Structural Analyses of gp45 Sliding Clamp Interactions during Assembly of the Bacteriophage T4 DNA Polymerase Holoenzyme

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The phage T4 gp45 sliding clamp is a ring-shaped replication accessory protein that is mounted onto DNA in an ATP-dependent manner by the gp44/62 clamp loader. In the preceding paper (Pietroni, P., Young, M. C., Latham, G. J., and von Hippel, P. H. (1997) J. Biol. Chem. 272, 31666–31676), two gp45 mutants were exploited to probe interactions of the sliding clamp ring with the gp44/62 loading machinery at various steps during the clamp loading process. In this report, these studies are extended to examine the polarity of the binding interaction between gp45 and gp44/62. Three different gp45 mutants containing a single cysteine in three topographically distinct positions were used. Several different reporter groups, including extrinsic fluorophores, a photo-cross-linker, and a biotin linker for use in a novel “streptavidin interference assay,” were covalently attached to these cysteine residues. Since gp45 is a trimeric protein, these three different mutations allowed us to probe up to nine distinct local environments along the surface of the sliding clamp in the presence and absence of other replication proteins. The results show that the gp44/62-ATP clamp loader complex binds exclusively to the C-terminal (S19C) face of the gp45 ring.

DNA replication is an enormously complex process that requires the coordination of a number of specialized enzymatic activities involving many different proteins (1). These proteins include: (i) the replication helicase, which unwinds the duplex DNA to create single-stranded regions to serve as templates for DNA synthesis; (ii) the single-stranded binding protein, which protects the exposed single strands of DNA from nucleases and acts to stabilize the replication machinery; (iii) the primase, which synthesizes oligoribonucleotide primers that nucleate DNA polymerization on the leading and lagging strands; (iv) the DNA polymerase, which extends the RNA primers continuously against the leading strand template and discontinuously against the lagging strand template by means of a deoxyribonucleotide transferase mechanism; (v) the trimeric ring-shaped sliding clamp complex, which binds to the DNA polymerase to stabilize protein-DNA interactions and promotes highly processive DNA synthesis; and (vi) the clamp loader accessory proteins subassembly, which uses ATP hydrolysis to drive the loading of the sliding clamp onto the DNA (2–4). Recent structural studies have demonstrated that hexameric helicases and sliding clamp proteins from several different organisms adopt a ring-like structure (5–8), raising a number of questions regarding the relation of form and function. Why is a ring-shaped structure necessary for the function(s) of these proteins? What is the structural basis of the interactions of protein rings with other macromolecules? Are opposite sides of the protein ring functionally equivalent?

In the case of the sliding clamp proteins, their toroidal structure is generally believed to be essential for proper function. Prior to the determination of the first high resolution structure of a sliding clamp (the β dimer of Escherichia coli), considerable biochemical evidence existed to support the notion that the β-clamp must be topologically associated with the DNA (9). Thus, it was particularly gratifying to learn from crystallographic studies that the β-clamp is shaped like a doughnut, with a central hole of sufficient diameter to accommodate double-stranded DNA (7). Although the dimeric β-clamp has no meaningful sequence homology with the trimeric sliding clamp proteins (proliferating cell nuclear antigen) of eukaryotes that have been investigated, nor with the trimeric phage T4 gp451 clamp protein, structural studies have now revealed that these proteins, like β, exist as protein rings (8).2 This result is consistent with the fact that β, proliferating cell nuclear antigen, and gp45 have nearly identical functions. All three proteins are essential for highly processive DNA replication, can slide one-dimensionally on double-stranded DNA, and are lost to solution if the DNA is linearized. Significantly, gp45 is also known to act as a mobile enhancer (i.e. a DNA-tracking protein) in late transcription in phage T4 by stimulating the RNA polymerase machinery (11, 12).

From a structural perspective, one of the more interesting questions in DNA replication is how the sliding clamp is loaded onto the template-primer DNA at the replication fork. In the phage T4 system, gp45 loading is an ATP-dependent event that requires the action of the gp44/62 accessory proteins complex (13). Both gp45 and, separately, template-primer DNA, stimulate the rate of ATP hydrolysis of the gp44/62 clamp loader (13, 14). This stimulation is synergistically activated when both

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1 The abbreviations used are: gp45, T4 gene 45 protein; gp44/62, a 4:1 complex of the T4 gene 44 protein and gene 62 protein; gp43, T4 gene 43 protein; IA, S-[(2-iodoacetamido)ethyl]amino-ethyl]amino-naphthalene-1-sulfonic acid; TFPAM-3 or T3, N-[1-pyrene]maleimide; FM, N-[4-azido-2,3,5,6-tetrafluorobenzyl]-3-maleimidopropionamide; SA, streptavidin; DTT, dithiothreitol; Biotin-BMCC, 1-biotinamido-4-[4-['maleimidomethyl]-cyclohexane-carboxamido]-butane.

2 I. Moarefi and J. Kuriyan, manuscript in preparation.
gp45 and template-primer DNA are combined with gp44/62-ATP (13, 14). Biochemical studies indicate that one gp44/62 molecule can act catalytically to load many gp45 molecules onto DNA (15, 16), with the release of ADP from gp44/62 probably representing the rate-limiting step in the reaction (14, 17). In the preceding paper (18) we have used photochemical cross-linking experiments with specific mutant cysteine residues of gp45 that had been covalently labeled with a photochemical cross-linker to probe specific interactions of the gp45 trimer ring with the gp44/62 clamp loading machinery at various stages of the ATP-driven loading process. These experiments have permitted us to begin to build some topographical details into the clamp loading reaction pathway that we (14, 17) and others (16, 19) had previously tried to define largely from a kinetic perspective. In this paper, we build on these results to further examine the interaction polarity and relative positioning of the two accessory proteins subassemblies during the clamp loading process.

In an attempt to better understand how the gp45 DNA sliding clamp interacts with other DNA replication proteins and DNA itself, we previously described the creation of a gp45 mutant that contains a single cysteine residue (gp45-S19C) (17). This mutant protein was labeled at the S19C position with an extrinsic fluorophore, which in turn reported ATP-dependent environmental changes when gp45-S19C was bound to the gp44/62-ATP complex to form the gp45-gp44/62-ATP loading complex. Significantly, we discovered that the fluorescence of the 5-(1-pyrene)maleimide (PM), and N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide (TFPAM or T3) were purchased from Molecular Probes (Eugene, OR). Biotin-BMCC was obtained from Pierce. Streptavidin was purchased from Fisher.

Preparation of Proteins—Site-directed mutagenesis of the pOP45 plasmid containing the gp45 gene was accomplished by polymerase chain reaction amplification of the plasmid using primers containing an altered sequence in the region of interest, as described previously (17). For each protein, the desired change in the gp45 coding sequence was determined by Bradford assay (using wild-type gp45 as the protein standard), and the stoichiometry of PM covalently attached to gp45 was determined spectrophotometrically. For each protein, the desired change in the gp45 coding sequence was accomplished by polymerase chain reaction amplification of the plasmid using primers containing an altered sequence in the region of interest, as described previously (17).

Fluorescent Modification of Mutant gp45 Proteins—Both the IA-gp45-S19C and IA-gp45-S45C proteins were prepared as described previously (14). Briefly, the gp45 mutants were first passed through a Bio-Spin 30 column (Bio-Rad) to remove DTT from the storage buffer and then equilibrated in 50 mM sodium phosphate (pH 7.0) with 15% glycerol. An ~5-fold excess of IA (in 50 mM sodium phosphate (pH 7.0)) over the concentration of gp45 sulfhydryl groups was added, and the reaction was incubated at 4 °C overnight. The next day, the unreacted IA was quenched with a vast excess of 2-mercaptoethanol, and the free label was separated from the gp45-bound label by applying the sample to a second Bio-Spin 30 column. The extent of protein labeling by the IA fluorophore was determined spectrophotometrically, after a slight correction for the IA absorbance at 280 nm, and the labeled proteins were diluted into gp45 storage buffer (20 mM HEPES (pH 7.5), 50 mM potassium acetate, 0.2 mM EDTA, 0.5 mM DTT, 50% glycerol), resulting in a final glycerol concentration of 30% in the sample. Cysteine modification of the gp45 proteins by PM was accomplished in a similar manner, except that the gp45 concentration was determined by Bradford assay (using wild-type gp45 as the protein standard), and the stoichiometry of PM covalently attached to gp45 was determined spectrophotometrically.

T3 and Biotin-BMCC Labeling of Mutant gp45 Proteins—A 5–6-fold excess of either T3 (in Me2SO) or biotin-BMCC (in Me2SO) was added to ~50 μg gp45 (trimer concentration) in buffer containing 50 mM sodium phosphate (pH 7.0) and 15% glycerol. The reaction was incubated at room temperature (~22 °C) for 1 h and then quenched with a final concentration of 10 mM cysteine for 20 min. The unreacted label in each case was removed by applying the sample to a Bio-Spin 30 column. The final glycerol concentration of the T3- and biotin-BMCC-modified gp45 proteins was adjusted to ~30%, and the stocks were stored at ~20 °C until needed.

To confirm that the gp45 sulfhydryl sites had been modified by the T3 and biotin-BMCC groups, an Ellman assay (20) was performed to measure the concentration of free sulfhydryls in each of the mutant proteins. This assay showed that the unmodified gp45 proteins contained the expected number of free sulfhydryls per trimer. However, the T3- and biotin-BMCC-labeled gp45 showed no evidence of free sulfhydryl groups, indicating that the three sulfhydryl sites of the gp45 trimer had been quantitatively modified. A gel mobility shift assay was performed with the biotin-BMCC-derivatized gp45 mutants to confirm that streptavidin (SA) could bind to the biotin tether on gp45. In the presence of SA, all three of the gp45 mutants (gp45-S19C-biotin, gp45-S45C-biotin, and gp45-K81C-biotin) were quantitatively shifted to a much slower mobility form on a 3.5% (native) polyacrylamide gel, consistent with complete covalent attachment of the biotin-BMCC groups to each of these mutation sites in the gp45 trimer.

ATPase Assays—The gp45-dependent stimulation of the gp44/62 ATPase activity was measured at 37 °C under the following conditions: 500 mM gp45 (as trimer), 1.2 μM gp44/62 (as a 4:1 complex), 600 μM ATP, 10 μCi of [γ-32P]ATP, 30 mM Tris acetate (pH 7.6), 160 mM potassium acetate, 6 mM magnesium acetate, 1 mM DTT, and 10% glycerol. Inorganic phosphate was separated from ATP by thin layer chromatography as described previously (13) and quantified on the Storm model 860 PhosphorImager (Molecular Dynamics). The steady-state rate of ATP turnover was calculated from the slope of the linear portion of the fitted kinetic curve.

Steady-state Fluorescence Measurements—The instrumentation and conditions used in the fluorescence experiments have been described previously (17). Briefly, fluorescence assays with IA-gp45 and PM-gp45 were conducted at 22 °C, using an excitation wavelength of 340 nm and an emission time of 0.5 s. Polarization studies were performed at 30 °C, and each data point was integrated over a time of 10 s. A

**EXPERIMENTAL PROCEDURES**

**Materials—**IA, N-(1-pyrene)maleimide (PM), and N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide (TFPAM or T3) were purchased from Molecular Probes (Eugene, OR). Biotin-BMCC was obtained from Pierce. Streptavidin was purchased from Fisher.
are marked by mutations in the gp45 protein structure of the S19C, K81C, and S45C amino acid positions. As a reference point for differentiating the two faces of the ring, the locations of the N terminus (M1) and C terminus (F227) are also shown. Although gp45 is a homotrimer, for clarity the amino acid changes are shown only within one monomer.

The three mutations allow us to probe up to nine distinct regions in the gp45 molecule (depending on the symmetry of the inter-monomer interface (Fig. 1). Since gp45 is a trimer in solution, this set of three mutations is sufficient to bathe the Eppendorf caps), and ~10% glycerol. Samples (20 µl) were added to the caps of sterile Eppendorf tubes that were taped down to the base of a shallow container. A small volume of room temperature water (sufficient to bathe the Eppendorf caps) was added to stabilize the temperature of the samples over the course of the cross-linking reaction. Cross-linking was initiated upon exposure to 366-nm UV light, supplied by a Blak-Ray lamp (model UVL-56, UVP, Inc.) placed 5 cm away from the sample. The proteins were irradiated for 40 min, after which time the cross-linked species were resolved by 10% SDS-polyacrylamide gel electrophoresis and stained with silver. (For further details of this photochemical cross-linking method see Ref. 18).

RESULTS

Rationale for the Creation of the gp45 Mutant Proteins—In an attempt to “map” local environments in the gp45 ring that are important for the binding of the gp44/62-ATP complex, we created and purified three mutants of gp45, each containing a single, defined cysteine residue per gp45 monomer (i.e. three cysteines per gp45 trimer ring). The residues of wild-type gp45 that were chosen for substitution with cysteine were selected on the basis of their reported positions within the gp45 crystal structure2 and are positioned on three different surfaces of the gp45 ring. The S45C residue lies on the same face as the N terminus; we refer to this side as the N-terminal face of the ring (Fig. 1). In contrast, the S19C residues lie on the opposite (C-terminal) face of the ring, near the hole that passes through the gp45 torus. The third mutation, K81C, is situated in the middle of the edge of the ring near the gp45 monomer-monomer interface (Fig. 1). Since gp45 is a trimer in solution, this set of three mutations allows us to probe up to nine distinct regions in the gp45 molecule (depending on the symmetry of the interaction of gp45 with other proteins). By placing specific reporter groups at each of these mutation sites, we sought to determine which regions on the surface of the gp45 ring are important for binding the gp44/62 clamp loader.

Fluorescently Labeled gp45-S19C, but Not Labeled gp45-S45C or gp45-K81C, Reports a Change in Environment upon Binding gp44/62-ATP—The three cysteine mutants were modified with extrinsic fluorophores to determine whether the environment in each position is altered on binding the gp44/62-ATP complex. Initially IA (see structure in Fig. 2) was used as a fluorescent probe, based on its success in previous experiments 17. Both the gp45-S19C and gp45-S45C proteins were efficiently labeled by IA; IA-gp45-S19C was found to carry 2.5 IA groups/trimer, whereas IA-gp45-S45C contained 2.2 IA fluorophores/trimer. When increasing amounts of gp44/62 were added to 83 nM IA-gp45-S19C containing 1 mM ATP, a linear increase in the emission maximum was observed until a 1:1 stoichiometry between gp44/62 and IA-gp45-S19C was reached (Fig. 3). This behavior is in excellent agreement with the previously characterized fluorescence properties of IA-gp45-S19C under these reaction conditions 17. In contrast, the IA-gp45-S45C protein failed to report any measurable increase in fluorescence, even when saturating amounts of gp44/62-ATP were added (Fig. 4). Similarly, modification of gp45-K81C by several different fluorophores failed to reveal any change in the fluorescence spectrum upon the addition of gp44/62-ATP 17.

It is of obvious importance to ensure that the fluorescently modified gp45 proteins retain the properties of the wild-type protein. Consequently, the binding of both IA-gp45-S19C and IA-gp45-S45C to gp44/62-ATP was analyzed using fluorescence polarization methods. Since fluorescence polarization is not dependent upon environmental changes (i.e. hydrophobic or hydrophilic changes) but relies instead on the ability of a fluorescent molecule to rotate, or depolarize, plane-polarized light, this technique can report binding between two macromolecules even if the steady-state fluorescence is unaffected by the binding interaction (as is the case for the IA-gp45-S45C). Thus, a change in the rate of rotational diffusion of the fluorescent protein on complex formation is all that is required to change the measured anisotropy of the fluorophore.

As Fig. 5 shows, the dissociation constants for the binding of IA-gp45-S19C to gp44/62-ATP (Kd (app) = 2 nM) and for the binding of IA-gp45-S45C to gp44/62-ATP (Kd (app) = 1 nM) are the same within the error of the measurement. Although these experiments were performed at gp45 concentrations (80 nM) well above the Kd, we note that these conditions are comparable with those used in the fluorescence intensity experiments.
shown in Figs. 3 and 4. Thus, it is clear that essentially all of the IA-gp45 is bound to gp44/62-ATP when both are present at 1:1 stoichiometries at these concentrations. As a result, the lack of fluorescence enhancement exhibited by the IA-gp45-S45C protein in the presence of gp44/62-ATP cannot be attributed to a lack of binding between the mutant clamp and the clamp loader. Consistent with this conclusion, we find that both IA-gp45-S19C and IA-gp45-S45C significantly stimulate the ATPase activity of gp44/62 in kinetic assays and, further, that the kinetics of ATPase stimulation by the labeled proteins is comparable with that induced by the wild-type (unmutated) gp45 protein (data not shown). Last, we find that the modified, mutated gp45 proteins also behave comparably with wild-type gp45 in stimulating DNA polymerase holoenzyme activity in replication assays (21).

To provide additional evidence that the S19C and S45C residues lie in very different protein environments in the accessory protein complex, we labeled gp45-S19C and gp45-S45C with PM (Fig. 2). PM has a large, multicyclic ring structure and, like IA, is known to be a sensitive indicator of polarity changes in the local environment (22). As Fig. 6 shows, the results were qualitatively the same with this fluorophore in that PM-gp45-S19C revealed a fluorescence enhancement upon the addition of gp44/62-ATP, whereas the PM-gp45-S45C fluorescence was completely unaffected by this addition. Anisotropy measurements confirmed that both PM-derivatized gp45 proteins bind to the gp44/62-ATP clamp loader (with values of $K_{d, app} \approx 10$ nM; data not shown). Thus, the failure to observe a fluorescence change in IA-gp45-S45C does not appear to be an anomaly of the IA fluorophore, since this negative result, and the positive result obtained with IA-gp45-S19C, could be reproduced when both of the gp45 trimers were labeled with PM.

**T3-gp45-S19C Cross-links to both gp44 and gp62, whereas T3-gp45-S45C Fails to Cross-link to either gp44 or gp62**—Cross-linking is an established technique for detecting protein-protein interactions. To determine whether the S19C and/or S45C positions on gp45 proteins bind to the gp44/62-ATP clamp loader (with values of $K_{d, app} < 10$ nM; data not shown). Thus, the failure to observe a fluorescence change in IA-gp45-S45C does not appear to be an anomaly of the IA fluorophore, since this negative result, and the positive result obtained with IA-gp45-S19C, could be reproduced when both of the gp45 trimers were labeled with PM.

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The intensity of these cross-linked species decreased when ATP was omitted (Fig. 7, lane 3). This result suggests that ATP is essential to promote optimal binding between gp45 and gp44/62, consistent with previous findings (17). In contrast, when T3-gp45-S45C was used in the cross-linking experiments, no protein-protein cross-links were observed, even when the concentrations of T3-gp45-S45C and gp44/62 were increased 3-fold (Fig. 7, lane 6).

A Streptavidin Interference Assay Reveals That gp44/62-ATP Binds to gp45 on the C-terminal (S19C) Face and Not to the Ring Edge or the N-terminal (S45C) Face—To further demonstrate that gp44/62-ATP binds to gp45 with a defined polarity, we developed a technique that we call the "streptavidin interference assay." If gp44/62-ATP binds primarily or exclusively to one face of the gp45 ring, a large portion of the available surface of the gp45 trimer should remain unobstructed. Consequently, if one could bind a very large molecule to this unoccupied surface, one would expect that binding would not be perturbed (assuming the blocking molecule is not so large that it encroaches on the actual binding site). Since fluorescence and cross-linking studies had suggested that the N-terminal (S45C) face of the gp45 ring might not be involved in gp44/62-ATP binding, we labeled this mutant (and the gp45-S19C and gp45-K81C mutants) with biotin-BMCC (Fig. 2). Biotin-BMCC features both biotin and maleimide groups, separated by a 32-Å spacer arm, which allows the biotin moiety to be conjugated to sulfhydryl groups. After the three gp45 mutants were labeled with this reagent, Ellman assays were performed to determine the extent of biotin modification. The results revealed that the biotin-BMCC labeling blocked 90% of the previously free sulfhydryl groups. Moreover, the addition of SA to all three biotin-derivatized gp45 trimers caused the proteins to move with a markedly reduced mobility on native gels, consistent with the formation of a SA-gp45 complex. It is not clear, however, whether more than one SA molecule binds to the available biotin adducts; although approximately three biotin groups are present on the trimer ring, more than one biotin may bind a single SA tetramer (which has binding sites for four biotin groups). Conversely, SA binding to gp45 may be sterically constrained so that only one biotin site can react per tetramer, in which case perhaps more than one SA tetramer could bind to each gp45 trimer.

The binding of gp45 to gp44/62-ATP in the streptavidin interference assay was monitored by measuring the kinetics of gp44/62 ATP hydrolysis in the presence of biotin-gp45, with and without SA. As illustrated in Fig. 8, the addition of SA decreased the ATPase rate stimulated by biotin-gp45-S19C by
more than 10-fold. Similarly, ATPase stimulation by the SA-bound biotin-gp45-S19C decreased nearly 10-fold compared with the biotin-gp45-K81C.3 Significantly, the ATPase rate stimulated by biotin-gp45-S45C was not affected by the binding of SA.

**DISCUSSION**

In this report, we describe our efforts to “map” the surface regions on the gp45 sliding clamp that are important for binding to the gp44/62 clamp loader. The recently determined three-dimensional crystal structure of gp45 has proved invaluable in guiding our selection of residues that lie in different regions of the protein. In this regard, it is important to note that gp45 monomers self-assemble into trimers in solution, and thus each amino acid change is represented in triplicate in the biologically relevant structure. As a result, efficient labeling of the three mutated residues (S19C, S45C, and K81C) by reporter groups has allowed us to characterize up to nine potential unique sites within the gp45 trimer.

Protein-protein interactions between gp45 and gp44/62-ATP were probed by three distinct methods. First, extrinsic fluorophores were covalently attached to the lone cysteine residue, the positions of which differ within the trimer ring for the three gp45 mutants, and the steady-state fluorescence intensity and fluorescence polarization properties of these labeled gp45 trimers were examined. Second, photo-cross-linking groups were bound to the cysteine residues, and the nature of the protein-protein cross-links was analyzed by SDS-polyacrylamide gel electrophoresis. Finally, a novel streptavidin interference assay was developed that permits the occlusion of large regions of the protein. In this regard, it is important to note that both of these cysteine residues should be solvent-exposed. Consistent with the prediction from the gp45 crystal structure, labels/trimer for gp45-S19C, and 2.2 IA/trimer for gp45-S45C), 62-ATP. Both proteins were efficiently modified by IA (2.5 IA

3 Although the biotin-gp45-K81C complex stimulates the gp44/62 ATPase more than does biotin-gp45-S19C or biotin-gp45-S45C, we note that the gp45-K81C complex also stimulates the gp44/62 ATPase better than wild-type gp45. It is unclear whether this hyperstimulation of ATPase activity reflects an unusual property of the gp45-K81C or simply batch-to-batch variation between protein preparations.

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**FIG. 7.** T3-gp45-S19C cross-links to both gp44 and gp62, whereas T3-gp45-S45C fails to cross-link to either protein. Cross-linking was initiated with 366-nm light and continued for 40 min at room temperature (see “Experimental Procedures”). Lane 1, 1.3 μM T3-gp45-S19C plus 1.3 μM gp44/62, no irradiation; lane 2, 1.3 μM T3-gp45-S19C plus 1.3 μM gp44/62 plus 1 mM ATP; lane 3, 1.3 μM T3-gp45-S19C plus 1.3 μM gp44/62; lane 4, 1.3 μM T3-gp45-S45C plus 1.3 μM gp44/62 plus 1 mM ATP; lane 5, 1.3 μM T3-gp45-S45C plus 1.3 μM gp44/62; lane 6, 3.9 μM T3-gp45-S45C plus 3.9 μM gp44/62 plus 1 mM ATP. Approximately equal amounts of total protein were loaded in each lane.

**FIG. 8.** SA tethered to biotin-gp45-S45C does not affect the ability of gp45 to stimulate the ATPase of gp44/62. The strategy behind the streptavidin interference assay is diagrammed at the top. The size and shape of the gp45 trimer and the SA tetramer are taken from the crystal structures of both proteins, and the relative dimensions of each are to scale. The ATPase activity of gp44/62 was measured under the following conditions: 800 nM biotin-gp45, 1.2 μM gp44/62, 600 μM ATP, 160 mM potassium acetate, 30 mM Tris acetate (pH 7.6), 6 mM magnesium acetate, and 1 mM DTT. In experiments containing SA, a final concentration of 3 μM was used. The ATPase rates shown represent the average of three separate experiments.

Fluorescence Methods for Determining Binding between the Clamp Loader and the Sliding Clamp—Extrinsic fluorophores such as IA and PM are known to be very sensitive indicators of local polarity change (22). Gp45-S19C and gp45-S45C were labeled with both of these reagents to determine the changes in fluorescence, if any, that might be observed upon adding gp44/62-ATP. Both proteins were efficiently modified by IA (2.5 IA labels/trimer for gp45-S19C, and 2.2 IA/trimer for gp45-S45C), consistent with the prediction from the gp45 crystal structure that both of these cysteine residues should be solvent-exposed. In agreement with previous work (17), IA-gp45-S19C reported a 20% increase in fluorescence and a slight blue shift in the emission maximum in the presence of saturating concentrations of gp44/62-ATP (Fig. 3). This result suggested that the environment of the IA label becomes more hydrophobic in the presence of gp44/62-ATP, as would be expected if S19C is at or near the binding site for the clamp loader. In contrast, IA-gp45-S45C failed to report any measurable change in fluorescence intensity upon the addition of gp44/62-ATP (Fig. 4). The results were essentially the same when a second fluorophore, PM, was
used to label the two proteins. Fluorescently labeled gp45-S45C also failed to show a fluorescence change in the presence of gp44/62-ATP, suggesting that the local environment of K81C, like that of S45C, is not perturbed in the clamp loading interaction.

To ensure that binding between IA-gp45-S19C and gp44/62-ATP and IA-gp45-S45C and gp44/62-ATP is identical, the anisotropy of both fluorescent gp45 molecules was measured in the presence of increasing concentrations of gp44/62-ATP. As shown in Fig. 5, the dissociation constants calculated from curve fits (−1 nM) were equivalent for both IA-gp45-S19C and IA-gp45-S45C. This result clearly shows that both IA-derivatized proteins were completely bound to gp44/62-ATP at the concentrations used in the fluorescence intensity assays. We note also that our determination of the gp45-gp44/62-ATP dissociation constant yielded a value that is in good agreement with the previously reported value (17), although the experimental set up was quite different.

**Photo-cross-linking Methods for Determining Binding between the Clamp Loader and the Sliding Clamp**—Although the fluorescence intensity assays suggested that the S45C and K81C positions are not near the gp44/62-ATP binding site, we employed a photo-cross-linking technique to test this hypothesis. Our cross-linker of choice was T3, which is 13 Å in length and contains both a maleimide group (for attachment to sulf-hydryl groups) and an azide moiety (which is converted to a highly reactive nitrile by irradiation with UV light). The results (Fig. 7) show that T3-gp45-S19C cross-links to both gp62 and gp44 in the presence and absence of ATP (see also Ref. 18).

In contrast to the cross-linking observed with T3-gp45-S19C, the T3-gp45-S45C mutant failed to show any evidence of cross-linking, even at elevated protein concentrations. As a result, although the T3 label was covalently linked to about three S45C residues per gp45 trimer, none of these cross-linkers was observed within 13 Å of either gp44 or gp62. We note that the Benkovic laboratory has also been unable to cross-link gp45 to gp44/62 when cross-linkers are positioned at the T7C residue of gp45, which lies on the same face as S45C. Neither T3-gp45-S19C nor T3-gp45-S45C formed intermolecular gp45 cross-links (data not shown), nor would any be expected, since interatomic distance measurements taken from the gp45 crystal structure indicate that the S19C and S45C residues are >13 Å removed from the closest amino acid residue in the neighboring gp45 monomer.

The results of T3-gp45-K81C cross-linking to gp44/62-ATP are described in the first paper in this series (18). In summary, photo-cross-linking with T3-gp45-K81C revealed protein-protein cross-links between T3-gp45-K81C and gp44 but no significant cross-links between T3-gp45-K81C and gp62 in the absence of ATP. Although T3-gp45-K81C did cross-link to gp62 in the presence of ATP, the cross-linking efficiency between these two proteins was poor compared with the efficiency of cross-links formed between T3-gp45-S19C and gp62. Since gp62 is required to observe gp45 (but not DNA) stimulation of the ATPase activity of the gp44/62 complex (23), gp62 clearly plays a pivotal role in coordinating interactions between the gp44/62 clamp loader and the gp45 sliding clamp. As a result, we argue that the lack of T3-gp45-K81C cross-linking to gp62 when ATP is absent can be interpreted to mean that K81C is situated within 13 Å of gp44, whose position in space is determined by the disposition of gp44/62 at the gp45 binding site. In contrast, when ATP is available and weak cross-linking between T3-labeled gp45-K81C and gp62 is observed, we propose that conformational changes within the accessory protein complex (18) bring K81C close enough to the actual gp62-gp45 binding site to permit cross-linking between the two protein subunits. In short, the cross-linking observed between T3-gp45-K81C and gp44 in the absence of ATP (and T3-gp45-K81C and gp62 in the presence of ATP) probably reflects the ability of the T3 cross-linker to “reach around and grab” the clamp loader bound at a site distal to K81C.

**Utility of the Streptavidin Interference Assay**—As an additional test for the hypothesis that the N-terminal (S45C) face of the gp45 does not provide a binding surface for gp44/62-ATP, we developed a streptavidin interference assay. The purpose of this assay was to tether SA to biotin-derivatized gp45 and monitor the formation of the accessory protein complex by measuring the gp45-dependent stimulation of the gp44/62 ATPase. Since SA is a tetrameric protein with dimensions ~50 × 50 × 40 Å and the face of the gp45 trimer is ~90 Å in diameter, even one SA tethered to one face of gp45 would occlude roughly half of the available surface of the ring. The presence of a molecular obstacle the size of SA, combined with the flexibility of the long (32 Å) biotin-BMCC linker, would almost certainly prohibit gp44/62-ATP from docking to gp45 on the SA-bound face if that face contained the binding site for the clamp loader. This assertion is supported by the results of the experiment (Fig. 8). SA tethered to the S19C and K81C positions reduced the stimulation of the ATPase of gp44/62 by the gp45 cofactor to roughly the unstimulated level (~0.3 μm/min under these conditions; Ref. 14). In contrast, SA tethered to S45C had no statistically significant effect on the stimulation of the ATPase activity of gp44/62 by this mutant.

Structural analyses based on an examination of physical models scaled to the dimensions of gp45, SA, and the biotin-BMCC linker offer the following insights. (i) SA tethered to S19C can block the C-terminal face and a sizable portion of the gp45 ring edge. (ii) SA linked to K81C can occlude the edge of the gp45 ring and a significant portion of both the C-terminal (S19C) and N-terminal (S45C) faces. (iii) SA tethered to S45C can block the N-terminal face and a significant portion of the edge of the gp45 ring. The fact that gp45-dependent stimulation of the gp44/62 ATPase is abolished when SA is linked to the K81C residue is consistent with the observation from model building that SA can swing around from the protein edge and block the binding of the clamp loader to the C-terminal face of gp45. In fact, S45C is actually closer to the edge of the protein ring than S19C. Consequently, it is difficult to imagine how the gp44/62-ATP subassembly could bind primarily to the edge of the gp45 ring if SA was bound to S45C. The only scenario that offers a satisfactory explanation for these results is that gp44/62-ATP binds primarily, if not exclusively, to the C-terminal (S19C) face of gp45. This conclusion is fully supported by the results of the fluorescence assays and photo-cross-linking studies discussed above.

**Fluorescence Evidence for Asymmetric Binding between the Clamp Loader and the Sliding Clamp**—Nearly all of the experiments described in this report have utilized gp45 molecules that were labeled efficiently by reporter groups (more than two labels/trimer). Thus, the absence of an effect, as reported by others discussed above.

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4 D. Sexton, personal communication.
To address this question, we repeated the fluorescence intensity experiment described in Fig. 3 with IA-gp45-S19C labeled with fewer IA groups per gp45 trimer. When gp45-S19C contained an average of 1.4 IA labels/trimer (instead of the 2.5 labels/trimer used above), the fluorescence measured in the presence of gp44/62-ATP was enhanced by only 9%. Thus, a decrease in labeling efficiency (from 2.5 to 1.4 labels/trimer) caused a decrease in the relative percentage of fluorescence enhancement from 20 to 9% (data not shown). This finding suggests that each IA label on S19C does not make an equal contribution to the fluorescence increase upon binding gp44/62-ATP and that the loading complex interacts asymmetrically with the gp45 trimer, perturbing only one or two of the three available Cys-19 labeled fluorophores. This result is in good agreement with our photo-cross-linking studies (18), which also suggest binding asymmetry between gp45 and gp44/62.

Possible Sites of Recognition on the Surface of the Sliding Clamp Protein—It is intriguing to speculate about where on the gp45 surface gp44/62-ATP might bind. Structurally speaking, the C-terminal face of gp45 differs from the N-terminal face in many ways, but one of the more obvious differences is that the C-terminal face has several protruding loops, or “handle” structures (see Fig. 1) that are absent on the N-terminal face, which is nearly planar by comparison. Since these “handle” regions are clustered near the three monomer-monomer interfaces of gp45, it is reasonable to suggest that gp44/62-ATP might dock onto gp45 using some of these sites and, through conformational changes in the accessory protein complex (18), disrupt the clamp interface(s) to open the ring. If this is true, it would also help to explain why the attachment of a bulky group such as PM to gp45 does not seem to compromise binding by gp44/62-ATP. Since the S19C residue does not extend into solution as far as do the “handle” structures, binding to these loop regions would also be consistent with the finding that Stern-Volmer plots failed to show any difference between the extent of acrylamide fluorescence quenching of the IA-gp45-S19C-gp44/62-ATP and IA-gp45-S45C-gp44/62-ATP accessory protein complexes (data not shown).

Conclusions—Elegant studies by O’Donnell and colleagues (10) have shown that the C terminus of the E. coli β-clamp is essential for binding the clamp loader and the core polymerase. Moreover, the crystal structure of the β protein ring (7) indicates that the C terminus lies on one face of the β ring, which is consistent with our results suggesting the gp44/62 clamp loader binds to the C-terminal (S19C) face of gp45.

We have also determined that the gp43 polymerase, like gp44/62, binds to the C-terminal face of gp45 (21). Taken together, these findings indicate that a polarized binding interaction between the clamp loader and the sliding clamp may represent another example of the conservation of function and form between analogous DNA replication proteins across the human, yeast, E. coli, and T4 DNA replication systems.

Acknowledgments—We are very grateful to Dr. J. Kuriyan and Dr. I. Moarefi for providing the protein coordinates for the gp45 trimer prior to publication. In addition, we thank Dr. F. Dong for the polymerase chain reaction-amplified gp45-S45C plasmid and Drs. M. Young and K. Latham for comments on the manuscript, and we thank all members of our laboratory for helpful and stimulating discussions.

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6 The O’Donnell laboratory (10) has also reported that kinase protection by the δ subunit of the γ complex clamp loader, or the σ subunit of the core polymerase, is not observed when a protein kinase recognition tag is placed at the N terminus of the β-clamp. Since the N terminus of β lies on the edge of the ring, this finding is also consistent with our finding that significant interactions between gp45 and gp44/62-ATP do not occur at the rim of the gp45 ring.