Identification and Mapping of Protein-Protein Interactions between gp32 and gp59 by Cross-linking*

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The bacteriophage T4 59 protein (gp59) plays a vital role in recombination and replication by promoting the assembly of the gene 41 helicase (gp41) onto DNA, thus enabling replication as well as strand exchange in recombination. Loading of the helicase onto gp32 (the T4 single strand binding protein)-coated single-stranded DNA requires gp59 to remove gp32 and replace it with gp41. Cross-linking studies between gp32 and gp59 reveal an interaction between Cys-166 of gp32 and Cys-42 of gp59. Since Cys-166 lies in the DNA binding core domain of gp32, this interaction may affect the association of gp32 with DNA. In the presence of gp32 or DNA, gp59 is capable of forming a multimer consisting of at least five gp59 subunits. Kinetics studies suggest that gp59 and gp41 exist in a one-to-one ratio, predicting that gp59 is capable of forming a hexamer (Raney, K. D., Carver, T. E., and Benkovic, S. J. (1996) J. Biol. Chem. 271, 14074–14081). The C-terminal A-domain of gp32 is needed for gp59-oligomer formation. Cross-linking has established that gp59 can interact with gp32-A (a truncated form of gp32 lacking the A-domain) but cannot form higher species. The results support a model in which gp59 binds to gp32 on a replication fork, destabilizing the gp32-single-stranded DNA interaction concomitant with the oligomerization of gp59 that results in a switching of gp41 for gp59 at the replication fork.

Assembly of the gp41 replicative helicase at the bacteriophage T4 DNA replication fork requires the displacement of single-strand DNA-binding proteins (gp32) coating the lagging strand. The helicase assembly protein, gp59, is required to effectively load the helicase under these conditions, thus assuming a critical role in bacteriophage T4 DNA replication as well as recombination (1–3). Mutations in gene 59 result in arrested DNA synthesis and reduced phage burst size (1), indicating that the gene product is essential for recombination-dependent DNA replication (which occurs in the late stages of Escherichia coli T4 infection). Since gp32 is also involved in early, origin-initiated DNA replication, it is likely that gp59 also facilitates replication in the early stages of T4 infection. Furthermore, gp59 mutants exhibit recombination deficiency, UV sensitivity, and sensitivity to chemical mutagens, suggesting a role of gp59 in recombination and recombination-mediated DNA repair (2, 3).

gp59 is a basic (pI = 10.18), 26-kDa protein (4, 5). Hydrodynamic studies indicate that it exists as a monomer in solution (4, 5). It has a high affinity for DNA and has been shown to bind duplex DNA, single-stranded DNA, and forked DNA substrates (4, 6). Moreover, Mueser et al. (7) demonstrate that gp59 binds with a higher affinity to a DNA fork than ssDNA. The crystal structure of gp59 reveals that it is composed predominantly of α-helices and contains two domains, a C- and an N-terminal domain. Models generated from the crystal structure of gp59 and its structural similarity to DNA-binding proteins of the high mobility group family propose that duplex DNA binds to the N-terminal domain, whereas ssDNA binds primarily to the C-terminal domain (7).

gp59 acts via direct contacts with gp41 and has been shown to interact with the helicase both on and off DNA (5, 8). gp59 increases the ATP-hydrolyzing activity of the helicase by accelerating the rate at which it is loaded onto DNA but may also lower the K_m of the enzyme for ATP (8). In addition to interacting with gp41, gp59 has also been shown to bind tightly to gp32 in both the presence and absence of DNA (5, 9, 10). Morrical et al. (11) demonstrate that the C-terminal, acidic domain (A-domain) of gp59 mediates the interaction between the two proteins. A truncated form in which the A-domain has been removed (gp32-A) results in the loss of interaction between gp32 and gp59 (11). Furthermore, gp59 contains distinct sites for gp32 and gp41 binding (8). As such, the gp59-mediated assembly of gp41 onto gp32-coated DNA most likely involves a ternary complex between the three proteins.

Although gp59 is a monomer in solution, evidence suggests that it may exist as an oligomer in a complex with gp41 and gp52. Raney et al. (12) demonstrate that the enhancement of the ATP-dependent DNA unwinding ability of gp41 is maximal when gp59 is present in a 1:1 molar ratio of gp59 monomer to gp41 monomer. Since the helicase is hexameric under these conditions, gp59 may also form a hexamer. In E. coli, the DnaB helicase requires a protein analogous to gp59, DnaC, to promote loading. Cryoelectron micrograph image reconstructions indicate that DnaC also forms a hexameric ring associated with the DnaB ring (13). By analogy, we predict a similar arrangement of gp59 and gp41.

The actual mechanism of helicase assembly by gp59 has yet to be elucidated. gp59 has been shown to lower the affinity of gp32 for ssDNA, which may be a key factor in the removal of gp32 for helicase loading (9). A number of mechanisms have
been proposed to explain this action of gp59. There could be a conformational change in gp32 upon binding to gp59 that directly destabilizes the association between gp32 and ssDNA. The interaction between gp59 and gp32 could also lead to a loss in cooperative binding between gp32 subunits, which in turn would result in a change in affinity. On the other hand, the observed changes could be due to an ability of gp59 to alter the structure of the gp32-coated ssDNA, which may then lead to loss of cooperativity of gp32 subunits or a direct change in affinity for ssDNA (9).

The interaction between gp32 and gp59 was studied via a number of cross-linking methods to understand the mechanism of gp59-mediated helicase assembly onto gp32-coated DNA. To determine whether the changes in the affinity of gp32 for ssDNA in the presence of gp59 could be due to a direct interaction between the DNA binding core domain of gp32 and gp59, this domain was labeled with a cross-linker, and the interaction between the two proteins was analyzed. Furthermore, the oligomerization of gp59 in the presence of gp32 and DNA was also assessed by cross-linking. We have demonstrated that 1) the core domain of gp32 interacts with gp59, and the site of this interaction was localized to Cys-166 of gp32 and Cys-42 of gp59, 2) gp59 is capable of forming at least a pentamer in the presence of gp32, and 3) gp59 can interact with gp32-A, but the association is weaker than the gp32-gp59 interaction, and no gp59 oligomer formation is observed. These results support a model in which helicase assembly requires the initial binding of a gp59 monomer to both the replication fork and gp32 on the lagging strand. A direct interaction between the core domain of gp32 and gp59 could destabilize the gp32-ssDNA interaction, which when followed by gp59 oligomer formation and binding to the helicase, results in displacement of gp32 from the DNA and loading of gp41 in its place. The interactions between the core domain and gp59 as well as the interactions between the A-domain and gp59 play an important role in the removal of gp32 from ssDNA.

**EXPERIMENTAL PROCEDURES**

**Cloning and Purification of gp59**—The 59 gene was isolated from bacteriophage T4 genomic DNA (Sigma) by polymerase chain reaction amplification using the following primers: A, 5'-GGGAATCCCTGATAGGAAAATCCGGC-3' B, 5'-GGGTTTACTGCTTTGCGGAATCTTGCAAGGTC-3'. The product was digested with Bsl I and Sapl I and ligated into a custom IMPACT (New England Biolabs) vector placed under the control of a T7 promoter (14) digested with Nci I and Sapl I. This vector places a self-cleaving intein and chitin binding domain at the C terminus of gp59 and allows affinity purification on a chitin column (New England Biolabs). The vector was transformed into Escherichia coli strain BL21 (DE3) cells, and the expression was induced by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were centrifuged at 4,200 × g for 30 min and resuspended in 20 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, 0.1 mM EDTA (buffer A). The cells were subsequently lysed by sonication, clarified by centrifuging at 20,000 × g for 30 min, and applied to a 10-ml chitin column (New England Biolabs). The column was washed extensively with 100 column volumes of buffer A and incubated for 24 h in 100 mM DTT dissolved in buffer A. The protein was eluted and dialyzed into 20 mM Tris, pH 7.5, 100 mM NaCl, and 10% glycerol (buffer B).

**Labeling of gp32 and gp59**—To label all the cysteine residues in gp32, 15 μl of a 27 μM protein solution was denatured by boiling for 5 min, and 20 μl of 100 mM ATP (warmed to 37 °C) and 6 μM DTT solution were added to the samples. The reaction mixture was incubated at 37 °C for 12 h, and excess label was removed with a 1-ml Sephadex G-25 column. The native protein (15 μl of a 27 μM solution) was labeled with 10 eq of AMCA-HPDP for 12 h at 4 °C to label only the solvent-exposed cysteine residue(s). Excess label was removed as described above.

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**Label Transfer between gp32 and gp59**—Label transfer was initiated by exposure cysteine residue(s). Excess label was removed as described above.}

**Cyanochrome (CNBr, Aldrich) was prepared by dissolving a medium-sized crystal in 1 ml of 88% formic acid (J. T. Baker Inc.). The CNBr solution (100 μM) was added to the denatured and native gp32 solutions. The cleavage was allowed to proceed for 2 h at 25 °C in the dark. The resultant mixture was dialyzed in a speed vac and re-dissolved in 10 mM of H2O, and 5 μl of buffer C was added. The peptides were separated by SDS-PAGE using a 10–20% Tris-Tricine gel (Bio-Rad) and visualized with a Fluor-S Multiimager (Bio-Rad).**

**Activity Assay for gp32**—Activity of labeled and unlabeled gp32 was measured by its ability to inhibit the ATPase activity of gp41 by preventing it from binding to gp32-coated ssDNA in the absence of gp59. The rate of ATP hydrolysis at 37 °C was measured as previously described (17). The reaction mixture consisted of 60 μl of 5 × complex buffer (100 mM Tris, pH 7.5, 750 mM potassium acetate, 50 mM magnesium acetate), 15 μl of 50 mM phosphonopyruvate, 12 μl of 5 mM NADH, 6 μl of a mixture of pyruvate kinase (704 units/ml) and lactate dehydrogenase (1030 units/ml) enzymes (Sigma), and 58 μl of H2O. To this solution 3.6 μl of M13mp18 ssDNA (560 ng/μl) and 55 μl of gp32 (27 μM) were added, and the solution was incubated at 37 °C for 5 min. The background ATPase activity was measured optically at 340 nm after the addition of 10 μl of 10 mM ATP warmed to 37 °C. ATPase activity was then monitored after the addition of 15 μl of gp41 (8 μl). After 5 min, 10 μl of gp59 (12 μM) was added, and the rate of ATP hydrolysis was measured.

**The ATPase activity of gp41 was measured in the absence of DNA and gp32. The background rate of gp59 ATPase activity was similarly measured in the absence of gp41.**

**Ligand Transfer between gp32 and gp59—gp32 (25 μM) was dialyzed into buffer A and incubated with N-(6-(7-aminomethylcoumarin-3-acetamido)hexyl)-3'-2'-pyridylidithio)propionamide (AMCA-HDP) (Fig. 1F, Pierce) in 0.1 vol volume DMP. The protein was labeled for 12 h at 37 °C, and excess label was removed with a 1-ml Sephadex G-25 column. The native protein (15 μl of a 27 μM solution) was labeled with 10 eq of AMCA-HDP for 12 h at 4 °C to label only the solvent-exposed cysteine residue(s). Excess label was removed as described above.
dialyzed into buffer A and labeled with 30 equivalents of either BMH or DBB for 60 min. Excess cross-linker was removed by subjecting the mixture to a 1 ml of Sephadex G-25 column. Labeled gp32 was mixed with gp59 to a final concentration of 5 μM each in a volume of 20 μl either in the absence or presence of DNA. M13mp18 ssDNA was used in the reaction mixtures containing DNA at a concentration of 5 nM. The reaction was allowed to proceed for 10 min before quenching with DTT (10 mM final). The samples were denatured in 5 μl of buffer B and subjected to SDS-PAGE. The gel was stained with Gel Code Blue (Pierce).

A similar procedure was conducted to verify that the putative cross-link observed was an actual cross-link between gp32 and gp59. 1,4-Di-(3’-2-pyridyldithio)propionamidobutane (DPDPB, Fig. 1D, Pierce), a cleavable thiol-reactive cross-linker was used instead of DBB or BMH. Cross-linking was visualized by negatively staining the gel with a copper stain (Bio-Rad). The cross-link band was excised and placed in a 1% SDS solution containing 50 mM DTT for 12 h at 25 °C. The eluted proteins were separated by SDS-PAGE and stained with Gel Code Blue.

To visualize the gp59 oligomers, gp32 and gp59 were mixed to a final concentration of 5 μM each in a volume of 20 μl. Cross-linking was initiated by the addition of 1 μl of 1.5 mM DDB or BMH. After 10 min the reaction was quenched with 10 mM DTT. Cross-linking studies using gp32 lacking the A-domain (gp32-A, obtained as a generous gift from Dr. David Giedroc) were performed under the same conditions, except that the reaction was quenched after 30 min.

RESULTS

Labeling of gp32 with a Trifunctional Cross-linker and Cross-linking to gp59—The crystal structure of gp32 indicates that only one of its four cysteine residues, Cys-166, is free and exposed to solvent (18). The other three cysteine residues are involved in ligation of a zinc ion (18). A 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) assay (19) confirmed the presence of a single free cysteine residue (data not shown). To ensure that we labeled this cysteine (Cys-166) and not one of the three cysteines involved in zinc ligation in the cross-linking experiments described below, we mapped the site of thiol modification. gp32 was labeled with AMCA-HPDP either under denaturing or native conditions. The labeled proteins were then digested with CNBr and subjected to SDS-PAGE, and the fluorescently labeled peptides were identified with a Fluor-S Multiimager. If any of the cysteine residues involved in zinc ligation had been labeled, the expected CNBr fragment would be ~17 kDa, whereas modification at Cys-166 would have produced a fragment of 7 kDa. Fragmentation of the labeled, denatured protein, in which all cysteine residues should be labeled, yielded two fluorescently labeled peptides, 17 and 7 kDa, when separated by SDS-PAGE (Fig. 2A). Labeling of the native protein followed by CNBr digestion and separation by SDS-PAGE showed only one fluorescently labeled peptide with a molecular mass of 7 kDa, indicating that only Cys-166 was labeled (Fig. 2A).

We specifically labeled this cysteine residue with the trifunctional cross-linker shown in Fig. 1A (16). To ensure that the labeled protein was still active, an ATPase activity of gp41 assay was performed. When gp32 was mixed with gp32-coated ssDNA, gp41 does not hydrolyze ATP due to its inability to bind DNA. However, when gp59 is added, gp41 is loaded onto DNA and subsequently hydrolyzes ATP. This is demonstrated by the gp41 ATPase activity summarized in Table I, indicating that only Cys-166 was labeled (Fig. 2A).

Our trifunctional cross-linker contains a thiol-reactive group, a photoactivatable aryl azide, and the affinity probe biotin. B, the short, homobifunctional, thiol-reactive cross-linker DBB. C, BMH, a homobifunctional, thiol-reactive cross-linker. D, DPDPB, a cleavable, thiol-thiol cross-linker. E, biotin-HPDP, a biotin probe containing a thiol-reactive 2-thiopyridine mixed disulfide. F, the fluorescent, thiol-reactive probe AMCA-HPDP.

**TABLE I**

| Components added | Rate of ATP Hydrolysis (μM/s) |
|------------------|-----------------------------|
| gp41             | 0.07                        |
| gp41 and gp59    | 1.24                        |
| Labeled gp32 + M13 DNAα | 0.08                        |
| gp41 and gp59    | 1.08                        |
| gp41 in the absence of gp32⁰ | 0.06                        |
| M13 DNA          | 0.23                        |
| M13 DNA and gp59 | 0.90                        |
| gp59 background activity | 0.07                        |

α The rate of gp41 ATPase activity was measured in the presence of gp32-coated M13 DNA in the absence or presence of gp59.

⁰ The rate of ATP hydrolysis of gp41 was determined in the presence or absence of M13 DNA and gp59.

visualized by a shift in mobility on the gel corresponding to the sum of the molecular weight of the labeled protein plus that of the cross-linked target protein. Under reducing conditions, however, the biotin label will be transferred to the target protein after the cleavage of the disulfide bond in the cross-linker. In this case, the cross-link will no longer be visible by Western blot, but a band corresponding to the molecular weight of the target protein will be visible.

The labeled gp32 was incubated with gp59 in the presence and absence of ssDNA and subjected to ultraviolet light to initiate cross-linking between gp32 and gp59. No gp32-gp32...
cross-linking was seen either on or off DNA (Fig. 2B). There was some intramolecular gp32 cross-linking, as observed by the transfer of biotin to gp32 under reducing conditions (Fig. 2B, lane 9). When gp32 was mixed with gp59, a band corresponding to the molecular weight of gp32 plus that of gp59 was visualized. This indicates that Cys-166 in the core domain of gp32 is in close proximity to gp59. However, a band corresponding to labeled gp59 was also observed under these conditions. We did not expect to see any transfer of the label to the target protein under nonreducing conditions. The labeling of gp59 was not due to the presence of excess label, since lane 3 (in which gp32 was denatured with SDS followed by addition of gp59) shows no labeled gp59. The labeling of gp59 was most likely due to a disulfide exchange. Since gp32 was linked to the trifunctional cross-linker via a mixed disulfide, an interacting protein containing a cysteine residue in close proximity could undergo a disulfide exchange with the cross-linker, specifically labeling this cysteine residue of the interacting protein with the probe.

**Label Transfer between gp32 and gp59**—To determine if the observations were due to a specific label transfer, we labeled gp32 with biotin-HPDP (Fig. 1E), a biotin probe that reacts with a cysteine residue to form a mixed disulfide, and incubated the conjugate with gp59. The two proteins were incubated over time periods ranging from 0 to 15 min and then subjected to a Western blot using SA-HRP as a probe. At the 0 min mark, all of the label was localized to gp32 (Fig. 3, lane 1). Over time, the label was transferred onto gp59 so that by 15 min most of the label was removed from gp32. This result indicated that one of the two cysteine residues in gp59 is close to Cys-166 of gp32. This cysteine residue may be more basic than Cys-166 of gp32, so the overall equilibrium favors a complete transfer of label.

**Thiol-Thiol Cross-linking between gp32 and gp59**—To confirm the close proximity between the cysteine residues of the two proteins, we subjected them to thiol-thiol cross-linking (Fig. 4A). BMH, a homobifunctional thiol-thiol cross-linker (see Fig. 1C), and DBB, a shorter reagent with similar reactivity (see Fig. 1B), were employed. gp32 was labeled with each cross-linker individually and mixed with gp59 in the presence and absence of M13 ssDNA. The products of the cross-linking reaction were separated by SDS-PAGE and stained with Gel Code Blue. No gp32-gp32 cross-links were detected with either cross-linker.

Incubation of gp32 with gp59 resulted in a species that migrated with a mobility on SDS-PAGE corresponding to the sum of the molecular weights of gp32 plus gp59 regardless of which cross-linker was used (Fig. 4A). This indicates that Cys-166 of gp32 and one of the cysteine residues of gp59 must be very close, since DBB has a very short spacer region (6 Å) between the reactive ends. The cross-links were seen in the presence and absence of DNA.

We then confirmed that the putative cross-link band was indeed a cross-link between gp32 and gp59. Cross-linking between gp32 and gp59 using DPDPB (Fig. 1D) results in the formation of a mixed disulfide as a means of covalently linking the two proteins. As such, this cross-link can be cleaved under reducing conditions. The products of the cross-linking reaction were subjected to SDS-PAGE and visualized with a negative copper stain. The putative gp32-gp59 cross-link band was excised, and the proteins extracted from the gel under reducing conditions and then run on a second gel (Fig. 4B). Coomassie staining of this gel showed two bands, one corresponding to the molecular weight of gp32 and the other corresponding to the molecular weight of gp59, verifying the identity of the cross-link.

**Mapping the Interaction between gp32 and gp59**—contains two solvent-accessible thiols, Cys-42 and Cys-215 (7). We utilized a label-transfer experiment to determine which cysteine was interacting with gp32. Since gp32 labeled with a biotin probe via disulfide results in almost complete label transfer to gp59, we decided to use a similar technique to map the site of interaction. gp32 was labeled with the fluorescent probe AMCA-HPDP (Fig. 1F) and incubated with gp59. Most of the fluorophore was transferred to gp59 after 30 min as determined by separation of the proteins by SDS-PAGE followed by visualization of the fluorescent bands on a transilluminator (data not shown). The proteins were digested with trypsin and subjected to reverse phase HPLC. The labeled peptide was selectively collected by monitoring the absorbance of the fluorophore. The peptide was analyzed by reflector mode MALDI mass spectrometry to determine the site of label transfer (Fig. 5). A monoisotopic mass of 1289.58 ([M + H]+) was observed, which corresponds to a gp59 fragment containing amino acids 39–44, in which Cys-42 was labeled with the fluorophore. The
proteins were extracted, reduced with DTT, and separated by SDS-cross-linked with the cleavable thiol-thiol cross-linker DPDPB (data not presence of DNA (4). gp32 labeled with DBB and mixed with gp59 in the presence of DNA (5), gp32 labeled with BMH and gp59 added (6), and gp32 labeled with BMH plus DNA and gp59 (7). The proteins were also cross-linked with the cleavable thiol-thiol cross-linker DPDPB (data not shown). The band corresponding to the cross-link was excised, and proteins were extracted, reduced with DTT, and separated by SDS-PAGE (panel B).

The site of label transfer on gp59 was mapped by digesting the protein with trypsin and analyzing the labeled peptide by MALDI mass spectrometry. A monoisotopic mass of 1290.58 ([M+H]⁺) is observed, which is consistent with the tryptic fragment containing Cys-42 labeled with the fluorophore (theoretical [M+H]⁺ = 1290.55).

Oligomerization of gp59 in the Presence of gp32—It has been previously suggested that gp59 exists as a higher oligomeric species in a complex between gp41 and gp32 (12). Raney et al. (12) demonstrate that the maximal stimulation of DNA unwinding occurred when gp59 was in a 1:1 ratio with gp41 monomers. Furthermore, the analogous E. coli protein, DnaC, exists as a hexameric ring when associated with the DnaB helicase (13). gp59 is a monomer in solution but may associate to form higher species upon binding to gp32 and DNA. We subjected gp59 to thiol-thiol cross-linking in the presence and absence of gp32 (Fig. 6). The proteins were cross-linked with BMH and separated by SDS-PAGE. In the absence of gp32, there were no higher gp59 oligomers except for a small amount of gp59-gp59 dimer (Fig. 6, lane 5). This BMH cross-link is believed to be initiated as a consequence of non-specific disulfide bridge formation between gp59 monomers under nonreducing conditions. These disulfide-linked dimers may place the free cysteine residues on each subunit in close proximity, allowing cross-link formation. No species higher than the dimer is observable species migrated with a molecular weight consistent with pentamer, although higher oligomer formation may be possible. In the absence of gp32 but in the presence of ssDNA, gp59 also formed multimers (Fig. 6, lane 6), indicating that gp59 may bind ssDNA as a cluster. The most intense cross-link bands were observed when gp59 was in the presence of both gp32 and ssDNA.

We also subjected gp59 to cross-linking in the presence of gp32-A. An interaction is observed between gp59 and this truncated form of gp32, indicating that the two proteins can still interact without the A-domain (Fig. 7, lanes 5 and 6). The interaction does not appear to be as strong as the gp32-gp59 association, since the cross-link band was not very intense and required longer cross-linking times for visualization. No self-association of gp59 beyond dimer is observed. Cross-linking of gp59 on DNA in the absence of gp32-A resulted in the formation of higher oligomeric species (Fig. 7, lane 7). The addition of gp32-A to gp59 and DNA did not yield more oligomeric species than gp59 alone on DNA, indicating that gp32-A did not promote formation of the gp59 oligomer.

DISCUSSION

gp59 is needed to displace gp32 to load the gp41 helicase onto the replication fork. Formation of a ternary complex between gp59, gp32, and gp41 may be an important step in loading of the gp41 helicase. In the absence of gp59, gp59 has been shown to bind preferentially to a replication fork over ssDNA or duplex DNA alone (6). gp59 and gp32 have also been shown to co-occupy the same piece of ssDNA by contacting each other as well as the DNA (9). Taken together, this suggests that an initial step in the loading of the helicase involves the simultaneous binding of gp59 to the replication fork and gp32. After formation of this complex, gp41 binding and subsequent loading would follow. We have characterized the interactions between gp59 and gp32 by cross-linking and have demonstrated that 1) the DNA-binding core domain of gp32 interacts with gp59, and this site of this interaction has been mapped to Cys-166 of gp32 and Cys-42 of gp59; 2) gp59 is capable of forming at least a pentamer in the presence of gp32 and DNA, and 3) gp59 can interact with gp32-A, although weakly, and no oligomer formation is observed.

Interaction between gp32 and gp59—Our cross-linking and mapping experiments reveal an interaction between Cys-42 of gp59 and Cys-166 of gp32. Based on our results and the crystal structures of gp59 and gp32, we have proposed a model of gp32-gp59 association on DNA (Fig. 8). The gp59 protein has been shown to be a two-domain helical protein with an N-terminal domain that has significant structural similarity to
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The A-domain of gp32 (shown in blue) most likely interacts with the C-terminal domain of gp59.

The affinity of gp32-A for ssDNA is decreased in the presence of gp59, indicating that the interaction between the core domain and gp59 may be independent of the C-terminal sequence, and this interaction is sufficient to destabilize gp32-ssDNA binding (9). However, gp59 cannot effectively load the helicase onto ssDNA coated with gp32-A (11), suggesting that contacts between the A-domain and gp59 are required in addition to those between the core domain and gp59.

Oligomeric State of gp59 in the Presence of gp32—Although gp59 exists predominantly as a monomer in solution, it may form a higher oligomer to load the gp41 helicase. Studies demonstrate that the maximal stimulation of the unwinding rate of gp41 by gp59 occurs when the two proteins are in a one-to-one ratio, suggesting that gp59 is hexameric under these conditions (12). The DnaC protein in *E. coli* is functionally similar to gp59 and forms a hexameric ring that associates with the DnaB hexamer (13). Moreover, gp59 exhibits cooperativity in binding of ssDNA (4) and forms condensed clusters with gp32 on ssDNA (9), suggesting that oligomer formation may be an important requirement for the action of gp59. Our thiol-thiol-cross-linking studies showed that gp59 oligomerized in the presence of gp32 as well as DNA. The largest species seen migrated with a molecular weight consistent with a gp59 pentamer. Based on the predicted 6:6 stoichiometry of gp59:gp41, we expect that gp59 is capable of forming hexameric species. Interaction with the gp32-coated DNA or ssDNA most likely induces a conformational change in gp59 that allows it to self-associate.

Cross-linking experiments between gp32-A and gp59 established that gp59 can still interact with gp32, even when the A-domain is absent. The association was weak because the cross-link was difficult to detect and required longer cross-linking times for visualization. gp59 did not oligomerize under these conditions, indicating that the A-domain is necessary to induce this association. Most likely, the A-domain is responsible for most of the contact between gp32 and gp59, and binding to this region causes a conformational change that allows oligomer formation and may contribute to the destabilization of gp32-ssDNA binding.

The multimeric gp59 species observed with the thiol-thiol cross-linkers indicate that both of the cysteine residues in gp59 must be in close proximity to the gp59-gp59 subunit interface. Since gp32 interacts with Cys-42 of gp59, it may also be binding at the gp59-gp59 subunit interface. The stoichiometry of gp32 to gp59 was demonstrated to be one to one.² but the oligomeric

² S. W. Morrical, personal communication.
state of the complex may be much larger than a heterodimer. At least two to three gp32 proteins (each with a binding site size of 8 nucleotides) must be released for loading of the helicase (which has a binding site size of 12–20 nucleotides) (21). Formation of gp59-gp32 heterooligomers larger than dimers may not only facilitate the binding and loading of the helicase but may also allow the binding and removal of multiple gp32 proteins from the lagging strand. Replacement by the helicase and possible departure of gp59 from the replication fork would represent final events in the loading process that have strong parallels to the successional steps observed in loading of the gp45 clamp protein by the gp44/62 clamp loader (22, 23).

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