In the absence of other proteins, the DNA polymerase (Pol-8) of Kaposi's sarcoma herpesvirus incorporates only several nucleotides from a primer template. However, association with the Kaposi's sarcoma herpesvirus processivity factor (PF-8) enables Pol-8 to incorporate thousands of nucleotides. Unlike the well described sliding clamp processivity factors, eukaryotic proliferating cell nuclear antigen and Escherichia coli beta-subunit, PF-8 and other herpesvirus processivity factors do not require a clamp loader or ATP to bind to template DNA. To begin to understand the mechanism used by PF-8 to achieve processivity, we have now purified PF-8 and demonstrated that it is a dimer both in solution and on the DNA. Mutational analysis of the PF-8 protein (396R) indicates that residues between 277 and 304 as well as the N-terminal 21 amino acids are required for dimerization. The results further correlate PF-8 dimerization with binding to Pol-8 and stabilizing Pol-8 on primer template. Notably, although removal of only 26 residues from the C terminus of PF-8 does not affect its ability to form dimers on DNA or to bind Pol-8, only short DNA from the C terminus of PF-8 does not affect its ability to template. Notably, although removal of only 26 residues with binding to Pol-8 and stabilizing Pol-8 on primer template DNA (22). One common mechanism by which DNA polymerases achieve catalytic efficiency is by associating with processivity factors that help tether the polymerase to the DNA so that the rate of polymerase nucleotide incorporation exceeds the rate of polymerase dissociation from the template. The most conserved processivity factors are the ring-shaped sliding clamps, best characterized by the beta-subunit of Escherichia coli (1), and proliferating cell nuclear antigen (PCNA) of eukaryotic cells (2) and archaeabacteria (3) and gp45 of T4 bacteriophage (4). Remarkably, despite having both disparate primary amino acid sequences and different numbers of monomers (two for the beta-subunit and three for PCNA and gp45, respectively), these highly symmetric ring structures are essentially superimposable (reviewed in Ref. 5). ATP-dependent clamp loaders enable ring-shaped sliding clamps to encircle the template since they do not self-assemble around the DNA (reviewed in Ref. 6). Other processivity factors are of insufficient size to form encircling sliding clamps, as with the bacterial protein thioredoxin that forms a 1:1 complex with bacteriophage T7 DNA polymerase without the aid of a clamp loader (7, 8). Interestingly, through its interaction with the thumb domain of the T7 DNA polymerase, thioredoxin appears to order a structure that enables the T7 DNA polymerase to slide along the template without dissociating (7, 9).

The DNA polymerases of human herpesviruses also associate with processivity factors. These include UL42 of herpes simplex virus I (HSV-1) (10–12); BMRF1 of Epstein-Barr virus (13, 14); UL44 of human cytomegalovirus (CMV) (15, 16); p41 of human herpesvirus-6 (HHV-6) (17, 18); and PF-8 of Kaposi's sarcoma herpesvirus (KSHV or HHV-8) (19). The herpesvirus processivity factors exhibit high intrinsic affinity for DNA and do not require clamp loaders or ATP to associate with DNA. The furthest characterized herpesvirus processivity factor is UL42 of HSV-1. Remarkably, the crystal structure of UL42 bound to a peptide representing the C terminus of HSV-1 Pol reveals a structure that is very similar to a monomer of PCNA, despite the fact that these processivity factors have different biochemical properties and distinct amino acid sequences (20). Nevertheless, both structural evidence and biochemical evidence suggest that, unlike trimeric PCNA, UL42 resides as a monomer both in solution and in association with HSV-1 DNA polymerase (10, 20). One model describes UL42 as a structure that tethers HSV-1 Pol to the DNA such that the UL42 slides with its catalytic subunit during DNA replication (21). A second model suggests that UL42 undergoes a conformational alteration upon interaction with HSV-1 Pol to enable stable binding of the homocomplex to primer template DNA (22).

We previously discovered that PF-8 is the processivity factor of KSHV DNA polymerase (Pol-8) (19). KSHV is the causative agent of Kaposi's sarcoma (23), a neoplasm that occurs most frequently in AIDS patients and is strongly linked to primary effusion lymphoma (24) and multicentric Castleman disease (25). We initially cloned both PF-8 and Pol-8 and demonstrated that the DNA synthesis activity of Pol-8 was strongly depend-
ent on PF-8. Specifically, Pol-8 alone was able to incorporate only three nucleotides, whereas in the presence of PF-8, the Pol-8 generated an extended DNA product (7,248 nucleotides) corresponding to the full-length DNA template (19). Our results also suggested that PF-8 and Pol-8 are specific for one another since they could not be functionally replaced by other herpesvirus processivity factors and DNA polymerases (19). Thus, the PF-8 and Pol-8 association may serve as a specific drug target that could block KSHV infection and Kaposis sarcoma. In this study, to gain greater insight into how PF-8 functions in processivity, we purified PF-8 and determined that it forms dimers in solution and on the DNA. These same types of PF-8 dimers were also observed in infected cells. Consistent with its role in processivity, we show that PF-8 can stabilize Pol-8 on DNA primer-template. Mutational analysis revealed that regions of PF-8 required for dimerization are also required for binding to DNA and Pol-8. Importantly, although two C-terminal deletion mutants of PF-8 retained partial processivity function by enabling Pol-8 to synthesize short DNA chains (<100 nt), only full-length PF-8 enabled Pol-8 to completely synthesize the template (>7,000 nt).

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of PF-8 from E. coli.** The PF-8 gene (ORF59) (19) was cloned into pGEX4T-2 (Amersham Biosciences), where it was fused to an N-terminal GST tag with an adjacent thrombin cleavage site. The GST-PF-8 fusion protein was induced in E. coli BL21 (DE3)-CondonPlus cells (Stratagene). The E. coli were resuspended in PBS containing 10 mM DTT, 100 mM MgCl2, 0.5 mg/ml lysozyme, 2 units/ml DNase, and 0.86 mg/ml protease inhibitor mixture (Sigma) for 1 h at 4 °C and then lysed by freeze-thawing. The lysate was incubated at 4 °C for an additional 30 min, briefly sonicated on ice to reduce viscosity, and centrifuged, and the clarified supernatant containing GST-PF-8 was filtered (0.2-µm filter) and applied to a GSTrap column (Amersham Biosciences) equilibrated with PBS (pH 7.3) and 5 mM DTT. After being washed with 20 column volumes of the same buffer, the column was filled with thrombin protease, sealed, and incubated at room temperature for 16 h. Cleaved PF-8 protein was eluted using 20 ml of PBS, and bound GST and uncleaved GST-PF-8 were eluted with 20 mM reduced glutathione in Tris-HCl (pH 8.0). Further purification was achieved by chromatography at room temperature on a Superdex 200 (Amersham Biosciences) gel filtration column equilibrated with PBS (pH 7.3), containing 2 mM DTT and 1 mM NaCl. Fractions were analyzed by SDS-PAGE, and protein was visualized by staining with Coomassie Brilliant Blue. Column fractions containing purified PF-8 were filtered (0.2-µm filter) and applied to a TBE-urea gel sampling protocol. The proteins were fractionated on a 12% SDS-polyacrylamide gel followed by staining with Coomassie Brilliant Blue.

**Construction of PF-8 Mutants.** The PF-8 deletion mutant Δ371–396 was generated by cleaving a Pf-8 open reading frame (19) with Ncol and SacI followed by cloning into the pTM1. The PF-8 deletion mutants, Δ305–396; Δ277–396; and Δ1–21, were generated by PCR amplification using corresponding primers to create a 5′-Ncol site and a 3′-SacI site followed by subsequent cloning into pTM1. All the plasmid constructs were confirmed by DNA sequencing and/or restriction endonuclease digestion and were introduced into E. coli DH5α (Invitrogen).

**Co-immunoprecipitation.**—Co-immunoprecipitation was performed by incubating 10 µl of co-translated Pol-8 and PF-8 proteins with 20 µl of anti-PF-8 antibody at 4 °C for 2 h in the presence of 100 mM KCl, 5 mM MgCl2, 1% Triton-X100, 50 mM Tris-HCl (pH 7.6), and 0.1% Tween 20. Samples were then reacted with 30 µl of protein A-Sepharose at 4 °C for 1 h. After washing extensively with buffer containing 100 mM KCl, 5 mM MgCl2, 50 mM Tris-HCl (pH 7.6), and 0.5% Tween 20, the immunoprecipitates were eluted by boiling in Laemmli’s buffer and fractionated on 4–12% SDS-polyacrylamide gels.

**Preparation of Biotinylated Primer-Template (P-T).**—P-T was prepared for analyzing protein binding. A 5′-biotinylated oligonucleotide, Primer A (5′-tgacggctggagttggggtg-3′), and a non-biotinylated oligonucleotide, Primer B (5′-ctagttacgtggactaatc-3′), were used to amplify a 250-bp DNA fragment by PCR from pBSK64 followed by purification through a Qiagen PCR purification column. Approximately 2 µg of DNA was bound to 100 µl of streptavidin-coupled magnetic beads (Dynabeads M-280). The beads were washed twice with 10 mM Tris-HCl (pH 8.0), heated to 95 °C for 5 min, cooled down quickly on ice, and then washed twice again. The DNA on the beads was further denatured by incubating with 100 µl of 0.1 M NaOH for 10 min followed by washing twice with 10 mM Tris-HCl (pH 8.0). Single-stranded biotinylated DNA was annealed to Primer-T (in the presence of 1 mM MgCl2, 5 mM Tris-HCl, 1 mM EDTA, and 50 mM Tris-HCL (pH 7.6) by heating to 95 °C for 5 min and then cooled down slowly to room temperature. For preparing P-T for DNA synthesis assay, Primer B was preannealed at the 5′-end with γ-32P[ATP]. The biotinylated P-T-coupled magnetic beads were then washed and finally resuspended in 100 µl of 10 mM Tris-HCl (pH 8.0).

**DNA Synthesis Assays.**—The in vitro DNA synthesis assays were performed on dsDNA cellulose (Sigma) and biotinylated P-T-coupled streptavidin-coated magnetic beads (Dynabeads M-280). For all binding assays, 2.5 µl of each [35S]Methionine-labeled in vitro translated protein was incubated with the 50 µl of swollen cellulose or
Fig. 1. Expression and purification of functional PF-8 from E. coli. A, an SDS-PAGE analysis of PF-8 purified from E. coli. Lane 1, broad range protein standards; lane 2, whole cell protein from uninduced E. coli; lane 3, whole cell protein from induced E. coli; lane 4, supernatant of whole cell extract from induced E. coli; lane 5, affinity-purified PF-8 eluted from a GSTrap column after thrombin cleavage of bound GST-PF-8; lane 6, PF-8 chromatographed on a Superdex 200 column; lanes 7 and 8, GST-PF-8 and GST markers that had been purified from GSTrap and Superdex 200 columns, respectively. B, purified PF-8 enables Pol-8 to synthesize DNA processively. Labeled DNA products synthesized from an 18-oligonucleotide primer annealed to the M13 template (7,249 nt) in the presence and absence of Pol-8 and PF-8 were analyzed by alkaline agarose gel electrophoresis. Designated are in vitro translated (IVT) Pol-8 and in vitro translated PF-8, purified PF-8 from E. coli (E). The nucleotide (nt) lengths of the newly synthesized DNA products are indicated.

dDNA cellulose or the 100 ng P-T coupled to the beads for 30 min at room temperature in the presence of 20 mM Tris-HCl (pH 7.6). The DNA cellulose or P-T beads were washed extensively with 20 mM Tris-HCl (pH 7.6). Bound proteins were eluted stepwise with 20 mM Tris-HCl (pH 7.6) containing increasing concentrations (100, 250, and 500 mM) of NaCl, fractionated on 4–20% SDS-polyacrylamide gels and examined by autoradiography.

Electrophoretic Mobility Shift Assay (EMSA)—Varying amounts of GST-PF-8, PF-8, or a mixture of GST-PF-8 and PF-8 (prepared by thrombin protease partial digestion) were incubated with a radiolabeled 18-bp DNA (1.25 μm) (positive strand sequence: 5' gtaaagcagcagcagcagc') in DNA binding buffer (4% glycerol, 20 mM Tris-HCl, pH 8.0, 30 mM KCl, 5 mM MgCl₂, 100 μg/mL bovine serum albumin) for 10 min at room temperature and immediately loaded onto a 6% polyacrylamide gel (acrylamide to bis-acrylamide, 29:1, w/w). After electrophoresis in 90 mM Tris borate (pH 8.3), 1 mM EDTA (TBE), the gels were dried and autoradiographed.

RESULTS

Purification of Functional PF-8—Full-length PF-8 was expressed in E. coli as a GST fusion protein. As shown in Fig. 1A, GST-PF-8 constituted more than 20% of the total protein produced in induced cells (compare uninduced and induced cell lysates, lanes 2 and 3). It is noted that this high level of GST-PF-8 expression was attainable with a strain of E. coli (BL21-CodonPlus®-RIL) that overcomes the problem of codon bias. Purification of PF-8 was facilitated by the N-terminal GST tag and a juxtaposed thrombin protease site for removal of the GST tag. Essentially, GST-PF-8 from supernatant extracts of induced E. coli (lane 4) was captured on a GSTrap affinity column and treated in-column with thrombin protease to release full-length PF-8 (lane 5). Although PF-8 was greatly enriched, several small peptides, possibly truncation products of PF-8, were observed. To remove these peptides as well as other potential contaminants, including DNA possibly bound to PF-8, thrombin-cleaved PF-8 was concentrated and chromatographed on a Superdex 200 gel filtration column in the presence of high salt (1 M NaCl). As shown in lane 6, most of the smaller peptides no longer accompanied PF-8 after the Superdex 200 column. PF-8 was estimated to be 95% pure and was available for several of the assays presented below.

To ascertain whether purified PF-8 retained function, the processivity factor was tested in a DNA synthesis assay (Fig. 1B). As expected, in vitro translated PF-8 alone (lane 2) or purified PF-8 alone (lane 4) was incapable of synthesizing DNA from an 18-nucleotide primer annealed to single-stranded M13 template (7,249 nt). Pol-8 alone failed to produce any resolvable products on this gel system (lane 1), consistent with our previous study demonstrating that Pol-8 alone incorporates only several nucleotides (19). By contrast, a full-length DNA product was generated when either in vitro translated PF-8 or purified PF-8 was added to Pol-8 (lanes 3 and 5). The newly synthesized DNAs in the 80-nucleotide range (lanes 3 and 5) are shorter processive DNA products, typically observed in these assays. Thus, PF-8 purified from E. coli provides Pol-8 with the processive function that is needed to synthesize thousands of nucleotides from primer template DNA.

Recombinant PF-8 Protein Is a Dimer in Solution—Several independent methods were employed to examine whether PF-8 is a monomer or a multimer in solution. The in vitro methods used were gel filtration chromatography, cross-linking, and native gel electrophoresis.

Gel Filtration Chromatography—PF-8 comprises 396 amino acids with an actual molecular mass of 43 kDa. In our initial attempt to employ gel filtration chromatography as a means to
obtain highly purified PF-8, we utilized a Superdex 75 column. However, we observed that PF-8 eluted in the void volume, which suggested that the size of the non-denatured bacterial-expressed protein could be larger than 75 kDa. As seen in Fig. 2A, when a Superdex 200 HR 10/30 column was used, PF-8 eluted as a single peak with an apparent molecular size of 76 kDa, based on the logarithm of molecular size against $K_{av}$ of protein standards that had been chromatographed in the same column (Fig. 2A, inset). This finding suggested that PF-8 exists mainly as a dimer in solution. However, since the behavior of a
protein during chromatography on a gel filtration column correlates directly with its Stokes radius and not always with its true molecular weight, other methods of determining whether PF-8 forms dimers were employed, as described below.

Cross-linking—As a second approach, we asked whether dimers could be obtained by cross-linking purified PF-8. We used BS\(^3\), a water-soluble, homobifunctional N-hydroxysuccinimide ester, primary amine-reactive cross-linker with a spacer arm length of 11.4 Å. Fig. 2B compares the SDS-PAGE profiles of non-cross-linked PF-8 (lane 2) and cross-linked PF-8 (lane 3). Clearly, cross-linking revealed that purified PF-8 does form dimers. It is noted that in the SDS-gel, PF-8 monomer migrates to a position (~60 kDa) that is higher than its actual size (43 kDa). Consistent with these results using purified PF-8, in vitro translated PF-8 was also able to form dimers upon cross-linking (Fig. 2C, compare lanes 3 and 4).

Native Gel Electrophoresis—Native gel electrophoresis was used as a third independent method to corroborate that purified PF-8 is able to form dimers in solution. We employed blue-native polyacrylamide gel electrophoresis (BN-PAGE) in which Coomassie Blue G-250 confers a negative charge on proteins so that separation is based on molecular weight and is independent of pI. To generate size markers for the blue-native gel that contains monomeric, dimeric, trimeric, and tetrameric forms of PF-8, we intentionally left DTT out of the cross-linking reactions. Minor aggregation that occurs in the absence of DTT causes trimers and tetramers of purified PF-8 to form and cross-link. The quality of the recombinant purified PF-8, cross-linked in the absence of DTT, was visualized by SDS-PAGE prior to native gel electrophoresis (Fig. 2D, left panel, compare lanes 2 and 3). When examined by BN-PAGE (Fig. 2D, right panel), purified PF-8 resolved as two major species (lanes 6 and 7) that migrated to positions closely corresponding to the PF-8 monomer and cross-linked dimer marker proteins (lanes 4 and 5). Conformational changes due to cross-linking likely account for why, on this native gel, the cross-linked PF-8 marker proteins (lanes 4 and 5) migrate slightly faster than the non-cross-linked forms of PF-8 (lanes 6 and 7). Because gel filtration-purified PF-8 dimer was applied to BN-PAGE, we were surprised to observe monomer protein (lanes 6 and 7). This could reflect the kinetics of the dimer-monomer equilibrium of PF-8 under these experimental conditions. Nevertheless, BN-PAGE, gel filtration chromatography, and cross-linking all concur that recombinant PF-8 is capable of forming dimers in solution.

Free Dimers of PF-8 Occur in KSHV-infected Cells—The above in vitro studies made it essential to ask whether PF-8 exists naturally as dimers in KSHV-infected cells. Bcbl-1 cells, which harbor latent KSHV, were treated with TPA to induce lytic infection. Nuclear extracts from uninduced and induced cells were cross-linked, fractionated by SDS-PAGE, and analyzed by Western blot using anti-PF-8 antibody as probe (Fig. 3). Again, the cross-linking reaction containing purified PF-8 from E. coli served as a size marker for monomer and dimer (lane 1). As expected, no PF-8 was observed in uninduced cells, regardless of whether the nuclear extract was cross-linked (Fig. 3, lanes 2 and 4). Importantly, PF-8 from cross-linked nuclear extracts from induced cells formed a band that co-migrated with PF-8 dimers from purified PF-8 (compare lanes 1, 3, and 5). Interestingly, no other cross-linked bands that are indicative of interactions of PF-8 with other proteins, especially Pol-8, were revealed (lane 5). It may be that the cross-linking buffer is not favorable for Pol-8/PF-8 binding or that the quantity of Pol-8 is far less than that of PF-8. Regardless, what is clear from this result is that PF-8 produced in KSHV-infected cells naturally forms free dimers.

Recombinant PF-8 Binds DNA as a Dimer—The preceding experiments clearly established that PF-8 exists in solution as dimers and raised the question of whether PF-8 associates with DNA as dimers. To address this possibility, we utilized our GST-PF-8 fusion protein with a thrombin site that cleaves between the GST and PF-8 moieties and applied the strategy presented in Fig. 4A. Essentially, concentration-controlled digestion of GST-PF-8 by thrombin protease is expected to yield three different types of dimers: uncleaved homodimers of GST-PF-8; fully cleaved homodimers of PF-8; and partially cleaved heterodimers of GST-PF-8 and PF-8. If these dimers are able to associate with DNA, they should band-shift to three different positions in a polyacrylamide gel.

Fig. 4B shows undigested GST-PF-8 (lane 1) followed by partial digestion of GST-PF-8 with increasing concentrations of thrombin (lanes 2–4) and complete thrombin digestion of GST-PF-8 (lane 5). These undigested and thrombin-cleaved GST-PF-8 products were then mixed with a radioactively labeled 18-bp oligonucleotide, and the protein-DNA complexes were analyzed by EMSA. As shown in Fig. 4C, the band in lane 5 with the fastest mobility represents the PF-8 DNA complex derived from complete cleavage of fusion protein. It is noted that the cleaved GST tag is not capable of binding to DNA (data not shown). The band in lane 1 (Fig. 4C) with the slowest mobility represents the uncleaved GST-PF-8 DNA complex. In lanes 2–4 (Fig. 4C), three bands were seen. The only way to account for the new band of intermediate mobility is that it represents a complex containing DNA and a heterodimer of PF-8 and GST-PF-8. Thus, the fastest and slowest migrating complexes must represent homodimers of PF-8 and GST-PF-8, respectively. Very notably, the intensity of the PF-8 DNA complex is much greater than that of GST-PF-8 DNA complex (compare Fig. 4C, lanes 1 and 5). Steric hindrance by the GST tag and potential weak GST-GST interactions likely account
for why the GST-PF-8 homodimer binds to dsDNA much less strongly than the GST-PF-8/PF-8 heterodimer and PF-8 homodimer. Clearly, both the GST-PF-8/PF-8 heterodimer and the PF-8 homodimer DNA complexes result solely from the dimerization of PF-8 and not GST. It is important to note that the shortest length of DNA to which PF-8 is able to bind is 14 bp (data not shown). However, the slightly longer 18-bp DNA that still allows the binding of only one dimer of PF-8 over a broad concentration ratio of protein:DNA was used to ensure greater binding stability of the protein-DNA complex in this experiment (Fig. 4C).

PF-8 from KSHV-infected Cells Binds DNA as Dimers—We next examined whether PF-8 also binds to DNA as dimers in vivo by performing EMSA with nuclear extracts from KSHV-infected Bebl-1 cells using the labeled 18-bp oligonucleotide described above. Lytic infection in Bebl-1 cells, which harbor KSHV, is induced by TPA. As shown in Fig. 5A, a single protein-DNA band shift complex was formed with nuclear extracts from induced cells (lanes 4–6), whereas no band shift was formed using extracts from uninduced cells (lane 3). Significantly, this protein DNA complex formed using lytically infected cell extracts migrated to the same position in the EMSA gel as the PF-8 dimer protein-DNA complex formed using purified PF-8 protein from E. coli (Fig. 5A, compare lanes 1 and 2 with lanes 4–6). A slower migrating band representing the Pol-8/PF-8 DNA complex was not seen, likely because the 18-bp DNA is not long enough to bind both proteins. To demonstrate definitively that PF-8 is actually present in the band shift obtained with the lytically infected cell extract, an EMSA of induced and uninduced cell nuclear extracts performed with unlabeled 18-bp DNA was transferred to a membrane and probed with anti-PF-8 antibody. As revealed in Fig. 5B, the

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Fig. 4. Recombinant PF-8 binds to DNA as a dimer. A, schematic of limited thrombin digestion of GST-PF-8. The products of the partial digestion are GST-PF-8 homodimers, PF-8 homodimers, and GST-PF-8/PF-8 heterodimers. B, an SDS-PAGE analysis of thrombin digestion of GST-PF-8. GST-PF-8 was digested with 0, 0.5 × 10^−3, 0.5 × 10^−2, 0.5 × 10^−1, and 0.5 units/μl thrombin, lanes 1–5, respectively. The digestion was for 1 h at 25 °C followed by SDS-PAGE and Coomassie staining. For convenience, the GST tag is not shown. C, EMSA. DNA band shifts containing uncleaved, partial, and complete thrombin-cleaved GST-PF-8. The series of proteins from lanes 1–5 above (in B) were incubated with a labeled 18-bp DNA probe, and the protein-DNA complexes were resolved by electrophoresis on a 6% polyacrylamide gel and analyzed by autoradiography. Free probe, in excess, is not shown.
PF-8 antibody recognized a PF-8 protein-DNA complex when extracts from induced but not uninduced cells were used (Fig. 5B, compare lanes 2 and 4). The inability of the PF-8 antibody to recognize any complex in the absence of DNA (Fig. 5B, lane 3) confirmed that the PF-8 detected by EMSA (Fig. 5B, lane 4) was associated with the 18-bp oligonucleotide. The failure of the PF-8 antibody to detect PF-8 that was not bound to DNA (Fig. 5B, lane 3) is consistent with the fact that PF-8 has a pI of 8.9 and cannot enter the gel (pH 8.0) unless it is associated with negatively charged DNA. Taken together, these results clearly demonstrate that PF-8 from KSHV-infected cells binds to DNA as dimers.

PF-8 Domains Important for Dimerization in Solution Are Also Needed for Binding to DNA—Deletion mutants of PF-8 (Fig. 6A) were generated to gain insight into the regions of PF-8 required for dimerization in solution. The deletion mutants and full-length PF-8 were labeled by in vitro translation and were either untreated or treated with BS3 cross-linking reagent and examined by SDS-PAGE. As seen in Fig. 6B, the non-cross-linked deletion mutants were of the expected monomeric size relative to full-length PF-8 monomers (lanes 1, 3, 5, 7, and 9). Interestingly, cross-linking revealed that as many as 92 amino acids removed from the extreme C terminus of PF-8 did not affect dimerization, as demonstrated by the ability of dl371–396 and dl305–396 to form bands of twice the molecular weight as their respective monomers (compare lanes 2, 4, and 6). However, as observed with dl277–396, further deletion of 28 residues (or a total of 120 amino acids) from the C terminus completely abolished the ability of PF-8 to dimerize (lane 8). The N terminus appeared more sensitive to mutation since deletion of only 21 amino acids from the extreme N terminus completely eliminated the ability of PF-8 to form dimers (lane 10). It is important to note that when full-length PF-8 was co-translated with each of the deletion mutants, cross-linked heterodimers of full-length PF-8 and dl277–396 or dl1–21

**Fig. 5.** PF-8 from KSHV-infected cells binds to DNA as a dimer. A, EMSA was performed by incubating a 32P-labeled 18-bp DNA with purified PF-8 from E. coli (E) lane 1, 25 ng, and lane 2, 250 ng) or from extracts of Bcbl-1 cells that were uninduced (U) (lane 3, 1:20 diluted) or induced (I) by TPA to produce lytic infection (lanes 4–6, 1:80, 1:40, 1:20 diluted, respectively). B, EMSA was performed by incubating unlabeled 18-bp DNA with extracts from Bcbl-1 cells that were either uninduced (U, lane 2) or induced (I) by TPA (lanes 4) to produce lytic infection. As controls, DNA was omitted from uninduced (lanes 1) and induced (lane 3) extracts. The samples were loaded onto a native 6% polyacrylamide gel in Tris borate-EDTA buffer (pH 8.0). The gel was transferred to a membrane and probed with anti-PF-8 antibody.

**Fig. 6.** Domains of PF-8 required for dimer formation. A, PF-8 deletion mutant constructs. Numbers correspond to the amino acids in the full-length (FL) PF-8. B, dimerization analysis of PF-8 deletion mutants by cross-linking. The PF-8 proteins were labeled by in vitro translation and were either untreated or cross-linked (+) (lanes 2, 4, 6, 8, and 10). The proteins were analyzed by SDS-PAGE. Indicated on the left are molecular size markers, and indicated on the right are the different monomeric and dimeric forms of full-length and mutant PF-8 proteins.
failed to form; however, as expected, heterodimers of full-length PF-8 and dl371–396 or dl305–396 were formed (data not shown). This indicates that residues 277–304 as well as the N-terminal 21 residues are essential for PF-8 to form dimers in solution.

We then asked how the same deletions in PF-8 would affect DNA binding. As shown in Fig. 7A, PF-8 bound to dsDNA cellulose, where it eluted at 250 mM NaCl (right panel), but failed to bind to cellulose (left panel). When the PF-8 deletion mutants (Fig. 7B) were similarly tested, the two mutants (dl371–396 and dl305–396) that retained the ability to form dimers in solution proved to be as capable of binding DNA as full-length PF-8, i.e. they each eluted at 250 mM NaCl (Fig. 7B, left panel). Interestingly, the two PF-8 deletion mutants (dl277–396 and dl1–21) that were incapable of forming dimers in solution, essentially, completely failed to bind DNA (Fig. 7B, right panel). Although the deletions produced in dl277–396 and dl1–21 may have eliminated potential nonspecific DNA recognition motifs, the results strongly suggest that PF-8 needs to dimerize to associate with DNA.

PF-8 Stabilizes Pol-8 on DNA—Relevant to its function in processive DNA synthesis is whether PF-8 stabilizes Pol-8 on P-T DNA. We prepared a 5'-biotinylated single-stranded DNA template of 250 bases in length and coupled this template to streptavidin-coated beads. A primer oligonucleotide of 20 bases was then annealed to the 3'-end of the template as depicted in Fig. 8A. For each binding assay, [35S]methionine-labeled Pol-8 alone or together with labeled full-length or mutant PF-8 was incubated with P-T. The bound proteins were eluted stepwise with increasing concentrations of salt and analyzed by SDS-PAGE. As shown in Fig. 8B, when Pol-8 alone was analyzed, the majority of the protein eluted at 100 mM NaCl with only a residual amount eluting at 250 mM NaCl (compare lanes 1–3). By contrast, in the presence of PF-8, the bulk of Pol-8 eluted at 250 mM NaCl with residual amounts eluting at 100 and 500 mM NaCl (lanes 4–6). Importantly, PF-8 co-eluted with Pol-8 from P-T (lanes 4–6). Apparently, this increase in the DNA binding stability of Pol-8 is attributable to PF-8 alone, as opposed to a synergistic affect of both proteins, since 250 mM NaCl is also the same salt concentration that effectively eluted Pol-8 alone from the DNA cellulose column (Fig. 7A).

The PF-8 mutants were then analyzed for their abilities to stabilize the interaction of Pol-8 to P-T DNA. The PF-8 mutants dl371–396 and dl305–396, which are able to dimerize in solution and bind DNA cellulose (Fig. 6B and 7B), were also able to stabilize binding of Pol-8 to P-T, albeit dl305–396 was not as efficient as full-length PF-8 or dl371–396 (Fig. 8B, compare lanes 7–12 with lanes 1–6). Again, Pol-8 co-eluted with these PF-8 deletion mutants at 250 mM NaCl. Not surprisingly, the PF-8 deletion mutants dl277–396 and dl1–21, which are defective both in forming dimers in solution and in binding DNA (Figs. 6B and 7B), had almost no effect on Pol-8 binding to P-T (Fig. 8B, compare lanes 13–18 with lanes 1–3).

We previously demonstrated that PF-8 binds to Pol-8 independently of DNA (19). We thus inquired whether PF-8 mutants that stabilize Pol-8 on P-T DNA also retain their abilities to bind Pol-8. A Pol-8/PF-8 binding assay was performed by co-translating both proteins followed by immunoprecipitation with anti-PF-8 antibody. As shown in Fig. 8C, full-length PF-8 and the two dimerization-capable PF-8 deletion mutants
(dl371–396 and dl305–396) that stabilize Pol-8 to P-T were still capable of binding Pol-8 in solution (lanes 7–9). On the other hand, the two dimerization-defective PF-8 deletion mutants (dl277–396 and dl1–21) that fail to stabilize Pol-8 to P-T were largely incapable of binding Pol-8 (lanes 10 and 11). Interestingly, in the absence of a refined mutational analysis, the PF-8 mutants presented here suggest that the same domains of PF-8 that are important for binding DNA are also important for binding Pol-8. These results clearly demonstrate that PF-8 enhances the stability of Pol-8 on P-T DNA and indicate that to achieve this stability, PF-8 must be able to dimerize.

Dimers of Full-length PF-8 Are Required to Support Efficient Processivity—The fact that two of the PF-8 mutants (dl371–396 and dl305–396) retained their abilities to bind as dimers on DNA and stabilize Pol-8 on P-T raised the question of whether these C-terminal truncation mutants could also support pro-
cessivity. It was significant to resolve this distinction since, in essence, the PF-8 dimer-Pol-8 interaction on P-T (Fig. 8) reflects the complex in a static state, whereas processivity is a kinetic process that depends on PF-8 to keep Pol-8 associated with the template strand during DNA synthesis.

Each of the mutant PF-8 proteins was tested for its potential to enable Pol-8 to completely synthesize the entire 7,249-nt DNA on M13 primed template. As shown in Fig. 9A, Pol-8 alone (lane 1) or any of the PF-8 proteins alone (lanes 2–6) was unable to synthesize detectable DNA products. However, complete processive DNA synthesis of M13 DNA was achieved when Pol-8 was combined with full-length PF-8 (lane 7). It is noted that an abundance of smaller products of varying lengths (around 80 nt) are typically observed with full-length PF-8 and Pol-8 (lane 7). Not surprisingly, the PF-8 mutants dl277–396 and dl1–21 that fail to dimerize or bind to DNA or to stabilize Pol-8 were completely defective in providing processivity to Pol-8 (lanes 10 and 11). Interestingly, dl371–396 and dl305–396, which can self-dimerize and bind to both DNA and Pol-8, synthesized only very short DNA products (< 100 nt) from the primed M13 template in the presence of Pol-8 (lanes 8 and 9). To confirm that dl371–396 and dl305–396 truly retain some processive function, all of the PF-8 mutant proteins were examined for DNA synthesis with and without Pol-8 using a 5’-32P-labeled 25-nt primer annealed to a 250-nt-long template followed by sequencing gel electrophoresis. As shown in Fig. 9B, the synthesis of DNA in this short-product assay revealed that only full-length PF-8 and dl371–396 and dl305–396 were capable of processivity in the presence of Pol-8. Thus, in the absence of its extreme C terminus, PF-8 is capable of limited processivity. This indicates that in some manner, the C terminus of PF-8, either structurally or functionally, is needed to impart robust processivity to Pol-8.

The Processivity Factor of CMV but Not HSV-1 Forms Dimers upon Cross-linking—Unlike PF-8, the highly studied UL42 processivity factor of HSV-1 exists as monomers (10, 20, 21). As shown in Fig. 2C, in vitro translated UL42 remained as monomer following cross-linking (lanes 1 and 2) as compared with dimers that were generated with in vitro translated PF-8 (lanes 3 and 4). Of note, the slower migrating bands of lesser intensity that are present in both the UL42 cross-linked and non-cross-linked lanes of Fig. 2C, are the products of translational read-through of non-UL42 sequences resulting from incomplete HindIII digestion of plasmid pINGUL42 (29). Consistent with these results, a purified fusion protein of UL42 also remained monomeric after cross-linking (data not shown). By contrast, as shown in Fig. 2B, purified UL44 of CMV was capable of forming dimers after cross-linking (lanes 4 and 5), as was purified PF-8 (lanes 1 and 2). These results demonstrate that the processivity factors of the herpesvirus are not exclusively monomeric or polymeric.
PF-8 N terminus destroyed processivity. Our study strongly argues that PF-8 dimerization is a prerequisite for enabling Pol-8 to synthesize long strands of DNA needed for replication. Our results further suggest that in order for PF-8 to fulfill this processivity function, it must homodimerize to bind DNA and Pol-8 and thereby stabilize or tether Pol-8 on the template.

Interestingly, although PF-8, like many processivity factors, is a phosphoprotein (31), our study indicates that dimerization is not dependent on phosphorylation. This is based on the fact that our purified recombinant PF-8 was expressed in E. coli and was able to form dimers in solution when measured by a variety of techniques, including gel filtration, cross-linking, and native gel electrophoresis. Moreover, purified PF-8 derived from bacterial expression was also able to dimerize on template DNA. Although purified PF-8 was able to function efficiently as a processivity factor, we cannot rule out the possibility that PF-8 becomes post-translationally modified when it is combined with the in vitro translation reaction from which Pol-8 was synthesized. It is of note that UL42 of HSV-1 can be phosphorylated by cdcl2 kinase (32) and pUL44 of CMV can be phosphorylated by viral pUL97 (33), but the significance of these modifications to function is not clear. Based on the PCNA paradigm, PF-8 has the potential to interact with a number of cellular proteins as well as other viral replication proteins (34), in which case phosphorylation may prove to be especially significant (35).

PF-8 is the first herpesvirus processivity factor demonstrated to exist in solution as a dimer and on the DNA. It needs to be determined whether dimers of PF-8 encircle template DNA and how PF-8, Pol-8, and DNA template interact, given the mechanism by which PF-8 provides Pol-8 with the ability to bind DNA, stabilize and interact with Pol-8 on primer-template DNA, and function in processive DNA synthesis.

**DISCUSSION**

We originally demonstrated that Pol-8 of KSHV alone incorporates only a few nucleotides and is completely dependent on its processivity factor, PF-8, to synthesize DNA strands that are thousands of nucleotides in length (19). Key to understanding the mechanism by which PF-8 provides Pol-8 with the processivity required to replicate its DNA is knowing whether PF-8 functions as a monomer or a multimer and how this processivity factor contributes to the Pol-DNA interaction. In this study, we demonstrate that PF-8 forms homodimers both in solution and on the DNA and that the shortest length of DNA required to bind the PF-8 dimers is 14 bp. PF-8 dimers were observed in vitro, using purified recombinant PF-8, as well as in vivo, using KSHV-infected cell extracts. A mutational analysis revealed that the N terminus (residues 1–21) and an internal segment (residues 276–304) of PF-8 (396 residues) are required for dimerization. Importantly, PF-8 mutants that were incapable of forming dimers were also incapable of binding DNA, binding Pol-8, and providing stability and processivity to Pol-8 on primer template. A summary of these findings is presented in Fig. 10.

Significantly, C-terminal deletion mutants (dl305–396 and dl371–396) that eliminated up to one-fourth of PF-8 retained the ability to form dimers and bind DNA and Pol-8 and stabilize Pol-8 on primer template to the same extent as full-length PF-8. However, these same mutants were diminished in their capacity to provide efficient processivity to Pol-8 since only short DNA products were synthesized (less than 100 nt) but not long products corresponding to full-length M13 template (7,249 nt). Predictably, these C-terminal deletion mutants (dl305–396 and dl371–396) should be incapable of providing Pol-8 with processivity required to synthesize the 142-kb KSHV genome (30). This suggests that C-terminal regions of PF-8 that are not involved in dimerization contribute to efficient processivity, perhaps by lending the stability needed for movement of the PF-8/Pol-8 complex on the DNA. Recently, Chan and Chandran (31) concluded that C-terminal mutants of PF-8 that eliminate up to one-fourth of the protein are still capable of supplying processivity to PF-8. However, their results were solely based on examining short homopolymeric templates (246-base average length) rather than long-strand templates (e.g. the classic M13 DNA template) that measures effective processivity. By contrast to its C terminus, deletion of just 21 amino acids from the extreme N terminus disabled every property of PF-8 examined, i.e. homodimerization, binding to DNA and Pol-8, and stabilizing Pol-8 on primer template and processivity. In agreement, Chan and Chandran (31) also showed that deletion of the extreme N terminus disabled every property of PF-8 examined, i.e. homodimerization, binding to DNA and Pol-8, and stabilizing Pol-8 on primer template and processivity.
It is becoming increasingly apparent that most, if not all, DNA polymerases rely strictly on their cognate processivity factors to execute long-strand DNA synthesis, making them attractive drug targets to block viral infection and disease (36). The requirement of PF-8 to form dimers to function may aid in the design of therapeutic agents to prevent Kaposi’s sarcoma.

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