

The Cytomegalovirus DNA Polymerase Subunit UL44 Forms a C Clamp-Shaped Dimer

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Summary

The human cytomegalovirus DNA polymerase consists of a catalytic subunit, UL54, and a presumed processivity factor, UL44. We have solved the crystal structure of residues 1–290 of UL44 to 1.85 Å resolution by multiwavelength anomalous dispersion. The structure reveals a dimer of UL44 in the shape of a C clamp. Each monomer of UL44 shares its overall fold with other processivity factors, including herpes simplex virus UL42, which is a monomer that binds DNA directly, and the sliding clamp, PCNA, which is a trimer that surrounds DNA, although these proteins share no obvious sequence homology. Analytical ultracentrifugation and gel filtration measurements demonstrated that UL44 also forms a dimer in solution, and substitution of large hydrophobic residues along the homodimer interface with alanine disrupted dimerization and decreased DNA binding. UL44 represents a hybrid processivity factor as it binds DNA directly like UL42, but forms a C clamp that may surround DNA like PCNA.

Introduction

Replicative DNA polymerases are multiprotein machines that synthesize long stretches of DNA. The polymerase activity is contained within the catalytic subunit, but to prevent dissociation from the template, accessory proteins known as processivity factors are required. The best-described family of processivity factors remains the “sliding clamps,” which include the β subunit of *E. coli* DNA polymerase III (Pol III) (Kong et al., 1992) and proliferating cell nuclear antigen (PCNA) of eukaryotic DNA polymerases δ and ϵ (Gulbis et al., 1996; Krishna et al., 1994). These proteins assemble as homomultimers in the form of a toroidal ring-shaped structure with a central channel of sufficient diameter to accommodate double-stranded DNA. Because sliding clamps have no significant intrinsic affinity for DNA, their assembly onto DNA requires protein complexes known as clamp loaders and is dependent upon ATP hydrolysis

(reviewed in Jeruzalmi et al., 2002). This assembly results in a loose association on DNA, which permits the clamps to slide freely. Upon displacement of the clamp loader complex, sliding clamps topologically tether the polymerases to DNA and therefore confer processivity (Maga et al., 2000; Naktinis et al., 1996; Stukenberg et al., 1991).

Several other processivity factors have been described that do not assemble into toroidal clamps. The crystal structure of one of these, herpes simplex virus (HSV) UL42, displays a surprisingly similar fold to one monomer of PCNA even though these proteins share no obvious sequence homology and utilize remarkably different biochemistry (Zuccola et al., 2000). UL42 binds directly to DNA with relatively high affinity as a monomer in the nanomolar range in a manner that does not require clamp loaders or ATP (Gallo et al., 1989; Gottlieb and Challberg, 1994; Randell and Coen, 2004; Thornton et al., 2000; Weissbart et al., 1999). The DNA binding activity of UL42 is required for processivity inasmuch as mutations that specifically reduce the affinity of UL42 for DNA also reduce the ability of UL42 to stimulate long-chain DNA synthesis by its cognate catalytic subunit, UL30 (Chow and Coen, 1995; J.C.W. Randell and D.M.C., unpublished data). The high-affinity DNA binding activity of UL42 explains the increased affinity of the UL42-UL30 heterodimer for primer-template compared to UL30 alone (Gottlieb and Challberg, 1994; Weissbart et al., 1999). Despite the high affinity for DNA, UL42 can diffuse linearly along DNA in the absence of UL30 (Randell and Coen, 2001), leading to the proposal that electrostatic interactions between basic residues on the “back” face of the UL42 structure and the phosphate backbone of DNA (Zuccola et al., 2000) provide an electrostatic tether that prevents dissociation, but allows UL42 to “slide” freely along the helical backbone.

The β -herpesvirus, human cytomegalovirus (HCMV), also encodes a DNA polymerase consisting of a large catalytic subunit, UL54 (Heilbronn et al., 1987; Kouzarides et al., 1987), and a proposed processivity factor, UL44 or ICP36 (Ertl and Powell, 1992). The mechanism that UL44 uses to stimulate UL54 remains poorly understood. Although template challenge assays to demonstrate processivity in a rigorous manner have not yet been reported, UL44 stimulates long-chain DNA synthesis by UL54 (Ertl and Powell, 1992; Weiland et al., 1994) and is widely assumed to be a bona fide processivity factor. The N-terminal two-thirds of UL44 have been predicted to adopt a fold like that of UL42 and PCNA (Zuccola et al., 2000) and, subsequently, the β subunit of Pol III (Novotny et al., 2001), even though UL44 shares no apparent sequence homology with these proteins. Finally, UL44 may promote processivity by a mechanism similar to that of UL42 since it binds to DNA in an apparently sequence-independent manner (Gibson et al., 1981; Weiland et al., 1994) and does not require clamp loaders or ATP hydrolysis.

Here, we report the crystal structure of the N-terminal 290 residues of UL44 (UL44 Δ C290). Although UL44

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shares a fold with UL42 and PCNA, in the crystal structure it forms a C clamp-shaped, head-to-head homodimer, rather than a monomer like UL42, or a head-to-tail homotrimer like PCNA. The observation that UL44 forms a dimer in the structure was confirmed by analytical ultracentrifugation and gel filtration. The mutation of hydrophobic residues along the homodimer interface was sufficient to decrease both dimerization and affinity for DNA. Like UL42, UL44 shares a basic, α -helical face, which may facilitate sliding along the DNA backbone via electrostatic interactions. In summary, UL44 may function as a hybrid processivity factor that can partially surround DNA (like PCNA) and form favorable electrostatic interactions (like UL42).

Results

Crystallization of UL44

Initial attempts to crystallize full-length UL44 yielded crystals that diffracted weakly to approximately 10 Å resolution (H. Zuccola, D.M.C., and J.M.H., unpublished data). UL44 contains several glycine-rich strings in its C-terminal 143 residues, which begin at residue 291 (Ertl and Powell, 1992). The majority of the C terminus, including these glycine-rich strings, is predicted to be unstructured using PSIPRED (McGuffin et al., 2000; B.A.A., D.M.C., and J.M.H., unpublished data), which could impede the formation of well-ordered crystals. Furthermore, the C-terminal one-third of UL44 (residues 310–433) is dispensable for it to bind DNA and stimulate long-chain DNA synthesis by UL54 (Weiland et al., 1994) while the N-terminal two-thirds of UL44 are predicted to fold in a manner similar to UL42 and PCNA (Zuccola et al., 2000). Therefore, we adopted a strategy to define a truncation of UL44 that lacked the majority of the unstructured C terminus while retaining the ability to bind DNA and UL54. A series of mutants with truncations from the C terminus were cloned as glutathione S-transferase (GST) fusion proteins, including UL44 Δ C290 (residues 1–290), which lacks all five glycine-rich strings. This construct retains the ability to bind DNA, bind UL54, and stimulate long-chain DNA synthesis by UL54 at levels that are indistinguishable from full-length UL44 (Loregian et al., 2004b).

Wild-type UL44 Δ C290 was crystallized in space group P6₃22 with one molecule per asymmetric unit. The structure was solved by multiwavelength anomalous dispersion (MAD) using data collected from two selenomethionyl crystals to 2.9 Å resolution, and refined using data from one native crystal to 1.85 Å resolution (Table 1). Using MAD data sets from both crystals, SOLVE (Terwilliger and Berendzen, 1999) located four of five possible selenium sites and calculated a figure of merit of 0.66. Furthermore, this strategy produced experimental electron density maps of higher quality than maps produced from either single crystal alone. A model for the structure was initially built into the experimental maps and refined against the experimental phases to 2.9 Å, before extending the resolution to 1.85 Å with refinement against the native data. The final native model is reported with an R_{cryst} and R_{free} of 23.5% and 26.8%, respectively (Table 1).

UL44 Shares the Fold of UL42 and PCNA

The structure of UL44 can be divided into two subdomains with similar topology comprised of residues 10–128 and 143–270. The two subdomains are related by an approximate 2-fold axis (Figure 1A) and can be superimposed with an rmsd of 2.8 Å between structurally equivalent α carbons. As predicted (Zuccola et al., 2000), UL44 has a remarkably similar fold to UL42 and PCNA even though these three proteins share no obvious sequence homology (Figure 1B). Thus, all secondary structural elements of UL44 are designated according to the nomenclature that was originally adopted for PCNA and used for UL42 (Krishna et al., 1994; Zuccola et al., 2000). The β strands βD_1 and βI_2 from the N- and C-terminal domains, respectively, are hydrogen bonded to create a central, nine-stranded β sheet (Figure 1A). Two additional β sheets are found at the top and bottom of UL44. On one face of the central sheet, the two subdomains are connected by a long loop (“connector loop,” residues 129–142) that traverses the molecule from the bottom right to the top left (Figure 1A). The other (“back”) face of the central sheet is flanked by four α helices (Figure 1B). The “back” face, including the flanking helices, is rich in basic residues, especially lysine, and may be involved in DNA binding (see Discussion). Recent data have demonstrated that the replacement of any residue from 133 to 136 of the connector loop with an alanine reduces the physical and functional interaction with the catalytic subunit, UL54, which binds UL44 via its extreme C terminus (Loregian et al., 2004a, 2004b). The analogous regions of the HSV DNA polymerase subunits are also crucial for their interaction (Bridges et al., 2001; Digard et al., 1993; Zuccola et al., 2000) even though the segments have no discernible sequence homology with their HCMV counterparts. Finally, three regions of UL44 Δ C290 are not visible in the map, namely the first 9 residues of the N terminus, the last 20 residues of the C terminus, and residues 163–174 (Figure 1B).

UL44 Forms a Dimeric C Clamp

In strong contrast to UL42, which is a monomer in solution and in the crystal structure (Crute and Lehman, 1989; Gottlieb et al., 1990; Randell and Coen, 2004; Zuccola et al., 2000), UL44 forms a head-to-head C clamp-shaped homodimer in the crystals. This also contrasts with PCNA (Gulbis et al., 1996; Krishna et al., 1994), which forms a toroidal, head-to-tail homotrimer (Figure 2). UL44 dimerizes across one of the crystallographic 2-fold axes with the βI_1 strands of the monomers forming an antiparallel β interaction that extends the bottom β sheets of the component monomers across the dimer interface. This interaction involves six main-chain-to-main-chain hydrogen bonds and extensive packing of hydrophobic side chains at the interface (Figure 3). F121 of each monomer is buried against a hydrophobic loop composed of P85, L86, and L87 of the other monomer. L86 and L87 also pack against M123 and L93 of the opposite monomer. These interactions bury a surface area of approximately 1100 Å². The Sc statistic (as defined by Lawrence and Colman, 1993) is 0.79, which indicates a very high degree of shape complementarity between the monomers. Both the extent and the nature of the interactions suggest that the dimerization is likely

Table 1. Data Collection and Refinement Statistics

Data Collection								
Space Group	P6 ₁ 22							
	Native	MAD Data Set 1			MAD Data Set 2			
Unit Cell (Å)	a = b = 53.93 c = 340.1	a = b = 53.95 c = 340.9			a = b = 53.90 c = 340.2			
		Peak	Inflection	High Energy	Peak	Inflection	High Energy	Low Energy
Wavelength (Å)	0.9000	0.9786	0.9788	0.9500	0.9786	0.9787	0.9500	1.010
Resolution (Å)	30–1.85	30–3.0			30–2.9			
Unique reflections	26,592	6,690	6,651	6,695	7,345	7,360	7,349	7,336
Total measurements	766,922	71,294	67,389	71,806	128,482	126,350	130,950	119,720
Completeness (%) ^a	99.8 (100)	99.7 (98.0)	99.4 (97.8)	99.7 (99.8)	99.7 (99.4)	99.7 (99.9)	99.7 (99.9)	99.5 (97.6)
R _{sym} (%) ^{a,b}	5.6 (26.7)	9.3 (13.6)	7.6 (11.3)	7.6 (11.3)	7.0 (13.8)	6.7 (14.5)	6.8 (14.4)	6.2 (15.6)
<I>/<σ(I)> ^a	58.4 (14.2)	25.0 (7.5)	31.5 (10.5)	32.8 (12.3)	32.9 (13.3)	33.3 (11.3)	34.5 (13.6)	37.0 (9.9)
Refinement								
Resolution (Å)	12–1.85							
R _{cryst} ^c	0.235							
R _{free} ^c	0.268							
Nonhydrogen atoms								
Protein	1,941							
Solvent	95							
Average B factor (Å ²)	29.9							
Rmsd bonds (Å)	0.015							
Rmsd angles (°)	1.58							
Phi-psi plot ^d								
Most favored (%)	90.7							
Disallowed (%)	0							

^a Values in parentheses refer to data in the highest resolution shell.

^b $R_{\text{sym}} = \sum_{(hkl)} |I(hkl)| / \sum_{(hkl)} \langle I(hkl) \rangle$, where $\langle I(hkl) \rangle$ is the mean intensity of symmetry related observations of a unique reflection.

^c $R_{\text{cryst}} = R_{\text{free}} = \sum_{(hkl)} |F_o| - F_c / \sum_{(hkl)} |F_o|$, where R_{free} represents 10% of the data selected randomly.

^d PROCHECK (Laskowski et al., 1993).

to be biologically relevant and not an artifact of crystallographic packing. Interestingly, PCNA also utilizes strand β_1 to form an intersubunit β sheet. In contrast to UL44, where the interaction results in a symmetric head-to-head dimer, in PCNA the interaction is with strand βD_2 of the next subunit, resulting in a head-to-tail trimer (Gulbis et al., 1996; Krishna et al., 1994).

UL44 Δ C290 Forms a Dimer in Solution

Although molecular interactions in crystal structures often provide important clues regarding the quaternary state of a protein, it is important to test this property in solution to exclude the possibility of artificial interactions arising from the high protein concentrations used in crystallization experiments (typically 100 μ M or greater). Thus, we investigated the quaternary state of UL44 Δ C290 in solution through a combination of analytical ultracentrifugation and gel filtration studies. We employed sedimentation equilibrium analysis to determine the quaternary state of wild-type UL44 Δ C290 in solution and determine the dissociation constant (K_d) for any observed interactions. UL44 Δ C290 was analyzed at three concentrations (typically, 1.3, 2.1, and 3.8 μ M) and each concentration was sedimented to equilibrium at three rotor speeds (13,500, 18,000, and 24,000 rpm) to produce nine independent data scans. These nine data scans were fit globally by nonlinear least squares minimization using UltraScan. The data fit best to a monomer-dimer model as determined by the low variance

and randomness of the residuals (Figure 4A). We also attempted to fit the data with other models, including an ideal single species and a monomer-dimer-trimer model, but these models did not fit the data in anything approaching a statistically acceptable fashion (B.A.A., D.M.C., and J.M.H., unpublished data). The sedimentation equilibrium experiment was repeated twice as described above to produce three independent sets of nine data scans, which were each fit as a monomer-dimer model. We used the global fit to assign a K_d for dimerization of 243 ± 63 nM for the three data sets.

Gel Filtration Analysis of Residues Crucial for Dimerization

For the gel filtration experiments, all UL44 Δ C290 samples were loaded in triplicate onto a calibrated, analytical Superdex 200 HR 10/30 column at an approximate starting concentration of 20–25 μ M. Wild-type UL44 Δ C290 is predicted to have a molecular weight of 33 and 66 kDa for monomer and dimer species, respectively. Wild-type UL44 Δ C290 eluted at an apparent molecular weight of 52 ± 1 kDa, which is approximately 80% of the predicted molecular weight for a dimeric species of UL44 Δ C290 (Figure 4B). We then used this assay to determine if mutation of residues at the dimer interface of the structure would affect dimerization. We constructed three mutant constructs, F121A, L86A/L87A, and P85G. The F121A and L86A/L87A mutations were predicted to have potentially detrimental effects as

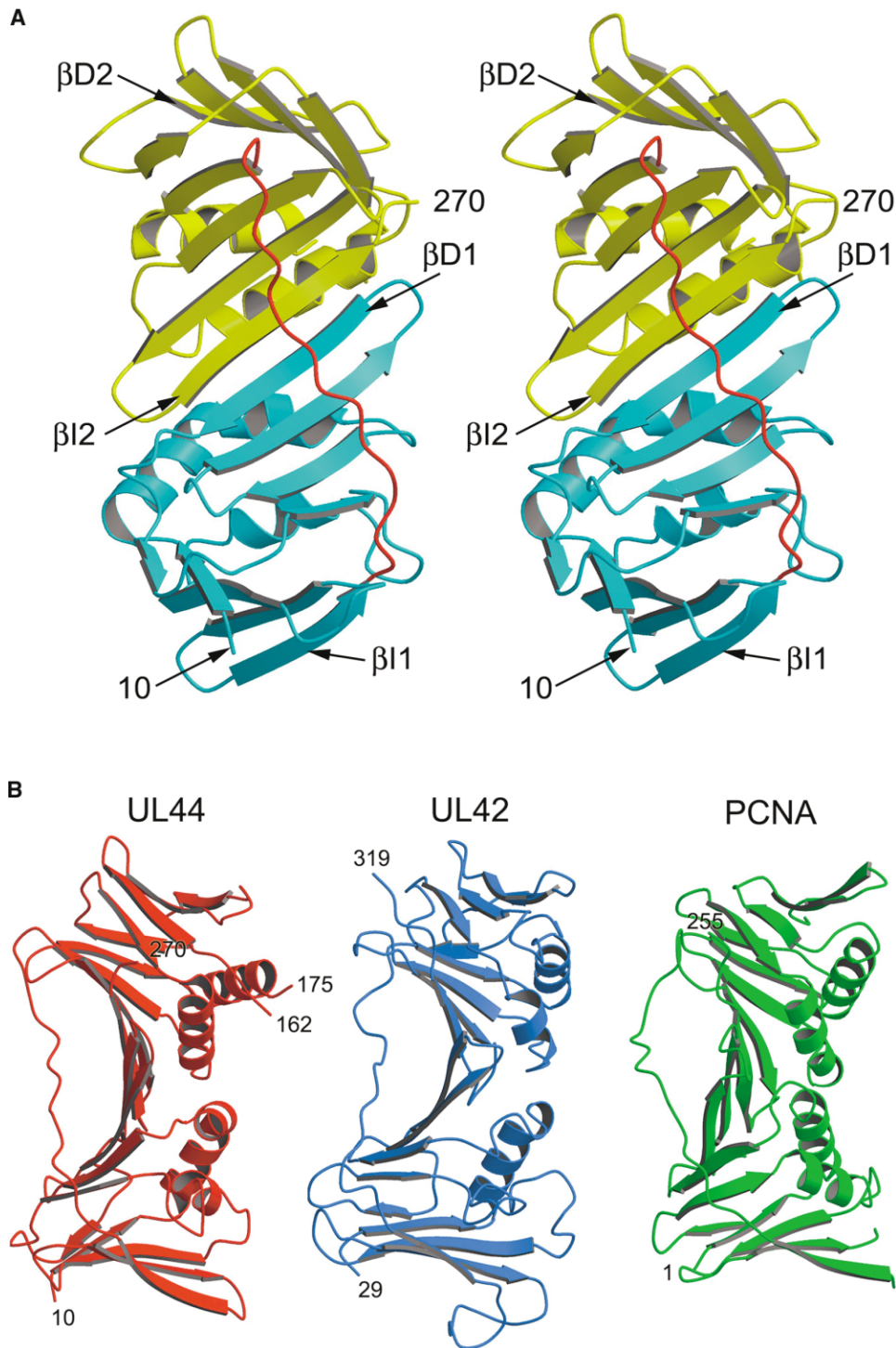


Figure 1. The Structure of the UL44 Monomer in Comparison to the PCNA Monomer and HSV UL42

(A) Stereoscopic ribbon diagram of a monomer of HCMV UL44 with the N- and C-terminal domains colored cyan and yellow, respectively. The connector loop, which connects the two domains, is shown in red. Residues 10 and 270 represent the last visible amino acids in the N and C terminus, respectively. All elements of secondary structure are named using the nomenclature originally adopted for yeast PCNA (Krishna et al., 1994). For example, the nine β strands found in the N-terminal domain are sequentially named from βA_1 to βI_1 . Strands βD_1 and βI_2 from the N- and C-terminal domains, respectively, are hydrogen bonded to create the central β sheet. UL44 Δ C290 forms a head-to-head dimer using hydrogen bonds between strands βI_1 from each subunit. In contrast, PCNA forms a head-to-tail homotrimer via hydrogen bonds between βI_1 and βD_2 of the next subunit (Gulbis et al., 1996; Krishna et al., 1994).

(B) Ribbon diagram representations of UL44, HSV UL42 (Zuccola et al., 2000), and human PCNA (Gulbis et al., 1996) are shown in red, blue, and green, respectively. These three proteins share a remarkably similar fold despite having no obvious sequence homology. Note the differences in the curvature along the α -helical or proposed DNA binding face among the structures. The curvature of this face of UL44 is

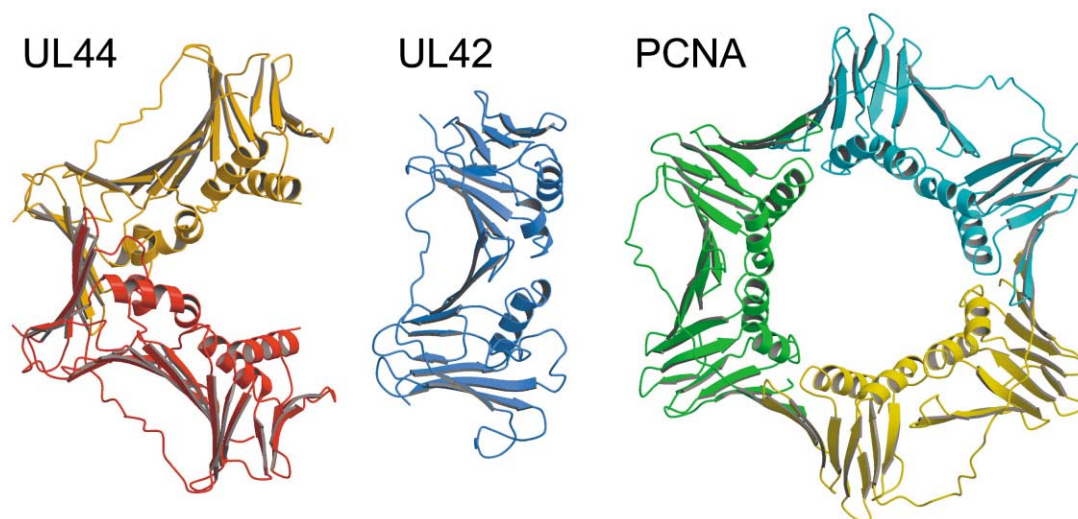


Figure 2. Comparison of the UL44 Dimer, UL42 Monomer, and PCNA Trimer

The dimer form of UL44 (red and orange for the subunits) is compared to the UL42 monomer (blue) (Zuccola et al., 2000) and the human PCNA homotrimer (yellow, green, and cyan for the subunits) (Gulbis et al., 1996).

these residues make extensive contacts with hydrophobic residues along the dimer interface. In contrast, the proline to glycine substitution was expected to maintain the β turn, but have relatively minor effects as P85 is not as significantly buried at the dimer interface. Mutant P85G eluted with an apparent molecular weight of 48 ± 0.4 kDa, which is similar to that of wild-type; however, the mutants F121A and L86A/L87A eluted with an apparent molecular weight of 29 ± 0.2 and 29 ± 2 kDa, respectively (Figure 4B). The values for F121A and L86A/L87A mutants are approximately 90% of the estimated value for monomeric UL44 Δ C290. The observation that the apparent molecular weights of both dimer and monomer and species is consistently less than the predicted weight is expected, given the observed elongated shapes of the monomer and dimer in the crystal structure. Thus, we conclude that wild-type and P85G UL44 Δ C290 behave as dimers, while F121A and L86A/L87A behave as monomers at a loading concentration of 20–25 μ M.

We also wished to rule out that the mutant proteins failed to dimerize and/or eluted at the observed molecular weights because they were misfolded. We have recently described the use of isothermal titration calorimetry to quantitatively measure the binding of UL44 to a peptide corresponding to the 22 C-terminal residues of UL54 (Loregian et al., 2004a). This peptide was sufficient to bind full-length UL44 at a 1:1 ratio with a K_d of 0.7 μ M. The wild-type and mutant UL44 Δ C290 constructs displayed K_d values for the peptide that ranged from 0.6 to 0.7 μ M, which was similar to the K_d determined for full-length UL44 (A.L., B.A.A., J.M.H., and D.M.C., unpub-

lished data). This indicates that the mutant proteins are not misfolded and suggests that dimerization is not required to bind UL54.

Dimerization of UL44 Δ C290 Correlates with DNA Binding

UL44, like HSV UL42, is unusual among processivity factors as it binds directly to DNA (Gallo et al., 1988; Gibson et al., 1981; Weiland et al., 1994). However, the DNA binding activity of UL44 has not been quantified. (UL42 binds to DNA with an apparent K_d in the nanomolar range [Gottlieb and Challberg, 1994; Thornton et al., 2000; Weisshart et al., 1999]). Thus, we investigated the affinity of UL44 Δ C290 for a double-stranded, 30 base pair oligonucleotide using a filter binding assay previously described for UL42 (Weisshart et al., 1999). An apparent K_d of approximately 1 nM was calculated for wild-type UL44 Δ C290 (Figure 5). Next, to determine whether dimerization of UL44 affects DNA binding, we investigated the ability of the UL44 Δ C290 mutants that are altered at the dimer interface to bind to the oligonucleotide. The mutant P85G, which was not defective in dimerization, displayed an apparent K_d of approximately 1 nM, similar to that of wild-type. In contrast, the F121A and L86A/L87A mutants, which behaved as monomers in gel filtration experiments, had apparent K_d values of 8–10 and 80–100 nM, respectively (Figure 5). It is unclear why these mutants displayed different affinities for DNA. Perhaps the F121A mutant displays weak dimerization upon DNA binding. Regardless, mutations that specifically affect dimerization also reduce the ability to bind dsDNA.

more reminiscent of PCNA than that of UL42. The numbers "162" and "175" indicate the location of a missing 12 residue loop in the C-terminal domain of UL44 (see Discussion and Figure 6). Other numerical values indicate the locations of the last visible residue in the N and C termini of each structure. All figures with ribbon diagrams were created with Molscript (Kraulis, 1991) and Raster 3D (Merritt and Bacon, 1997). Diagrams of UL42 and PCNA were built using the coordinates 1DML.pdb and 1AXC.pdb (Protein Data Bank). UL42 and human PCNA were cocrystallized with peptides, which were omitted from this illustration for simplicity.

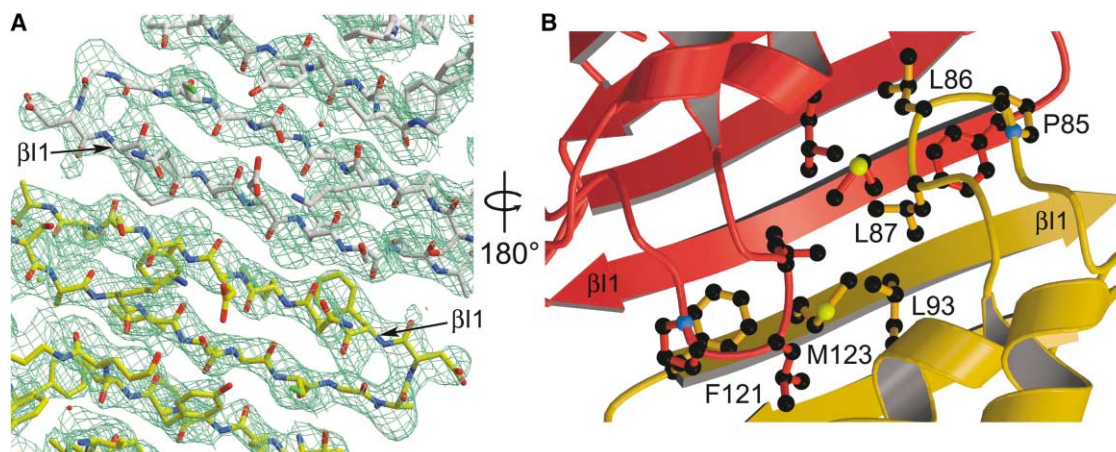


Figure 3. Molecular Details of the UL44 Homodimer Interface

(A) The final atomic model at the homodimer interface is shown in the 2.9 Å solvent-flattened experimental electron density map contoured at 1σ . The carbon backbone of the two subunits is colored yellow and gray, respectively, while oxygen and nitrogen atoms are colored red and blue, respectively. This view looks down the crystallographic 2-fold symmetry axis from outside the C clamp. Note the six main-chain-to-main-chain hydrogen bonds formed between strand $\beta 1$, of the respective monomers, which extends the bottom β sheet of each subunit. This figure was created with XtalView (McRee, 1999) and Raster 3D (Merritt and Bacon, 1997).

(B) Ribbon diagram of the homodimer interface looking down the 2-fold symmetry axis from inside the central cavity. This view is rotated 180° about the vertical axis compared to the view shown in (A). Six hydrophobic residues (P85, L86, L87, L93, F121, and M123) are represented as ball-and-stick models from each monomer. For clarity, only the residues from the orange subunit are labeled. Carbon, nitrogen, and sulfur atoms are colored black, blue, and yellow, respectively.

Discussion

The Interaction of UL44 with DNA

Like UL42, but unlike the sliding clamps and the T7 DNA polymerase processivity factor, thioredoxin (Huber et al., 1986; Stukenberg et al., 1991; Thornton et al., 2000; Weisshart et al., 1999) UL44 displays a relatively high affinity for DNA (Figure 5). This suggests that UL44 may confer processivity by a mechanism similar to that of UL42. Like UL42 and UL44, the mitochondrial DNA polymerase, pol γ B, binds DNA; however, the DNA binding activity of pol γ B evidently is not required to stimulate the polymerase (Carrodeguas et al., 2001, 2002). In contrast, the DNA binding activity of UL42 appears to be essential for processive DNA synthesis by UL30 (Chow and Coen, 1995; J.C.W. Randell and D.M.C., unpublished data), supporting the hypothesis that this activity tethers UL30 to DNA (Gottlieb and Challberg, 1994). Thus, it is possible that DNA binding by UL44 tethers UL54 to the primer-template thereby promoting processive DNA synthesis.

The structural and biochemical characterization of UL42 led to the proposal of an electrostatic sliding model to describe the ability of UL42 to tether UL30 to DNA without impeding its progress during DNA synthesis (Zuccola et al., 2000). In this model, electrostatic interactions between the basic “back” face of UL42 and the negatively charged phosphate backbone of DNA inhibit the dissociation of UL42 from DNA with minimal effects on its ability to slide freely along the DNA backbone. The model is based on the following observations. (1) UL42 does not slow the rate of DNA synthesis by UL30 (Weisshart et al., 1999). (2) The putative DNA binding “back” face of UL42 is highly basic (Zuccola et al., 2000). (3) UL42 slides on DNA in the absence of UL30 (Randell and Coen, 2001). (4) The mutation of key basic residues

on the “back” face leads to a reduction of the affinity of UL42 for dsDNA and a reduction in the ability to stimulate long-chain DNA synthesis (J.C.W. Randell and D.M.C., unpublished data).

Like UL42, UL44 also displays a “back” face that is remarkably basic in character (Figures 6A and 6B). However, the “back” face of UL44 is lysine rich while that of UL42 is arginine rich. These differences may have evolved for UL44 and UL42 to function as dimeric and monomeric processivity factors, respectively. Although lysine and arginine can form favorable electrostatic interactions with the negatively charged backbone of DNA, the guanidinium group of arginine is capable of forming stronger interactions with nucleic acids in comparison to the epsilon amino group of lysine. Thus, despite a similar distribution of positive charge on the DNA binding faces of UL42 and UL44, the affinity for DNA would be expected to be higher for a monomer of UL42 than for a monomer of UL44. Indeed, wild-type monomeric UL42 (Weisshart et al., 1999; J.C.W. Randell and D.M.C., unpublished results) and dimeric UL44 (Figure 5) display similar affinities for DNA while mutants of UL44 that fail to dimerize exhibit lower affinities for DNA (Figures 4B and 5). As optimal function would require a balance between tethering and sliding, we speculate that evolution would select a monomeric processivity factor such as UL42 to contain arginine residues on its DNA binding face and a dimeric processivity factor such as UL44 would contain lysine residues. Perhaps, arginine residues would make UL44 bind DNA too tightly and thus slow DNA sliding. These speculations are testable.

The Dimer Interface

The homodimeric interface of UL44 consists largely of hydrophobic residues that are buried against the other monomer. The hydrophobic nature of these amino acids

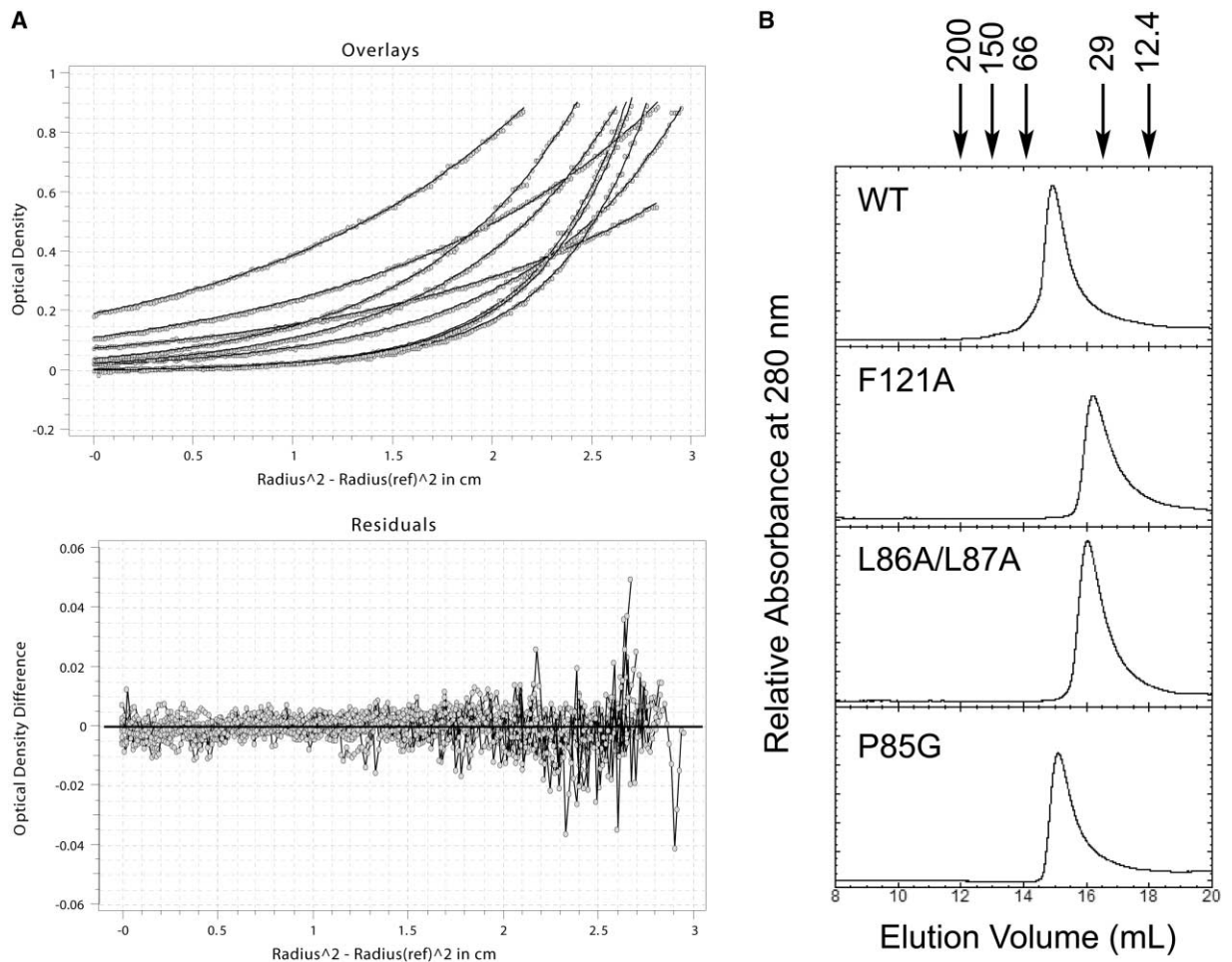


Figure 4. Analytical Ultracentrifugation and Gel Filtration of UL44 Δ C290

(A) Sedimentation equilibrium data for wild-type UL44 Δ C290 at loading concentrations of 1.3, 2.1, and 3.8 μ M, in which each concentration was sedimented to equilibrium at three rotor speeds of 13,500, 18,000, and 24,000 rpm to produce nine scans of data. The scans were fit globally to a monomer-dimer model by nonlinear least squares minimization using UltraScan. This experiment was repeated twice and produced an average dissociation constant (K_d) for dimerization of 243 ± 63 nM. The overlay of nine scans (top) and the corresponding residual plots (bottom) from the monomer-dimer fitting session are shown from one of the three experiments.

(B) Gel filtration data for wild-type, F121A, L86A/L87A, and P85G UL44 Δ C290 constructs are shown from top to bottom. Arrows represent the elution volumes for the indicated molecular weight standards used to calibrate the column. For clarity, the elution profiles are truncated from 8 to 20 ml.

is largely conserved in the β family of herpesviruses, including human herpesvirus-6, human herpesvirus-7, and cytomegaloviruses from other species. For example, L86 and L87, which appear to be essential for dimerization (Figure 4B), are conserved as large, hydrophobic residues (L, M, V, or F) in UL44 homologs from other reported β -herpesviruses. In contrast, hydrophobic amino acids are not found in analogous regions of UL42 (Zuccola et al., 2000), which is from an α -herpesvirus, HSV. L86 and L87 are part of a loop in UL44 that is analogous to a loop in UL42 composed of several charged, hydrophilic residues ($_{93}$ DQKR $_{96}$), and if UL42 is modeled as a head-to-head C clamp, it creates an orientation in which these charged residues would create potentially repulsive interactions (B.A.A., D.J.F., D.M.C., and J.M.H., unpublished data). Interestingly, it has been reported that PF-8, which is the processivity factor for the γ -herpesvirus Kaposi's sarcoma herpesvirus (KSHV), also dimerizes (Chen et al., 2004). In agree-

ment, the processivity factor for another γ -herpesvirus, Epstein-Barr Virus (EBV) BMRF1, stimulates the catalytic subunit at a ratio of 2:1, suggesting that BMRF1 might dimerize (Tsurumi et al., 1993). However, as the γ -herpesvirus processivity factors share no obvious sequence homology with any processivity factor from the α - or β -herpesvirus family, it is difficult to determine whether PF-8 or BMRF1 contain similar hydrophobic residues in the locations that were described for UL44. Regardless, it appears that the oligomeric state of the β - and γ -herpesvirus processivity factors has diverged from α -herpesvirus processivity factors during evolution.

It should be noted that our studies have focused on UL44 Δ 290. Full-length UL44 behaves poorly under the conditions used for analytical ultracentrifugation and gel filtration analyses. However, both full-length GST-UL44 and GST-UL44 Δ C290 associate with cell-free translated, full-length UL44 in "pulldown" experiments, sug-

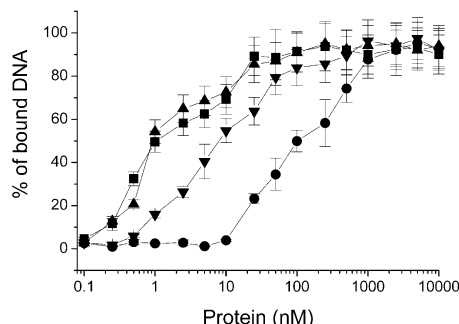


Figure 5. DNA Binding Activity of Wild-Type and Mutant UL44 Protein

Increasing amounts of wild-type UL44ΔC290 (squares), UL44ΔC290 P85G (triangles), UL44ΔC290 L86A/L87A (circles), or UL44ΔC290 F121A (inverted triangles) were incubated with 1 fmol of radiolabeled ds 30 bp DNA. Free and protein-bound DNA were quantified by filter binding assays, and the fraction of protein-bound DNA was plotted against the protein concentration.

gesting that full-length UL44 dimerizes (A.L. and D.M.C., unpublished data). Also, we do not know what fraction of UL44 in cells is dimeric. However, UL44 is abundant in virus-infected cells and concentrates in subnuclear compartments where viral DNA replication occurs (Iwiyama et al., 1994). Given the K_d for dimerization, it seems likely that much of UL44 is dimeric in these compartments.

UL44 May Surround DNA like PCNA

Even though UL44, UL42, and PCNA share a similar fold, there are significant differences in the structures, including the curvature along the α -helical face that is believed to be responsible for interactions with DNA (Figure 1B). Due to the rotation between the N- and C-terminal domains, both UL44 and PCNA appear more concave in comparison to UL42 (Gulbis et al., 1996; Zuccola et al., 2000). As judged by the superposition of 163 structurally equivalent α carbons, UL44 displays an rmsd value slightly (3.4 Å) more similar to PCNA than is UL42 (3.7 Å). In contrast, the relatively flat appearance of UL42 would make it exceedingly difficult for UL42 to form a toroidal-shaped ring. The curved shape of UL44 creates a cavity (Figure 2) that may be analogous to the central channel of PCNA that surrounds DNA (Gulbis et al., 1996; Krishna et al., 1994). This cavity of UL44 is approximately 25 Å in diameter and thus appears to be of sufficient size to accommodate dsDNA (Figure 6C). In contrast, the cavity of sliding clamps is larger (35–35 Å), which is expected since they loosely encircle DNA. The GRASP representation shown also places the lysine-rich faces near the center cavity. Interestingly, these faces are partially rotated away from the cavity, suggesting that the dimer would rest at an approximately 30° angle, rather than perpendicular to the DNA axis (Figure 6C).

UL44 also contains a unique 12 residue loop ($_{163}\text{HTRVKNRVKAP}_{174}$) between αA_2 and βB_2 in the C-terminal domain, which contains 5 additional lysine and arginine residues and is not visible in the electron density maps (Figures 6A and 6C). This disordered re-

gion, which sits above the α -helical face, is found near the tip of each monomer of the C clamp and could potentially extend the clamp around the phosphate DNA backbone via electrostatic interactions. In contrast, UL42 lacks a long loop in this position and contains a 24 residue disordered loop between βD_2 and βE_2 (Zuccola et al., 2000), which is not rich in basic residues and appears to project away from the arginine-rich, α -helical face (Figure 6A).

In summary, UL44 uses the same fold as UL42 and PCNA to build a novel dimeric structure that is a hybrid of these processivity factors. Like UL42, UL44 binds DNA with nanomolar affinity and displays a remarkably basic, α -helical face that may slide along DNA via electrostatic interactions. However, the nature of these basic residues is strikingly different and may have diverged as UL42 and UL44 evolved into monomer and dimer species, respectively. The C clamp architecture creates a channel that is reminiscent of PCNA with one subunit removed. However, the partially open shape of the UL44 C clamp allows for self-assembly onto DNA without the need of clamp loaders, as is the case for PCNA. Finally, PCNA and sliding clamps interact with a wide array of other cellular proteins in addition to their interactions with the catalytic subunits of DNA polymerases (reviewed in Vivona and Kelman, 2003). HCMV DNA replication is presumed to require a coordinated assembly of multiple proteins (Anders and McCue, 1996). Given its similarity to PCNA, the dimeric nature of UL44 could allow for the second subunit of the complex to act as a scaffold to recruit other viral or cellular proteins during viral DNA replication.

Experimental Procedures

Plasmids

The pD15-UL44ΔC290 plasmid, which expresses the N-terminal 290 residues of UL44 as a glutathione S-transferase (GST) protein, was described previously (Loregian et al., 2004a). This plasmid was constructed using a His-tagged UL44 template plasmid, pRSET-UL44 (gift from P.F. Ertl, GlaxoSmithKline, UK). All UL44 mutants were obtained using the Quick-Change Mutagenesis kit (Stratagene) by amplification of the pD15-UL44ΔC290 plasmid with appropriate primer pairs to generate the desired nucleotide change(s). For primer sequences used to create these mutants, please see Supplemental Data at <http://www.molecule.org/cgi/content/full/15/2/233/DC1>. All plasmids were sequenced to exclude the presence of unwanted mutations.

Protein Purification

Recombinant GST-UL44ΔC290 wild-type or mutant protein was expressed at 16°C in *E. coli*, pelleted, and stored at –80°C as described (Loregian et al., 2004a). The cell pellets were lysed with two to three passages through an Emulsiflex-C5 apparatus (Avestin) and centrifuged at 43,000 × g for 1 hr. All chromatography resins and prepacked columns were purchased from Amersham-Pharmacia Biotech. The lysate was initially passed over Glutathione Sepharose 4 Fast Flow resin equilibrated in buffer A (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 20% glycerol, 5 mM EDTA, and 2 mM DTT) and eluted in buffer A + 15 mM glutathione. Fractions containing GST-UL44ΔC290 were pooled and cleaved with PreScission Protease to remove the GST tag, diluted 1:1 in buffer A without NaCl, and applied to a Heparin HiTrap column equilibrated in buffer B (50 mM Tris-HCl [pH 7.5], 10% glycerol, 1 mM EDTA, 2 mM DTT) + 250 mM NaCl. Proteins were eluted from the column with a linear gradient from 250 to 1000 mM NaCl in buffer B. Fractions containing UL44ΔC290 were concentrated using Vivaspins concentrators (Vivascience AG, Germany) and applied to either a Sephacryl S-100 HR

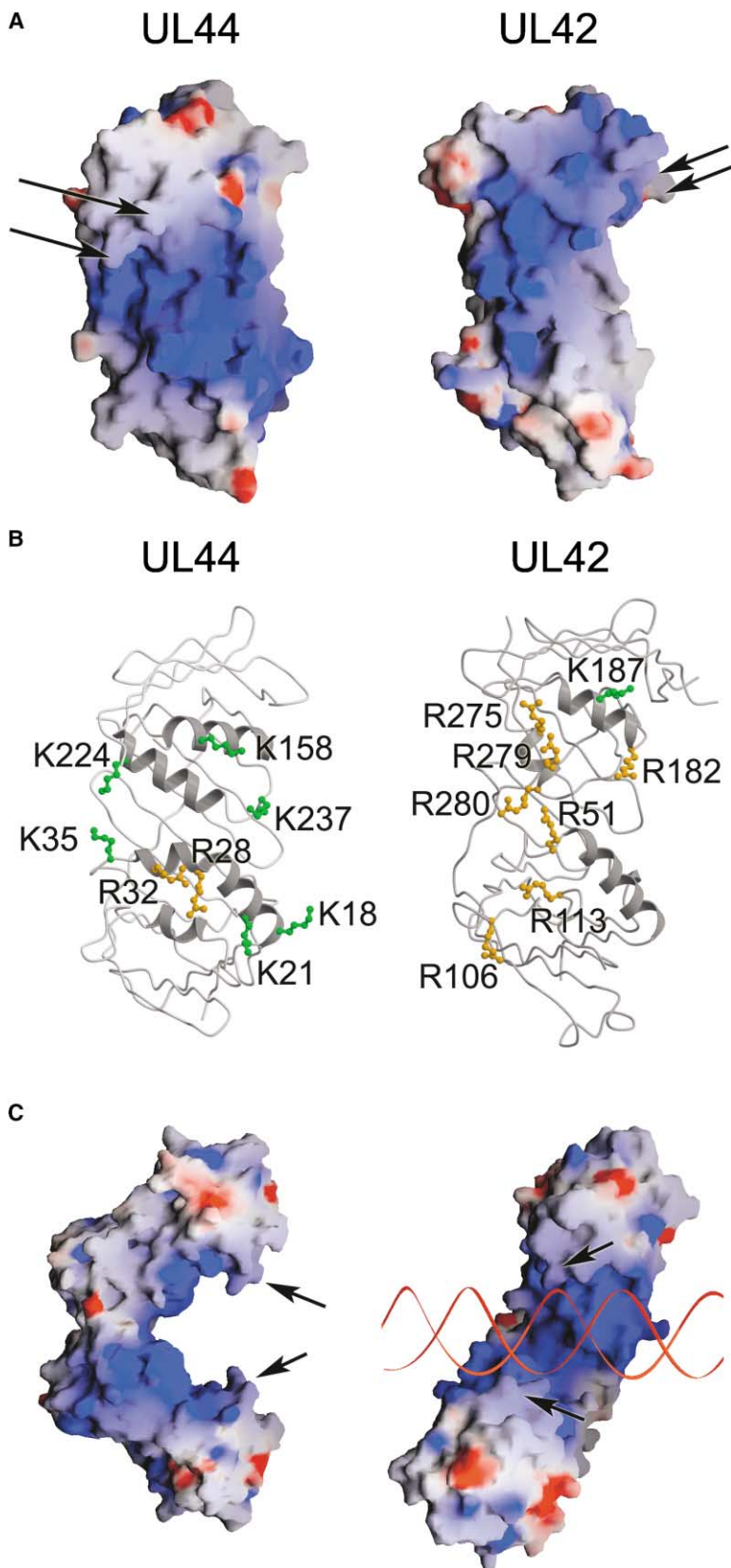


Figure 6. The Proposed DNA Binding Face of UL44 and UL42

(A) The solvent accessible surfaces along the α -helical face of UL44 (left) and UL42 (right) are colored according to the electrostatic potential using GRASP (blue, positive; red, negative) (Nicholls et al., 1991). Like UL42, UL44 displays a remarkably basic α -helical face that may bind to DNA via electrostatic interactions. However, in contrast to UL42, UL44 contains a unique 12 residue loop (₁₆₃HTRVKRNVKKAP₁₇₄), which has several basic residues and is not visible in the electron density maps. This region could potentially extend over the basic face and contact the DNA backbone via electrostatic interactions. UL42 does contain a 24 residue disordered loop, which is not rich in basic residues and is located to the right of the α -helical face. The locations of these missing loops are marked with arrows.

(B) Even though UL44 (left) and UL42 (right) share a remarkably basic face, UL44 contains predominantly lysine residues while UL42 contains mostly arginines. Arginine and lysine side chains are colored in orange and green, respectively, as ball-and-stick models.

(C) GRASP representation of the UL44 dimer. The cavity of the C clamp (left) is approximately 25 Å in diameter, which is sufficient to accommodate double-stranded DNA. In the right panel, DNA (red ribbon) is modeled into the C clamp in an orientation to form potential electrostatic interactions with the basic faces. The basic faces are rotated away from the central cavity, suggesting that the dimer does not sit perpendicular to the DNA axis. This view is rotated 90° about the y axis and approximately 30° about the z axis from the left panel. Arrows indicate the missing loop that is discussed in (A).

or Superdex 200 HR column equilibrated in buffer B + 500 mM NaCl. UL44ΔC290 fractions were dialyzed against buffer B + 150 mM NaCl, applied to a SP-sepharose HiTrap column, and eluted with a linear gradient from 150 to 750 mM NaCl in buffer B. UL44ΔC290 was dialyzed in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 20% glycerol, 0.1 mM EDTA, and 2 mM DTT and concentrated with Vivaspinn concentrators to approximately 10–13 mg/ml as estimated by Bradford assay using γ -globulin as a reference standard. Quantitative amino acid analysis (performed by the Molecular Biology Core Facility, Dana-Farber Cancer Institute) indicated that the actual protein concentration was 4–6 mg/ml or 40% of the value estimated by Bradford assay. Samples were flash frozen in liquid nitrogen and stored at -80°C . For samples used in gel filtration experiments, the final SP-sepharose column was omitted and samples were dialyzed and stored as described. Selenomethionyl (SeMet) UL44ΔC290 was expressed according to previously described protocols (Van Duyne et al., 1993) and purified in a similar manner to native protein, except that all buffers included 5 mM DTT.

Crystallization

UL44ΔC290 was crystallized in hanging drops at 22°C by vapor diffusion. UL44ΔC290, which was concentrated and stored as described above, was thawed and mixed 1:1 with a well solution composed of 30% PEG 4000, 1000 mM NaCl, 100 mM sodium acetate (pH 4.1–4.25), and 20 mM DTT. DTT was essential for crystallization of SeMet-UL44ΔC290 and improved the quality of native crystals. Crystals appeared overnight and reached approximately $100 \times 150 \times 400 \mu\text{m}$ around 7 days. The drop solution was gradually replaced with a cryosolution until the final concentration was approximately 20% ethylene glycol, 20% PEG 4000, 1000 mM NaCl, 67 mM sodium acetate (pH 4.1–4.25), and 20 mM DTT. The original well solution was replaced with a solution that matched the final cryosolution and crystals were allowed to equilibrate overnight. Crystals were flash frozen in liquid nitrogen.

Data Collection, Phasing, and Refinement

Experimental phase information was obtained from MAD experiments on two isomorphous SeMet-UL44ΔC290 crystals at Beamline X12C at the National Synchrotron Light Source (Brookhaven, NY). All images were processed with DENZO and merged with SCALEPACK (Otwinowski and Minor, 1997). Data were collected near the selenium K-edge (Table 1). When all seven selenomethionyl data sets were included, SOLVE (Terwilliger and Berendzen, 1999) located four of five possible selenium sites. Density modification was performed with RESOLVE (Terwilliger, 2000). Atomic models were built into experimental phased maps using XtalView (McRee, 1999). Native data were collected to $1.85 \text{ \AA}$ on one crystal at Beamline 14-BMC at the Advanced Photon Source (Argonne, IL). Beyond $1.85 \text{ \AA}$, R_{sym} values were nearly random. Atomic models built from the selenomethionyl and native data were each refined using REFMAC 5 (Murshudov et al., 1997) with experimental phases maintained as refinement targets until late in the structure solution.

Analytical Ultracentrifugation

Sedimentation equilibrium experiments were performed in a Beckman XL-A analytical ultracentrifuge at 4°C . Because UL44ΔC290 contains only one tryptophan and thus displays a relatively low extinction coefficient at 280 nm, we chose to perform experiments at 230 nm where the extinction coefficient is higher. UL44ΔC290 was extensively dialyzed against buffer C (10 mM MOPS [pH 7.0], 150 mM NaCl, and 0.25 mM TCEP [Tris(2-carboxyethyl)-phosphine]). Cells were typically loaded with protein concentrations of 1.3, 2.1, and $3.8 \mu\text{M}$ using buffer C in the reference chambers. Samples were centrifuged sequentially at 13,500, 18,000, and 24,000 rpm and scanned every 2–4 hr. Samples were considered to be at equilibrium when no significant differences were observed between consecutive scans. Values for protein partial specific volume and solvent density (ρ) were determined with SEDNTERP (<http://www.jphilo.mailway.com>). Each data set was evaluated globally using nonlinear least squares minimization with different self-associating models in UltraScan 6.0 (<http://www.ultrascan.uthscsa.edu>). The best fit was obtained with a monomer-dimer model as judged by minimization of the variance and randomness of the residuals.

Analytical Gel Filtration Chromatography

An analytical Superdex 200 HR 10/30 column was equilibrated in buffer D (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 0.5 mM TCEP) at 4°C . Void volume (V_0) was determined with blue dextran (Sigma). The column was calibrated with the following standards (Sigma): β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa). The ratio of elution volume to void volume (V_e/V_0) was plotted versus the Log(MW) for each standard to generate a linear standard curve. Wild-type or mutant UL44ΔC290 was dialyzed from frozen stocks into buffer D, diluted to a concentration of 20–25 μM , and injected onto the column at a volume of 100 μl . Each UL44ΔC290 construct was injected onto the column in triplicate. The apparent MW of each UL44ΔC290 construct was determined from the standard curve.

Filter Binding Assays

The apparent K_d values of UL44ΔC290 constructs and a 30 bp DNA fragment were investigated using a filter binding assay and saturation isotherm analysis essentially as described (Weissbart et al., 1999), but with the following modifications. The double-stranded (ds) 30 bp DNA used in these assays was constructed by annealing the DNA oligonucleotide 5'-CTAGCTGTAGTCAGCTGACTGTAGA TCGCT-3' and its complement. The dsDNA was radiolabeled with [γ - ^{32}P]ATP (DuPont NEN) and T4 polynucleotide kinase (New England Biolabs) and purified as previously described (Randell and Coen, 2001). 1 fmol of labeled (10,000 cpm/fmol) dsDNA was incubated at room temperature with various amounts of wild-type or mutant UL44ΔC290 for 10 min in a 20 μl volume in binding buffer (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 4% glycerol, 3 mM MgCl_2 , 0.5 mM DTT, 0.1 mM EDTA, 40 μg of bovine serum albumin per ml). At the end of the incubation, samples were passed through two filters. The top and bottom filters were nitrocellulose and DE81, which retain protein-bound and unbound oligonucleotides, respectively. Filters were washed in binding buffer and dried, and radioactivity was measured by liquid scintillation counting.

Isothermal Titration Calorimetry

Isothermal calorimetry (ITC) experiments were performed as previously described (Loregian et al., 2004a) with protein concentrations of 5–10 μM and UL54 peptide 1 concentrations of 100–250 μM . UL54 peptide 1 corresponds to the last 22 residues of UL54 and was synthesized by the Biopolymers Laboratory in the Department of Biological Chemistry and Molecular Pharmacology at Harvard Medical School. The data were analyzed as previously described (Bridges et al., 2001).

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Accession Numbers

The coordinate data has been entered in the Protein Data Bank under ID code 1TGL.