG program (Wisconsin package version 9.1; Genetics Computer Group, Madison, WI). Consensus regions were generated with the PRETTY program and are in bold. The putative zinc binding residues (C-H-C-C) are in italic. The accession numbers for each virus, as obtained from the NCBI database, are as follows: HSV-1, NP044655; HSV-2, NP044523; human herpesvirus 4 (HHV4) or Epstein-Barr virus, P03193; HHV6, NP042936; HHV7, AAC40757; varicella-zoster virus (VZV), P09270; murine CMV (MCMV), Q69153; equine herpesvirus (HVS), P14346; human CMV (HCMV), P17149; bovine herpesvirus (EHV), NP 041016; and herpesvirus saimiri (BHV), NP 045306.

tegrity (1, 36, 43, 52). For example, residues in the zinc finger motif of the T7 primase have been shown to be involved in the specificity of primase recognition (30, 34, 35). Mutations in the conserved Cys and His residues of the HSV-1 UL52 zinc finger motif abolish the ability to complement a UL52 null virus in tissue culture and result in reduced biochemical activities of the subcomplex (4). Comparison of the UL52 sequence with those of other herpesvirus primase homologues has led to the identification of additional conserved residues flanking the Cys and His residues, and we have shown that mutations in several of these conserved residues exhibit variable effects on complementation and biochemical activities. Some mutants are unable to complement a null virus and are defective in helicase, primase, and DNA binding activities. Other mutants exhibit partial complementation ability and retain residual biochemical activities (4, 10, 12).

In this paper we report the analysis of a UL52 mutant, L986F, which complements the UL52 null virus more efficiently than the wild type (WT). This mutant is of interest because while most of the herpesvirus primase homologues contain a phenylalanine residue at position 986, HSV-1 and HSV-2 contain a leucine residue at this position. The L-to-F substitution thus results in the HSV-1 UL52 becoming more like all the other UL52 homologues, and it was expected that the L986F mutant would exhibit higher primase activity than WT HSV-1 UL52, as it can complement the UL52 null virus more efficiently than the WT. To our surprise, L986F showed a severe defect in primase activity, possessing less than 25% of the WT level of primase activity.

We previously reported that the recruitment of the HSV-1 polymerase to replication complexes in infected cells required the presence of an active primase (10). In this study, we show that although it is severely defective in primase activity, L986F is capable of recruiting the viral polymerase UL30 and forming replication compartments. These results prompted an analysis of the nature of the interaction of the HSV-1 polymerase

within the replication compartments. Treatment of infected cell nuclei with detergents has widely been used to determine how proteins are tethered within the nucleus (15). We now report for the first time that UL30 is extracted from replication compartments by such treatment, indicating that the HSV-1 polymerase catalytic subunit UL30 may be tethered to the replication fork via protein-protein interactions. These results will be discussed in the context of how replication proteins are recruited to replication forks.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney fibroblasts (Vero, from the American Type Culture Collection) were propagated and maintained as described previously (7). G418-resistant BL-1 cells, containing the UL52 gene under the control of the ICP6 promoter, and the *hr114* UL52 null virus have been described previously (23). KOS was used as the WT virus.

Reagents. Supplemented Grace's medium, 10% fetal bovine serum, penicillin-streptomycin solution, and Lipofectamine Plus reagent were purchased from Gibco/Invitrogen. Protease inhibitor cocktail and HIS-Select nickel affinity gel were purchased from Sigma. DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). [γ - 32 P]ATP and [α - 32 P]CTP were purchased from Perkin-Elmer Life Science (Boston, MA). NTPs were purchased from Amersham Biosciences (Piscataway, NJ).

Construction of point mutation L986F. The UL52 mutant L986F was constructed by a two-step PCR method as previously described (10). The PCR product was cloned into the pF1'CMV-UL52 amplicon vector using the KpnI and HindIII restriction sites, and the presence of the mutation was verified by restriction digest analysis and sequencing. Amplicon vectors are defined by the presence of an HSV origin of replication as well as packaging sequences such that they can be replicated and packaged if introduced into cells which are infected with a helper virus to provide the transacting replication and structural proteins.

Protein expression and purification. A recombinant baculovirus containing UL52 L986F was constructed using the Bac-to-Bac system (Invitrogen). The UL5/8/52 complex was expressed in insect cells coinfecting with recombinant baculoviruses capable of expressing WT UL5, His-tagged WT UL8, and either WT or mutant UL52. The UL5/8/52 complex was purified with a HIS-Select nickel affinity column as previously described (12). The purified UL5/8/52 protein complex was more than 95% pure as judged by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

Biochemical assays. Biochemical assays were performed as previously described (12), unless otherwise stated. All quantification was based on data from at least three independent experiments. ATPase assays were performed using Malachite Green. The amount of inorganic phosphate released per reaction was determined from a standard curve generated using KH_2PO_4 as a substrate. The helicase assay was performed using a ^{32}P -labeled forked DNA molecule as a substrate. Products were resolved by 10% nondenaturing PAGE, visualized, and analyzed using a Storm PhosphorImager (Amersham Biosciences) and ImageQuant software (version 2.1). To measure DNA binding activity, an electrophoretic mobility shift assay was performed. Products were resolved by 4% nondenaturing PAGE. The primase assay was performed using a 49-base DNA oligonucleotide containing a preferred primase recognition site as a substrate (5' GTTGGGTGCACGAGTGGGCCTTCCTGAACTGGATCTCAACAGC GTAAGA 3'). The primase assays were performed under two different conditions. The first set of reaction mixtures contained 40 mM *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS; pH 8.5), 3.5 mM MgCl_2 , 1 mM ATP, 1 mM GTP, 1 mM UTP, 2.5 μM radiolabeled CTP, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 10% glycerol, 40 nM DNA substrate, and 0.2 μM of the UL5/8/52 complex (WT and L986F mutant). The second set of reaction mixtures were identical except that the labeled CTP was diluted with cold CTP such that the total CTP concentration was 1 mM and included 2.5 μM radiolabeled CTP. Reactions were allowed to proceed for 30 min at 37°C and terminated using 2 $\times$ termination dye (10 mM EDTA [pH 8.0], 90% [vol/vol] formamide, and 0.1% each of xylene cyanol and bromophenol blue). Products were boiled for 5 min, resolved by 18% denaturing PAGE, visualized, and analyzed using a Storm PhosphorImager (Amersham Biosciences) and ImageQuant software (version 2.1).

Transfection of mammalian cells. Vero cells were transfected with 1 μg of plasmid DNA using the Lipofectamine PLUS reagent as directed by the manufacturer (Invitrogen).

Transient-complementation assay. Transient-complementation assays were performed as previously described (10). In brief, Vero cells were transfected with amplicon plasmids bearing the WT or mutant version of UL52, followed by superinfection with the UL52 null virus *hr114* at a multiplicity of infection of 3 PFU/cell. Cells and medium were harvested at 20 to 24 h postinfection (p.i.). Virus titers were determined on the UL52-complementing cell line BL-1, and the results were averaged from at least three independent repeats.

IF. Indirect immunofluorescence (IF) was performed as previously described (10). Vero cells were plated on glass coverslips, transfected, and superinfected as described above for the transient-complementation assay. For the in situ extraction assay, KOS-infected Vero cells (10 PFU/cell) at 6 h p.i. were treated for 2 min with either 0.5% Triton X-100 cytoskeletal extraction buffer (15) or 1 $\times$ phosphate-buffered saline (PBS) on ice. Following fixation and permeabilization in 1:1 acetone-methanol solution (-20°C) and blocking in 5% normal goat serum, infected Vero cells were labeled either with the primary mouse anti-UL30 1051b antibody (a gift from Charles Knopf, German Cancer Research Center) or mouse anti-UL42 antibody at 1:200 (Abcam). Coverslips were washed extensively with 1 $\times$ PBS, followed by incubation with Alexafluor 488 secondary antibodies (Molecular Probes) diluted 1:200 for 30 min. Coverslips were inverted onto microscope slides with glycerol gelatin mounting medium containing 2.5% 1,4-diazabicyclo-[2,2,2]octane (DABCO) to retard photobleaching. Imaging was performed on a Zeiss LSM410 inverted confocal microscope using a 63 $\times$ 1.4 objective lens. Images were arranged using Photoshop 7.0 and Adobe Illustrator software.

Detergent extraction of infected cells for Western analysis. Vero cells were plated to 80% confluence in 100-mm plates and mock infected or infected with 10 PFU/cell KOS the following day, when the plates were about 100% confluent. The plates were harvested at 6 h p.i., whereupon the Vero cell monolayer was washed in cold 1 $\times$ PBS three times and cells were gently scraped into conical tubes. Vero cells were pelleted by centrifugation and then exposed to either 0.5% Triton X-100 cytoskeletal extraction buffer (15) or PBS for 2 min on ice. Cells were washed, pelleted, resuspended in 2 $\times$ SDS loading buffer, and boiled for 5 min in preparation for Western analysis. Samples were subjected to SDS-PAGE analysis (10% bisacrylamide), transferred to polyvinylidene difluoride (Millipore) membranes, and blocked in 5% milk diluted in 1 $\times$ Tris-buffered saline-Tween 20. Membranes were incubated with mouse monoclonal anti-UL30 1051c primary antibodies (a gift from Charles Knopf) or mouse anti-UL42 antibodies at 1:1,000 (Abcam) for 1 h at room temperature. Following 45 min of extensive washing in 1 $\times$ Tris-buffered saline-Tween 20, goat anti-mouse-alkaline phosphatase secondary antibody (Promega) was diluted to 1:3,000 for detection by the alkaline phosphatase method as recommended by the manufacturer.

FIG. 2. Transient complementation of the UL52 null virus *hr114*. Complementation abilities of the WT and L986F were determined as described in Materials and Methods. The complementation index of WT UL52 was set at 100%, which represents an approximately 100-fold increase in viral yield with the WT over that seen with noncomplementing mutants. Mock represents cells transfected with empty vector without the UL52 gene insert.

RESULTS

HSV-1 UL52 contains a putative zinc finger motif at its C terminus that is highly conserved among herpesviruses primases. Figure 1 shows that this region contains the well-conserved Cys-His-Cys-Cys residues typical of this motif and several other highly conserved residues surrounding and between the conserved Cys and His residues ("consensus sequence" in Fig. 1). We have previously shown that several of these conserved residues play important roles in supporting viral replication and the biochemical activities of the helicase/primase complex (4, 12). Interestingly, at position 986, although HSV-1 and HSV-2 contain a leucine (Leu) residue, most other herpesviruses primase zinc finger homologues contain a phenylalanine (Fig. 1). The divergence between the two HSVs and the other herpesviruses at this position prompted us to ask whether replacement of Leu 986 with phenylalanine would alter its biological and biochemical properties.

HSV-1 UL52 L986F shows a higher ability to complement a UL52 null virus than the WT. The L986F mutant was constructed and expressed under the control of the cytomegalovirus (CMV) promoter in an amplicon vector as described in Materials and Methods. We determined its ability to complement a UL52 null virus in a transient-complementation assay and found that L986F can complement the UL52 null virus better than the UL52 WT, showing a 2.5-fold increase in complementation index compared to that of the WT (Fig. 2). We had previously shown that several other UL52 zinc finger mutants exhibited total or partial defects in complementation ability (10); therefore, we were surprised at the increase in the complementation ability exhibited by L986F. Western blot analysis indicated that the WT and the mutant were able to produce similar levels of UL52 protein (data not shown). This indicates that the difference we observed in the transient-complementation assay is not due to altered protein synthesis and/or protein stability.

Protein expression and purification. The increased ability of HSV-1 UL52 L986F to complement the UL52 null virus sug-

FIG. 3. Purified WT and mutant UL5/8/52 proteins. UL5/8/52 complexes were purified from insect cells coinfecting with recombinant baculoviruses carrying WT UL5 and His₆-UL8 and either WT or L986F UL52 as described in Materials and Methods. Proteins were resolved by 10% SDS-PAGE subsequently stained with Coomassie blue.

gested that one or more of the enzymatic activities of the helicase/primase complex UL5/8/52 might be altered. To test this hypothesis, we generated a recombinant baculovirus containing the UL52 L986F mutation. Both the WT and L986F UL5/8/52 complexes were expressed from insect cells coinfecting with recombinant baculoviruses capable of expressing WT UL5 and His-tagged UL8, as well as either WT or mutant versions of UL52. The UL5/8/52 complex was then purified by affinity chromatography on a HIS-Select nickel affinity column. The proteins were shown to be more than 95% pure by SDS-PAGE (Fig. 3), and the identity of each band was confirmed by Western blotting using antibodies generated against UL5, UL8, and UL52, respectively (data not shown).

L986F exhibits a severe defect in primase activity. We previously showed that mutations in the zinc finger motif resulted in severe defects in the primase activity of the UL5/8/52 complex which correlated with their inability to complement a UL52 null mutant virus (12). Since UL52 L986F can complement the UL52 null virus better than the WT, we hypothesized that L986F would exhibit increased primase activity compared to the WT. A primase assay was performed using a DNA oligonucleotide that contains a preferred primase initiation site (27). Radiolabeled [α -³²P]rNTP was used to follow the de novo synthesis of the RNA primer (Fig. 4A). The primase assay was performed under two different assay conditions as described in Materials and Methods. Figure 4B shows the results of an assay performed under conditions in which only hot CTP was present in the reaction mixture. The synthesized products were resolved on a sequencing gel (Fig. 4B). In the absence of the enzyme, no primer was produced. Both the WT and L986F were able to synthesize an RNA primer of 10 to 16 nucleotides in length in a 30-min reaction. To our surprise, however, L986F exhibited a severe defect in primase activity, which was less than 10% of the WT level (Fig. 4B). We repeated the assay under conditions in which the labeled CTP was diluted with cold CTP such that the total CTP concentration in the reaction mixture was identical to that of the other ribonucleotides. Under these conditions, L986F exhibited 25% of the WT level of primase (data not shown). In the first set of experiments, the CTP levels were suboptimal; therefore, the 25% seen at the higher CTP concentration probably reflects a more accurate representation of the activity of the mutant protein. The WT also showed higher primase activity when the

FIG. 4. L986F exhibits a severe defect in primase activity. RNA primer synthesis by either WT or L986F UL5/8/52 was measured in a 30-min reaction, using DNA template containing a preferred primase initiation site as described in Materials and Methods. (A) Schematic of the reaction. DNA template is in black, and the RNA primer synthesized is in gray. Asterisks indicate the incorporated radiolabeled [α -³²P]CTP. (B) Primase assay with only radiolabeled CTP. Synthesized RNA primers were resolved on a 20% sequencing gel. Lane M, 10-bp DNA ladder (Invitrogen). Lane Ctrl, control reaction in the absence of protein. Relative primase activity is shown below the lanes.

template concentration was dropped from 40 nM to 0.4 nM (data not shown). On one hand, this result was consistent with previous observations that mutations in the zinc finger motif could affect primase activity; however, the severe defect in primase activity was not expected considering the increased ability of L986F to complement the null mutant.

L986F displays a robust helicase activity. We next asked whether the UL52 L986F mutation affects the helicase activity of the UL5/8/52 complex. WT and mutant complexes were tested for their ability to unwind either a forked (Fig. 5) or three-way-junction (data not shown) substrate. L986F exhibited a robust helicase activity in a 30-min reaction, especially at lower enzyme concentrations (Fig. 5A and B). At 50, 100, and 200 nM enzyme concentrations, L986F showed a higher-than-WT level of helicase activity by a factor of 2, resulting in unwinding efficiencies of 22%, 36%, and 70%, respectively. At higher enzyme concentrations (400 nM and 800 nM), the differences were not as significant. Both WT and mutant complexes unwound the substrates very efficiently; however, even under these conditions, L986F still exhibited a higher level of helicase activity than the WT. Similar results were observed during a 60-min time course at an enzyme concentration of 50 nM (Fig. 5C and D). We and others have previously shown that the ssDNA binding protein ICP8 stimulates the helicase activity of UL5/8/52 (12, 49). Figure 5C and D show that ICP8 did stimulate the helicase activity of both the WT and L986F by twofold, although ICP8 itself did not show any unwinding activity (data not shown). In the presence of ICP8, L986F still exhibited higher helicase activity than the WT, by a factor of 2. Overall, results from the helicase analyses indicate that although

FIG. 5. L986F displayed a robust helicase activity. (A) The ability to unwind a forked substrate was examined in a 30-min reaction using WT or L986F complexes at different concentrations. Heat-denatured forked DNA and ssDNA were used as controls for the positions of substrate and products, respectively. Lane Ctrl, control reaction in the absence of protein. (B) Quantification of the unwinding results shown in panel A. (C) Time course of the helicase assay at an enzyme concentration of 50 nM, in the absence (–) or presence (+) of ICP8. Lane C, control reaction in the absence of protein. (D) Quantification of the unwinding results shown in panel C.

the L986F mutant displays a severe defect in primase activity, it possesses a robust, higher-than-WT level of helicase activity.

L986F exhibits elevated levels of ssDNA-dependent ATPase activity but similar levels of ATPase activity in the absence of ssDNA. Since ATP hydrolysis is required for DNA unwinding, we surmised that L986F might display more robust levels of ATPase activity as well. Indeed, at several different enzyme concentrations, L986F exhibited increased ssDNA-dependent ATPase activity compared to WT enzyme in a 30-min reaction (Fig. 6A). Similar results were observed during a 30-min time course. At 100 nM enzyme (Fig. 6B), L986F exhibited a twofold increase in ssDNA-dependent ATPase activity compared to that of the WT enzyme. At the 30-min time point, WT hydrolyzed 2.8 nmol of ATP, while L986F was able to hydrolyze 5.8 nmol of ATP. Like

other helicases, the HSV-1 helicase/primase shows a low intrinsic ATPase activity in the absence of ssDNA (12). At least 10-fold-larger amounts of the complex were required in order to achieve the levels of ATP hydrolysis seen in the presence of ssDNA (Fig. 6C). Interestingly, in the absence of ssDNA, the WT and L986F displayed similar levels of intrinsic ATPase activity (Fig. 6C). At the 30-min time point, in the absence of ssDNA, 1 μ M WT complex hydrolyzed 1.9 nmol of ATP, while L986F hydrolyzed 2.1 nmol of ATP. Overall, these data suggested that the enhanced level of ATPase activity of the mutant protein was seen only in the presence of ssDNA.

L986F displays elevated DNA binding ability. Based on the observations that the WT and L986F possessed similar levels of intrinsic ATPase activity and that L986F displayed an enhanced level of ssDNA-dependent ATPase activity, we hypothesized that L986F would exhibit an increased ability to bind ssDNA. Electrophoretic mobility shift analysis showed that L986F in fact did display an increased ability to bind ssDNA (Fig. 7), especially at lower enzyme concentrations. For example, the WT showed efficiencies of binding to ssDNA of 3% and 9% at 50 nM and 100 nM, respectively. On the other hand, L986F displayed increased efficiencies of binding of 6% and 21% to ssDNA at 50 nM and 100 nM, respectively. This experiment indicates that the increased ssDNA-dependent ATPase activity of L986F may be a result of its increased ability to bind ssDNA.

Despite a defect in primase activity, L986F is capable of recruiting viral polymerase and forming replication compartments efficiently. We have previously shown that the presence of five replication proteins (ICP8, UL9, and the three members of the helicase/primase complex, UL5, UL8, and UL52) is required to recruit the viral DNA polymerase UL30 to prereplicative sites and replication compartments (7, 8, 10, 39, 54); furthermore, UL30 recruitment requires the presence of a functional UL52 protein, implying that an active primase may be required to recruit polymerase (10). We have proposed that polymerase recruitment may require the presence of an RNA primer; alternatively, polymerase may be recruited to a complex containing an active primase by protein-protein interactions. Since L986F exhibited a severe defect in primase activity, we asked whether UL30 can be recruited to viral foci. Vero cells were transfected with the L986F mutant and superinfected with the UL52 null virus, and IF and confocal microscopy of cells stained with antisera raised against ICP8 or UL30 were performed. In cells transfected with an empty plasmid, no staining was observed (Fig. 8, top panels). In cells transfected with plasmids bearing WT UL52 (middle panels) or L986F (bottom panels), ICP8 (green, left) and UL30 (red, center) colocalized in replication compartments (see merged image on the far right). The observation that replication compartments could form in cells transfected with either the WT or L986F suggests that even though L986F exhibits 25% of the WT levels of primase activity, UL30 can still be recruited to viral foci. The observation that UL30 can be recruited to replication foci by the L986F mutant complex may suggest that UL30 may be recruited by the synthesized primer and that the low level of primase activity exhibited by this mutant helicase/primase complex is sufficient to recruit UL30. Alternatively, UL30 may be recruited by protein-protein interactions.

FIG. 6. L986F exhibits elevated levels of ssDNA-dependent ATPase activity compared to the WT. (A) ssDNA-dependent ATPase activity of the WT and L986 complexes in a 30-min reaction was measured as described in Materials and Methods. (B) Time course of ATP hydrolysis in the presence of ssDNA at an enzyme concentration of 100 nM. (C) Time course of ATP hydrolysis in the absence of ssDNA at an enzyme concentration of 1 μM. The amount of inorganic phosphate was determined from a standard curve.

The viral polymerase UL30 is detergent extracted from HSV-1-infected cells. In order to address whether UL30 is tethered to viral foci by protein-protein interactions or protein-nucleic acid interactions (i.e., to the RNA primers themselves), we took advantage of the observation that detergent treatment has been shown to extract nucleosolic proteins but not proteins bound to chromatin or to the nuclear matrix (15). If HSV-1 polymerase is recruited and tethered to the replication fork by binding to nucleic acid alone, it would be expected to remain at the fork despite detergent extraction. On the other hand, it would likely be extracted from the infected cells if it is recruited by protein-protein interactions. Vero cells were infected with KOS and harvested at 6 h p.i. Cells were exposed to either ice-cold 0.5% Triton X-100 cytoskeletal extraction buffer (15) or 1× PBS and subjected to SDS-PAGE and Western analysis as described in Materials and Methods. Figure 9

shows that most of the UL30 (top panel) was extracted from infected cells by detergent treatment but not by PBS treatment. On the other hand, the viral polymerase accessory protein UL42 (middle panel) and ssDNA binding protein ICP8 (data not shown) remained detectable in both detergent-treated and PBS-treated cells. This result was also confirmed by IF microscopy (Fig. 10). Infected Vero cells (6 h p.i. at a multiplicity of infection of 10) were labeled with either monoclonal mouse anti-UL30 antibody 1051b or monoclonal mouse anti-UL42.

FIG. 7. L986F displays elevated DNA binding activity. ssDNA binding affinity was examined by gel mobility shift assay at room temperature in a 30-min reaction and quantified.

FIG. 8. L986F is capable of recruiting viral polymerase and forming replication compartments efficiently. Vero cells were transfected with plasmids bearing empty plasmid (top panels), the WT (middle panels), or L986F (bottom panels) and then superinfected with the UL52 null virus *hr114* for 6 h. Cells were stained with the ICP8 antibody (left panels, green) or the UL30 antibody (middle panels, red). Merged images (orange) are shown on the right.

FIG. 9. The viral polymerase UL30 was detergent-extracted from HSV-1-infected cells. Vero cells were either mock infected or infected with HSV WT strain KOS for 6 h. Cells were treated with either PBS or 0.5% Triton X-100 extraction buffer, followed by Western blotting using antisera raised against UL30 (top), UL42 (middle), or γ -tubulin (bottom) as described in Materials and Methods.

Each image in Fig. 10 shows a representative KOS-infected cell, with images taken at the same settings for each primary antibody. We found that UL30 was extracted from the replication compartments of infected cells (Fig. 10C), while UL42 remained bound to matrix in extracted cells (Fig. 10D). If cells were cross-linked with 4% paraformaldehyde prior to detergent extraction, no differences were detected between cells that were cross-linked and cells that were not treated with detergent (data not shown). Thus, these experiments indicate that UL30 can be extracted by detergent treatment and that extraction can be blocked by prior treatment with a cross-linking agent. These results suggest that UL30 may be tethered to the fork via protein-protein interactions, while UL42 and ICP8 may be tethered by binding to nucleic acids.

DISCUSSION

The UL52 subunit of the HSV-1 helicase/primase contains a highly conserved zinc finger motif of the CHCC type that is reminiscent of zinc finger regions in other primases such as the T7 bacteriophage primase (29, 45). We have shown previously that the introduction of single amino acid substitutions in this motif not only affects the ability of the mutant UL52 to complement the growth of a UL52 null virus in vivo (10) but also can affect all of the biochemical activities in vitro (4, 12). In this paper we have focused on residue 986 within this domain because in most herpesvirus primase homologues, the equivalent residue is a phenylalanine; however, HSV-1 and HSV-2 both contain a leucine residue at this position. In this study we have mutated residue 986 in the HSV-1 UL52 gene to a phenylalanine so that it more closely resembles the primase homologues of the other herpesviruses. In vivo complementation assays indicate that the mutant L986F can complement the growth of the UL52 null virus better than the WT. We speculated that the increased complementation ability of L986F may result from increased primase activity. To our surprise, biochemical studies indicated that L986F exhibited a severe defect in primase activity, 25% of that of the WT. On the other hand, L986F demonstrated elevated levels of helicase, ssDNA-dependent ATPase, and DNA binding abilities. These results challenge some of our assumptions about the role of the helicase/primase complex in HSV DNA replication.

Interdependence between the subunits of the helicase/primase. As mentioned in the introduction, the UL5 and UL52 subunits of the HSV-1 helicase/primase complex exhibit a com-

FIG. 10. UL30, but not UL42, is extracted by detergent from replication compartments. Vero cells were plated on glass coverslips and infected the following day with 10 PFU/cell KOS. At 6 h p.i., cells were incubated for 2 min in either cold PBS (A and B) or cold 0.5% Triton X-100 cytoskeletal extraction buffer (C and D), followed by fixation and permeabilization for IF analysis as described in Materials and Methods. Cells were probed with monoclonal mouse anti-UL30 antibody 1051b (A and C) or monoclonal mouse anti-UL42 (B and D). Each image shows a representative KOS-infected cell, with images taken at the same settings for each primary antibody.

plex pattern of interdependence. Mutations in conserved residues in the UL52 subunit have been shown to drastically affect primase activity (4, 12, 18, 32), and mutations in the helicase motifs of UL5 were seen to abolish helicase activity (26, 56). Interestingly, mutations in UL5 have been shown to stimulate primase activity (26). In addition, some mutations in the conserved residues of the UL52 zinc finger motif cause significant decreases in all the biochemical activities of the helicase/primase complex, including helicase, ATPase, and DNA binding activities (4, 12). These results indicate that the UL52 subunit may play a wider role in the helicase/primase complex than that of primase alone. In this study, we have shown that the L986F mutant exhibited a marked defect in primase activity but, interestingly, exhibited higher levels of all other enzymatic activities of the helicase/primase complex. These results confirm and extend previous studies suggesting a complex interdependence. Various scenarios can be envisioned to explain how these two subunits can have such drastic effects on each other's activities. Interestingly, in other systems, such as the T7 helicase/primase, both activities reside on a single polypeptide chain. If the HSV helicase/primase evolved from a common ancestor with T7, it is possible that at some point in evolution, these functions were separated onto two peptides. Thus, although the two functions reside on separate peptides, residues from one may still have functions in the activities of the other. Since both helicase and primase activities involve interactions with DNA, it is possible, for instance, that the helicase utilizes a DNA binding domain found on the UL52 subunit or even that a DNA binding domain itself spans the two peptides. The observation that UL5 and UL52 must be cotranslated to be active (16, 17) supports the idea of a complex evolution.

DNA binding ability of HSV-1 helicase/primase. As would be expected for an enzyme complex with helicase and primase activities, the HSV-1 helicase/primase is capable of binding DNA. Attempts to identify additional DNA binding regions on UL52 or on the putative helicase subunit UL5 have been complicated by the fact that neither protein can be expressed alone in an enzymatically active state. We have previously shown that this complex can bind ssDNA substrates and forked substrates which contain both ssDNA and double-stranded DNA (4, 5, 12). At least one DNA binding site likely resides within the zinc finger motif of UL52. Zinc finger motifs are known to coordinate zinc atom binding and are often found to participate in DNA binding. More specifically, the zinc finger motif of the T7 primase has been shown to be important for DNA binding specificity (30, 34, 35). We have shown that mutations in the HSV-1 UL52 zinc finger motif affect the DNA binding ability of the helicase/primase (4, 12), indicating a direct role of the zinc finger motif in DNA binding. Furthermore, in this study, we have observed that a mutation in residue 986 appears to increase the ssDNA-stimulated ATPase and helicase activities of the complex. These results suggest that the DNA binding ability conferred by the UL52 subunit of the helicase/primase may contribute directly to its helicase and ssDNA-dependent ATPase activities. It is possible that the DNA binding of UL52 through its zinc finger motif is required to load UL5 onto DNA. Alternatively, it is possible that helicase/primase binds to DNA through an interface that is created by the interaction between UL5 and UL52. The mapping of the helicase/primase inhibitor drug resistant mutations to both UL5 and UL52 subunits (3, 31, 38) may support the latter possibility. One way to distinguish between these possibilities would be to ask whether the zinc finger motif by itself is capable of sequence-specific DNA binding; these experiments are in progress.

By analogy with other superfamily I helicases (33, 48, 53), it is expected that one or more DNA binding sites will be identified within the UL5 protein itself, and we are currently using genetic and biochemical approaches to identify and map important residues. Given the unique configuration of this helicase/primase and its potential role as a target for antiviral therapy, it will be important to better understand how the enzyme is loaded onto its DNA substrates and how the presumed multiple DNA binding regions participate in the various enzymatic activities of this complex.

Polymerase recruitment. Another important unanswered question in HSV DNA replication relates to how the viral DNA polymerase is recruited to the replication fork. We have previously shown that an active primase, but not helicase, is required to recruit the HSV-1 polymerase to prereplicative sites (10). That observation was consistent with studies of both bacterial and mammalian replication primases. In *Escherichia coli*, an active primase (DnaG) is required to load polymerase III holoenzyme onto the replication fork (2, 21). In mammalian cells, the recruitment of polymerases δ and ϵ to the replication fork requires polymerase α /primase to synthesize RNA/DNA primers. In this case, the length of the primer is important to load polymerase, suggesting that polymerase is recruited by the synthesized primer itself (46). We have been interested in whether the HSV polymerase is recruited by the newly synthesized primer itself or by protein-protein interac-

tions between the polymerase and the active primase. In this study, we show that although L986F displays a severe defect in primase activity, 25% of the primase activity of the WT, it can recruit viral polymerase UL30 and form replication compartments efficiently. Furthermore, we observed in this study that UL30 is extracted from the replication compartments of infected cells by detergent treatment, while UL42 remains bound to matrix in extracted cells. These results suggest that the UL30 polymerase subunit may be tethered to the replication fork via protein-protein interactions, while the accessory subunit UL42 may be tethered by nucleic acids. As UL8 has been shown to interact with UL30 (42), this interaction may play an important role in polymerase recruitment, although other proteins may also be involved in recruitment and retention of UL30 at the replication fork.

In summary, our results suggest that polymerase may be recruited and tethered to the replication fork via protein-protein interactions. In this case, the partially active primase of L986F may maintain an intact conformation sufficient for polymerase loading. We cannot rule out, however, that polymerase maybe recruited by the newly synthesized RNA primer itself and then stabilized by protein-protein interactions. In this case, a small amount of primer may be sufficient for initial polymerase loading. Additional studies are in progress in order to fully address these questions.

Roles of helicase and primase in HSV DNA replication. The observation that L986F is capable of supporting normal or even higher levels of viral replication with 25% of WT primase activity was initially quite surprising. It is clear that both helicase and primase activities are essential for HSV DNA replication. UL5 mutants that are defective in helicase activity while retaining WT levels of primase activity fail to complement a UL5 null virus (56). Similarly, a UL52 DID motif mutant that is defective in primase activity but not helicase activity was unable to complement a UL52 null virus (32). One possible explanation for the fact that a primase-deficient mutant has such robust growth ability is that the primase activity may be required only for the initiation stage of replication, while at later stages, alternative priming pathways are utilized. Multiple lines of evidence suggest that other mechanisms could provide primers for polymerase. For example, recombination plays an important role at later stage of HSV replication (55); it is possible that recombination provides DNA primers for replication.

The observation that most herpesvirus primase homologues contain a phenylalanine at position 986 while HSV-1 and HSV-2 have evolved to contain a leucine is intriguing, especially in light of our finding that L986F was able to complement a null mutant more efficiently than WT UL52. The mutation caused a decrease in primase activity, and somewhat surprisingly, the increased complementation correlated with a more robust helicase activity. This may indicate that helicase plays a more vital role in DNA replication than primase itself. During the course of replication, helicase is needed continuously to unwind the duplex DNA (56). The role of primase in infection is not entirely clear. It is possible, as mentioned above, that primase activity is needed only transiently during initiation and that at later times alternative priming pathways, such as recombination, may provide primers for DNA polymerase (22). Additional experiments will be needed to unambiguously clar-

ify the role of primase during HSV-1 DNA replication. For instance, it will be of interest to look at the length of Okazaki fragments in infected cells and to ask whether RNA primers can be detected at early and late times of infection.

The fact that UL52 homologues from HSV-1 and HSV-2 have evolved a leucine at position 986 is also intriguing in light of the strong conservation of phenylalanine in most other herpesvirus primase homologues. Given the higher efficiency of complementation of the L986F mutation, one might ask why a phenylalanine substitution has not been observed in nature for HSV-1 and HSV-2. It is possible that other factors would provide a counterselection, thus maintaining a leucine at this position. An answer to this question will require a better understanding of the precise role or roles of helicase and primase during infection, in both cell culture and animal models.

Overall, we have shown that a point mutation in the UL52 zinc finger motif affects all the biochemical activities of the helicase/primase complex. These results confirm that the zinc finger region plays an important role in all the activities of this complex and support our previous suggestion of a strong interdependence between the subunits of the helicase/primase complex. In addition, we have shown that a primase-defective mutant is capable of recruiting the viral polymerase and forming replication compartments. The viral polymerase UL30 may be recruited and tethered to the replication fork by protein-protein interactions. Alternatively, it may be recruited by newly synthesized primers and then stabilized by protein-protein interactions. If the latter is true, our results suggest that a small amount of RNA primer is sufficient for polymerase recruitment under these conditions.

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