Structural analyses of the bacterial primosomal protein DnaB reveal that it is a tetramer and forms a complex with a primosomal re-initiation protein

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The DnaB primosomal protein from Gram-positive bacteria plays a key role in DNA replication and restart as a loader protein for the recruitment of replisome cascade proteins. Previous investigations have established that DnaB is composed of an N-terminal domain, a middle domain, and a C-terminal domain. However, structural evidence for how DnaB functions at the atomic level is lacking. Here, we report the crystal structure of DnaB, encompassing the N-terminal and middle domains (residues 1–300), from Geobacillus stearothermophilus (GstDnaB1–300) at 2.8 Å resolution. Our structure revealed that GstDnaB1–300 forms a tetramer with two basket-like architectures, a finding consistent with those from solution studies using analytical ultracentrifugation. Furthermore, our results from both GST pulldown assays and analytical ultracentrifugation show that GstDnaB1–300 is sufficient to form a complex with PriA, the primosomal reinitiation protein. Moreover, with the aid of small angle X-ray scattering experiments, we also determined the structural envelope of full-length DnaB (GstDnaB1–2782) in solution. These small angle X-ray scattering studies indicated that GstDnaB1–2782 has an elongated conformation and that the protruding density envelopes originating from GstDnaB1–300 could completely accommodate the GstDnaB C-terminal domain (residues 301–461). Taken together with biochemical assays, our results suggest that GstDnaB uses different domains to distinguish the PriA interaction and single-stranded DNA binding. These findings can further extend our understanding of primosomal assembly in replication restart.

Accurate and timely DNA replication is essential for cell proliferation in all organisms, which relies on a number of inter-protein interactions (1–3). Sequential assembly of the primosome complex is one of the most important processes that drives DNA replication in all bacterial species. In bacteria, DNA replication is initiated when DnaA recognizes and binds to the origin of replication (oriC) (4, 5) where all the DNA unwinding machinery is assembled. Subsequently, the hexameric helicase-loader complex is recruited followed by joining of the primase DnaG to complete primosome and replisome assembly (6–8).

During the elongation phase of DNA replication, replication forks are frequently challenged by mutations, nicks, base modifications, and other factors that could result in fork arrest or collapse (3, 9, 10). Once the DNA replication process is stalled, it undergoes a reinitiation process, and the primosome re-initiation protein PriA is recruited at the respective sites (11). The proteins DnaA, PriA, and the replicative helicase are well-conserved in both Gram-negative bacteria (such as Escherichia coli) and Gram-positive bacteria (such as Bacillus subtilis) (12–14). However, DnaA and PriA in E. coli and B. subtilis differ in their recruitment of subsequent loader proteins before recruitment of the replicative helicase. During the normal replication process in Gram-negative E. coli, DnaA recognizes oriC sites and recruits DnaB helicase with the help of the helicase loader protein DnaC and promotes the association of the primase DnaG (2, 15–18). In instances of abnormal DNA repair and restart, PriA recognizes the stalled forks and sequentially recruits three other primosomal reinitiation proteins (PriB, PriC, and DnaT) and a loader protein DnaC to trigger re-loading of hexameric replicative helicase as a helicase-loader complex DnaB–DnaC to restart the replication process (13). These two distinct primosome reinitiation processes share the same helicase (DnaB) and primase (DnaG) in the replisome system.

However, in Gram-positive bacteria homologues of PriB, PriC, and DnaT are absent. Instead, only the primosomal loader proteins DnaB and DnaD are found, which are mediated by both DnaA and PriA to initiate and restart the replication process at oriC and stalled forks, respectively (12, 19, 20). Assembly of DnaB and DnaD preloader proteins at replication sites promotes loading of the helicase DnaC (the replicative helicase homologous to E. coli DnaB) in complex with the loader protein DnaI and DnaD (the helicase loader homologous to E. coli DnaC) in an ATP-dependent manner (6, 21) to unwind the parental DNA template.

Previous studies suggested that Gram-positive bacterial loader protein DnaB is a multifunctional protein. Its interaction with DnaD on single-stranded DNA-binding protein-coated DNA has been demonstrated (22), and its cooperative role with DnaI in acting as a “co-loader” for the recruitment of DnaC helicase has also been shown (23). Furthermore, the cell mem-

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This article contains supplemental Table S1 and Figs. S1–S9.

The atomic coordinates and structure factors (code 5WTN) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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brane attachment properties of DnaB have been described (24–27), and these have been shown to regulate the recruitment of DnaD to the membrane-attached oriC region of bacterial DNA in a supercoiled state (28, 29). This process is thought to be essential for regulating DNA replication and the re-initiation process (30). Although previous low resolution structural studies using atomic force microscopy (AFM)² and electron microscopy (EM) showed that DnaB from *B. subtilis* (BsuDnaB) assembles as a non-globular tetramer (in a square or spiral shape) (7, 29, 31), the functional significance of adopting this conformational state and its structural details remain unclear. Due to the lack of full-length structures of DnaB or its homologues, our understanding of the mechanics involved in effective DNA processing during the replication initiation and restart processes are limited. This shortcoming warrants further structural and functional studies on DnaB at higher resolution to address its functional involvement in the association of proteins such as PriA and DnaD and to aid our understanding of the regulation of bacterial replisome priming and the process of DNA replication and restart.

In this study we determined the crystal structure of the truncated form (residues 1–300) of DnaB from *Geobacillus stearothermophilus* (*Gst*DnaB<sub>1–300</sub>) at 2.8 Å resolution. The *Gst*DnaB<sub>1–300</sub> crystal structure describes a unique tetrameric organization comprising two dimers arranged in a domain-swap conformation. The results from our GST pulldown assays and analytical ultracentrifugation (AUC) suggest that GstDnaB physically interacts with GstPriA. Furthermore, small angle X-ray scattering analyses (SAXS) of both *Gst*DnaB<sub>L</sub> and *Gst*DnaB<sub>1–300</sub> corroborate the consistency of the structural conformation in solution. Our results enhance understanding of the domain arrangement of DnaB<sub>L</sub>, and its biological role in association with the replication re-initiation protein PriA.

## Results

### Sequence alignment of DnaB from different species

The Gram-positive bacterial helicase loader protein DnaB from *G. stearothermophilus* (*Gst*DnaB) comprises 461 residues. Based on proteolysis assays using proteinase K and trypsin digestion studies of *B. subtilis*, BsuDnaB is structurally organized into three domains: residues 1–184 (N-terminal), 204–296 (middle), and 297–472 (C-terminal) (31). The corresponding domains in *Gst*DnaB comprise residues 1–144 (N-terminal), 201–290 (middle), and 304–461 (C-terminal) (described in further detail in the following sections). To identify the sequence variation between species, we aligned the *Gst*DnaB sequence with that of *Bsu*DnaB. As shown in supplemental Fig. S1, *Gst*DnaB exhibits 47.2% overall sequence identity with *Bsu*DnaB. The lowest sequence similarity was observed between residues 150 and 204 and in the extreme C-terminal region (residues 430 and 460) of DnaB.

²The abbreviations used are: AFM, atomic force microscopy; AUC, analytical ultracentrifugation; SAXS, small angle X-ray scattering; ITC, isothermal titration calorimetry; AFM, atomic force microscopy; SAD, single-wavelength anomalous diffraction; r.m.s.d., root mean square deviation; Se-Met, selenomethionine; NSRRC, National Synchrotron Radiation Research Center; *R*<sub>g</sub>, radius of gyration; ssDNA, single-stranded DNA.

### Crystal structure of DnaB protein

**GstDnaB loader protein interacts physically with the primosome restart protein GstPriA**

The DNA replication restart process and the underlying mechanics relating to the association of co-proteins in Gram-positive bacteria needs further clarification. As mentioned earlier, the primosomal loader protein DnaB is unique among Gram-positive bacterial species and plays a key role in both DnaA- and PriA-mediated pathways to initiate and restart the DNA replication process at oriC and stalled forks, respectively (7, 18–20, 22, 32, 33). In addition to localization at oriC (34), in *B. subtilis* the association of DnaB with loader proteins such as DnaD and DnaI at origin-independent loci is also considered to be essential for the replication restart process (19, 22). In this regard it is not clear whether the DnaB loader protein associates directly or indirectly with primosomal protein PriA and other loader proteins. Hence, we aimed to test for direct association of *Gst*DnaB with *Gst*PriA. To this end, we performed glutathione S-transferase (GST) pulldown assays using purified recombinant full-length *Gst*DnaB<sub>L</sub> and truncated forms (*Gst*DnaB<sub>1–300</sub> or *Gst*DnaB<sub>301–461</sub>) as fusion proteins containing an N-terminal GST moiety (*Gst*DnaB<sub>L</sub>, *Gst*DnaB<sub>1–300</sub>, or *Gst*DnaB<sub>301–461</sub>). As shown in Fig. 1A, *Gst*PriA specifically interacted with *Gst*DnaB<sub>L</sub> and *Gst*DnaB<sub>1–300</sub>, whereas interaction was completely abolished for the C-terminal region (*Gst*DnaB<sub>301–461</sub>). This suggests that the N-terminal and middle domains of DnaB are essential for interactions with *Gst*PriA.

To corroborate this interaction between *Gst*DnaB and *Gst*PriA, we performed sedimentation-velocity experiments through AUC to provide direct evidence of complex formation in solution. Five different fragments of *Gst*DnaB (*Gst*DnaB<sub>L</sub>, *Gst*DnaB<sub>1–300</sub>, *Gst*DnaB<sub>301–461</sub>, *Gst*DnaB<sub>1–200</sub>, and *Gst*DnaB<sub>201–300</sub>) were individually mixed with an equal molar ratio of *Gst*PriA (1:1) and then subjected to AUC. The sedimentation coefficient of *Gst*PriA alone was 5.1 S, which corresponds to the relative molecular mass of the monomer ~89.5 kDa (*green dashed lines*) in Fig. 1, *B–F*. The sedimentation coefficient of *Gst*DnaB<sub>L</sub> alone was 7.2 S, which corresponds to the relative molecular mass of the tetramer ~212 kDa (*red dashed lines* in Fig. 1B). With respect to *Gst*PriA interaction with full-length *Gst*DnaB, we noticed the formation of two distinct major peaks, denoted as *A* and *B* in the *Gst*DnaB<sub>L</sub>/*Gst*PriA mixture. Peak *A* has a sedimentation coefficient of ~5.2 S, which is similar to the 5.1 S for *Gst*PriA alone. However, the s value of peak B was 8.3 S, which is much greater than that for the *Gst*DnaB<sub>L</sub> tetramer alone, clearly indicating that this peak corresponds to a *Gst*DnaB<sub>L</sub>*Gst*PriA complex. Consistently, similar results were observed when *Gst*DnaB<sub>1–300</sub> was substituted for *Gst*DnaB<sub>L</sub> in the above experiment (Fig. 1C); the sedimentation coefficient of peak A (5.2 S) corresponded closely to *Gst*PriA alone, whereas the s value of peak B was also much higher than for the *Gst*DnaB<sub>1–300</sub> tetramer alone (7.1 S), indicating formation of a complex between *Gst*DnaB<sub>1–300</sub> and *Gst*PriA. However, consistent with our earlier GST pulldown experiments, we did not find an interaction between *Gst*DnaB<sub>301–461</sub> and *Gst*PriA as a shift in the protein peaks of the mixture (*blue line*, Fig. 1D) was not observed, and instead, the peaks were
much closer to those of both \textit{Gst}DnaB\textsubscript{301–461} and \textit{Gst}PriA alone (green and red dashed lines, Fig. 1D).

In addition, the \(s\) value of \textit{Gst}PriA-\textit{Gst}DnaB\textsubscript{1–300} complex (\textit{peak B} of Fig. 1C) was 7.1 S, which is similar to the \(s\) value of \textit{Gst}DnaB\textsubscript{FL} tetramer. This would give an estimated molecular weight of the \textit{Gst}PriA-\textit{Gst}DnaB\textsubscript{1–300} complex at 7.1 S was \(~212\) kDa. Therefore, this indicated that the stoichiometry between \textit{Gst}PriA and \textit{Gst}DnaB complex most likely is 1–4. To further corroborate the stability for the complex between \textit{Gst}DnaB and \textit{Gst}PriA in solution, we also performed isothermal titration calorimetry (ITC) experiments. As shown in the supplemental Fig. S2, the dissociation constant (\(K_d\)) between \textit{Gst}DnaB\textsubscript{1–300} and \textit{Gst}PriA was 47.2 \(\mu M\), and the \(C\)-value in ITC experiment is 1.4. The low \(C\)-value range from 1 to 5 often leads to a large error in fitting, a result of stoichiometry (N). Fortunately, the decoupled of \(K_d\) with the error in N at the low \(C\)-value permits the determination of \(K_d\) when the titration proceeds to a near saturate level (35). Due to the low \(C\) value (1.4) in our original setting, we also switched the order of syringe and cell component in the experiment by titrating high concentration of \textit{Gst}PriA (780 \(\mu M\)) to \textit{Gst}DnaB\textsubscript{1–300}. \textit{Gst}PriA did not form protein complexes, as the protein peaks of the mixture were not significantly shifted and were close to that of \textit{DnaB}\textsubscript{301–461} alone and \textit{PriA} alone as was the case for \textit{DnaB}\textsubscript{1–200} and \textit{DnaB}\textsubscript{202–300}.

![Sedimentation coefficient distributions derived from sedimentation velocity profiles. B and C, DnaB\textsubscript{FL}/PriA and DnaB\textsubscript{1–300}/PriA can form protein complex and shift to a higher S marked as peak B. D and F, DnaB\textsubscript{301–461} and PriA did not form protein complexes, as the protein peaks of the mixture were not significantly shifted and were close to that of DnaB\textsubscript{301–461} alone and PriA alone as was the case for DnaB\textsubscript{1–200} and DnaB\textsubscript{202–300}.](image)

Figure 1. Analysis of the interaction between \textit{Gst}DnaB and \textit{Gst}PriA. A, GST pulldown assay. SDS-PAGE was followed by Western blotting analysis using anti-His\textsubscript{6} and anti-GST antibodies showing the interaction between \textit{Gst}PriA and both \textit{Gst}DnaB\textsubscript{FL} and \textit{Gst}DnaB\textsubscript{1–300} (but not \textit{Gst}DnaB\textsubscript{301–461}). The positions of individual proteins and the anti-His\textsubscript{6}-bound proteins are labeled accordingly. B–F, sedimentation coefficient distributions derived from sedimentation velocity profiles. B and C, DnaB\textsubscript{FL}/PriA and DnaB\textsubscript{1–300}/PriA can form protein complex and shift to a higher S marked as peak B. D and F, DnaB\textsubscript{301–461} and PriA did not form protein complexes, as the protein peaks of the mixture were not significantly shifted and were close to that of DnaB\textsubscript{301–461} alone and PriA alone as was the case for DnaB\textsubscript{1–200} and DnaB\textsubscript{202–300}. AU, absorbance units.
concentration. Therefore, we only discussed the $K_d$ from ITC but did not take N into consideration. In addition, our ITC result indicated that the interaction between GdstDnaB and GstPriA is dynamic. This dynamic interaction may be crucial for the formation, rearrangement and dissociation of the primosomal complexes. To identify the GdstPriA-binding region in the GdstDnaB$_{1–300}$ protein segment, we prepared two truncated forms of the protein (GdstDnaB$_{1–145}$ and GdstDnaB$_{160–300}$). However, neither truncated form was soluble. Hence, we then generated two other soluble truncated forms (GdstDnaB$_{1–200}$ and GdstDnaB$_{202–300}$) and subjected them to sedimentation-velocity AUC. As shown in Fig. 1E, the mixture of GdstDnaB$_{1–200}$ and GstPriA produced a broader peak (blue line) covering tetrameric GdstDnaB$_{1–200}$ alone and monomeric GstPriA alone (red and green dashed lines), but a shift in the peak or a change to the sedimentation coefficient was not observed. This result indicates that GdstDnaB$_{1–200}$ is not sufficient to interact with GstPriA. We did not observe complex formation between GdstDnaB$_{202–300}$ and GstPriA; protein peaks of this mixture (blue line, Fig. 1F) did not shift noticeably, and its sedimentation coefficient was similar to that of GdstDnaB$_{202–300}$ and GstPriA alone (green and red dashed lines, Fig. 1F). In brief, our sedimentation-velocity AUC results further support the GST pulldown assays in demonstrating DnaB interaction with PriA. Both the N-terminal and middle domain regions are required to establish this interaction, and the connecting flexible linker might have some additional role in PriA interaction. We found that the C-terminal domain of GdstDnaB is not involved in PriA interaction, but it may have another role in DNA binding or in the recruitment of other primosome machinery for further DNA unwinding, and this topic warrants further study.

**Overall structure of GdstDnaB$_{1–300}$ monomer**

Previous structural studies of DnaB have been limited to a low resolution using atomic force microscopy (AFM) and EM (29, 31), which lack detailed structural information at the atomic level. To unravel the structural details of GdstDnaB to better understand its biological role in primosome assembly and replication restart, we first attempted to crystallize full-length GdstDnaB (GdstDnaB$_{FL}$). However, we could not obtain crystals, perhaps due to the flexibility of the linker region connecting the middle and C-terminal domains. Hence, we generated a truncated version of the protein encompassing the N-terminal and middle domains (residues 1–300: GdstDnaB$_{1–300}$), which we previously showed to be functionally important for associations with PriA. A previous study also showed that BsuDnaB is truncated at its C terminus in a growth-phase-dependent manner (34). These observations suggested to us that structural study of this portion of the protein (i.e. DnaB$_{1–300}$) would have biological implications for its functional importance in primosome assembly.

The crystal structure of GdstDnaB$_{1–300}$ was determined at 2.8 Å resolution via single-wavelength anomalous diffraction (SAD) phasing (Table 1). As expected, the GdstDnaB$_{1–300}$ monomer consisted of the N-terminal and middle domains connected by a long linker (Fig. 2A). The N-terminal domain (residues 3–144) was composed of three β-strands and eight α-helices, whereas the middle domain (residues 161–288) was composed of two β-strands and seven α-helices (Fig. 2, A and B). The three β-strands in the N-terminal domain were organized into a three-stranded β-sheet, in which β1 and β3 are parallel but β2 and β3 are antiparallel. In the middle domain, composed of seven helices (α9–α15) and two β-strands (β5 and β6), four α-helices (α11, α13, α14, and α15) were juxtaposed, and the two β-strands (β5 and β6) formed an antiparallel β-sheet. These two domains were connected by a long linker region (residues 145–160) containing one β-strand (β4), which forms a new β-sheet with β1-β3 of the neighboring monomer (supplemental Fig. S3). Although a well-defined electron density was observed for most of the GdstDnaB$_{1–300}$ structure, we did not see a continuous density between residues 172 and 189 in the middle domain (dashed line in Fig. 2B). Surprisingly, residues between 190 and 202 formed a long random structure that connected to α10 (Fig. 2A; supplemental Fig. S4). Residues Asp-190–Ile-192 form three main-chain hydrogen bonds with residues Phe-144–Arg-146 of the linker region to further stabilize this random structure. We speculate that this region may form a large loop with the unobserved region (residues 172–189), further highlighting the dynamic nature of the protein.

**Structural organization and assembly of GdstDnaB as a tetramer**

Although previous AFM and EM structural studies at low resolution had shown that BsuDnaB is a tetramer with a square-like architecture (29), atomic details of the arrangement of the functional tetramer are lacking. In agreement with previous observations, we observed a D2 tetrameric conformation (dimer of dimers) for the GdstDnaB$_{1–300}$ in our crystal structure.
Crystal structure of DnaB protein

(Fig. 3A). All four molecules showed almost identical conformations, with the root mean square deviation (r.m.s.d.) among these monomers ranging from 0.7 to 1.1 Å for 256 Ca atoms. Interestingly, the GstDnaB1–300 dimers (Chains A and B or Chains C and D) were assembled in a domain-swap conformation, and the overall dimer was basket-like (Fig. 3A).

Surprisingly, the overall tetrameric conformation of GstDnaB1–300 in our crystal structure differs significantly from those of previously observed low-resolution structures of BsuDnaB reported in AFM and EM studies (29, 31). AFM results revealed a square-like architecture of BsuDnaB, measuring 21.6 nm and with a distinct central space formed between the four monomers of the tetramer. In contrast, a 24 Å resolution EM reconstruction map demonstrated that the BsuDnaB tetramer adopted a spiral conformation with a distinct and large central space. Although both of these studies showed some similarity in the BsuDnaB tetramer conformation, differences between the low-resolution structures are suggestive of a highly flexible protein. Our GstDnaB1–300 crystal structure did not reveal a central space or hole between the four monomers of the tetramer. Instead, our overall structure had a completely new conformation in which two dimers of GstDnaB1–300 are aligned back-to-back and form a large interface at the center of the tetramer.

To resolve the structural differences and domain arrangement, we first calculated our GstDnaB1–300 X-ray structure to a 24 Å density map and compared it with the EM structure of BsuDnaB (the accession code EMD-1225 in the Electron Microscopy Data Bank) at the same resolution. As shown in supplemental Fig. S5, A–C, GstDnaB1–300 tetrameric structure (colored in pink) did not show square or spiral arrangement with distinct central holes as observed in the EM structure of BsuDnaB (colored in green). Instead, the GstDnaB tetrameric structure was arranged in a letter X-like manner and lacked any similarity in comparison with the structural arrangement with BsuDnaB. Interestingly, although the GstDnaB1–300 tetrameric structure lacks C-terminal domain, its overall volume appear to be comparable with that of the BsuDnaB EM structure at 24 Å resolution (supplemental Fig. S5, A–C). Meanwhile, as the AFM structural data of BsuDnaB (29) is not available in data bank and it has been shown that both EM and AFM low-resolution structures BsuDnaB share similarities (31), we did not further attempt to compare with our structure.

Structural interactions between monomers and dimers of GstDnaB

The dimeric interface of two monomers is located between chains A and B or chains C and D. The interface between the two dimers that forms the stable tetramer is formed by chain A/B and chain C/D (Fig. 3A). To determine the most likely interaction interface that could represent a major structural element in the formation of the tetramer, we used the PISA server (36) to measure the surface areas of these interfaces. The buried surfaces within the chains A and B dimer and the chains C and D dimer were 3542.1 Å² and 3431.5 Å², respectively (Interface-1 and Interface-2 of Fig. 3, A–C, respectively). The buried surfaces of the interfaces of chains A/C and chains B/D were 1548.4 Å² and 1490.5 Å², respectively (Interface-3 of Fig. 3, A and D). These results suggest that the dimeric interfaces between chains A and B and chains C and D are the most likely to play a major role in the formation of GstDnaB dimer, whereas interactions between chains A and C and between chains B and D contribute to assembly and stabilization of the overall tetramer.

Because GstDnaB1–300 dimer (chain A/B or chain C/D) is assembled in a domain-swap conformation, the DnaB dimeric interface is stabilized by Interface-1 and Interface-2 from two adjacent N-terminal and middle domains of DnaB (Fig. 3, B and C), with Interface-1 being formed between the two adjacent N-terminal domains of DnaB (Fig. 3B). This interaction is stabilized by reciprocal hydrogen bonds between residues Gln-23 and Glu-24 of chains A and B, and reciprocal salt bridges between residues Asp-26 and Arg-194 of chains A and B. In addition, evidence for several van der Waals interactions...
between both chains was found. Interestingly, the major interaction between the DnaB monomers in the dimer occurs in Interface-2 (see Fig. 3C), which might serve as a key factor in establishing the “domain-swap” to stabilize the dimer. We found that the N-terminal domain of chain A builds an interaction interface (Interface-2) with the linker region and middle domain of adjacent monomer chain B (Fig. 3, A and C). These interactions involve residues from α13 and α15 in chain A and residues from β2, α5, α6, and α7 in chain B. Residues Lys-235, Arg-267, and Glu-282 of chain A form salt bridges with Asp-121, Glu-122, and Arg-129 of chain B, respectively. In addition, hydrogen bonding between residues Gln-156, Arg-159, Glu-196, and Tyr-199 of chain A with Asp-91, Tyr-78, Glu-85, Gln-117, and Asp-121 of chain B further strengthens the dimerization interface, which could contribute to stabilization of the DnaB dimer. The nature of this dimerization interface and the corresponding residues involved in the interactions establish the dimer are also consistent in the other dimer of chains C and D.

As described earlier, the overall tetrameric conformation of DnaB is stabilized by the interaction interface between the two dimers (AB and CD) through Interface-3 (Fig. 3, A and D). The intermolecular interactions stabilizing this interface also involve several salt bridges, hydrogen bonds, and hydrophobic interactions. Residue Glu-54 of chain A/D forms a salt bridge with Lys-28 of chain B/C at one end of Interface-3. At the other end, hydrogen bonds are formed between residues Thr-48 of chain A/D and residue Gln-35 of chain B/C. The central part of Interface-3 possesses a distinctive hydrophobic core that is created by the hydrophobic residues Leu-55 and Leu-69 of one chain and Leu-32, Leu-136, and Phe-143 of the neighboring chain. We postulate that these hydrophobic interactions might play an additional role in the establishment or stabilization of the GstDnaB tetramer by maintaining the interdimer conformation. Surprisingly, there are 36 residues (9 residues of each monomer) involved in interface-3 interaction, and sequence alignment between GstDnaB and BsuDnaB shows that these residues involved in the GstDnaB tetramer interfaces share 44% sequence identity (56% sequence similarity). This result indicated that DnaB co-loader protein from different species may use these residues to form a stable tetramer structure. These structural interactions and the observed interfaces between DnaB monomers in the tetramer suggest that the N-terminal and middle domains of DnaB dictate the association of monomers to effect DnaB’s role as a tetramer.

The N-domain and linker regions are required for tetramerization of GstDnaB

Our earlier results suggested that residues 1–300 of GstDnaB play an important role in tetramerization and functional stability. We next prepared the three truncated forms GstDnaB1–144 (N-terminal domain alone), GstDnaB1–200 (N-terminal domain with linker region), and GstDnaB202–300 (middle domain alone) for AUC studies to determine which region guides formation of the functional tetramer. Unfortunately, GstDnaB1–144 showed impaired solubility, and we could not pursue further studies.

Figure 3. Tetrameric organization and interactions of GstDnaB1–300–A, the four molecules of the GstDnaB tetramer are shown in green, cyan, magenta, and yellow. The N-terminal and middle domains of each monomer are labeled as NTD and MD, respectively. The interactions between monomers or dimers are formed by Interface-1 (B), Interface-2 (C), and Interface-3 (D). Color coding is as for panel A, and residues involved in protein interactions are indicated as stick models.

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AUC results for \( \text{GstDnaB}_{1-200} \) and \( \text{GstDnaB}_{202-300} \) showed sedimentation coefficients of 4.8 S and 1.2 S, respectively (red lines in Fig. 1, E and F). The c(s) distribution analysis corresponded to the molecular mass of \( \sim 97.3 \) kDa for \( \text{DnaB}_{1-200} \), which closely matches the tetrameric conformation. The molecular mass of \( \text{DnaB}_{202-300} \) was \( \sim 12.5 \) kDa, which closely matches the monomeric conformation. Thus, our AUC analysis with different truncated forms of \( \text{GstDnaB} \) indicates that the N-terminal domain plus linker region contributes to dimer and/or tetramer formation.

**GstDnaB binds ssDNA substrate through its C-terminal domain**

As studies have shown that DnaB plays a prominent role in the DNA replication initiation process at both \( \text{oriC} \) and stalled forks, we next tested the DNA-binding ability of \( \text{GstDnaB}_{FL} \) and its truncated forms. We wondered if the C-terminal region plays an important role in DNA-binding activity of \( \text{Geobacillus} \) species. Hence, we tested purified full-length and two truncated forms of \( \text{GstDnaB} \) (\( \text{GstDnaB}_{1-300} \) and \( \text{GstDnaB}_{301-461} \)) for ssDNA (dT40)-binding activity by electrophoresis mobility shift assays (EMSA). As shown in Fig. 4A, EMSA results revealed that increasing concentrations of \( \text{GstDnaB}_{FL} \) exhibited increased ssDNA-binding activity. Surprisingly, we did not observe ssDNA-binding activity for \( \text{GstDnaB}_{1-300} \) even with a protein concentration of 240 \( \mu \)M (Fig. 4B). This observation conflicts with a previous study of ssDNA substrate binding in the \( \text{Bacillus} \) species (34). However, ssDNA-binding activity was observed for \( \text{GstDnaB}_{301-461} \) with increasing protein concentrations (Fig. 4C). This observation is in accordance with an earlier study (34). To calculate the dissociation constants of DnaB and ssDNA binding, we quantified the florescent signal in each DNA band. As shown in Fig. 4D, \( \text{DnaB}_{FL} \) had the highest ssDNA-binding affinity, with an apparent \( K_d \) of 34.9 \( \mu \)M followed by \( \text{GstDnaB}_{301-461} \) (\( K_d = 115.4 \mu \)M) and with no binding activity for \( \text{GstDnaB}_{1-300} \). These results indicate that DNA binding by \( \text{GstDnaB} \) is predominantly mediated by the C-terminal domain. Overall, these results highlight the functional difference between two truncated forms of \( \text{GstDnaB} \) representing the N-terminal plus middle domain and the C-terminal domain, respectively, in terms of PriA interaction and ssDNA binding.

**Comparisons with other protein structures**

To further understand the structure and function of \( \text{GstDnaB} \), we first performed a structural homology search for the N-terminal domain of \( \text{GstDnaB} \) (\( \text{GstDnaB}_{-NTD} \)) (residues 1–144) using the Dali server (37). As shown in Fig. 5A, the proteins RepA (38) from \( \text{Staphylococcus aureus} \) (SaRepA) (PDB ID code 4PT7) and TFE (39) from \( \text{Sulfolobus solfataricus} \) (SsTFE) (PDB ID code 1Q1H) showed the highest structural homology with \( \text{GstDnaB}_{-NTD} \) despite having lower sequence identities with it of 18 and 10%, respectively. We next superimposed \( \text{GstDnaB}_{-NTD} \) with \( \text{SaRepA}_{-NTD} \) and with \( \text{SsTFE}_{-NTD} \) and found that in both cases they were well-aligned, with r.m.s.d. of 2.6 Å and 2.1 Å, respectively (Fig. 5A). Both \( \text{SaRepA} \) and \( \text{SsTFE} \) lack the first \( -1 \) strand observed in \( \text{GstDnaB}_{-NTD} \), and their \( -2 \) and \( -3 \) strands are folded into a characteristic winged helix-turn-helix fold that is structurally well-conserved in both proteins. In RepA, the winged helix-turn-helix folded region is proposed to have DNA-binding activity and

![Figure 4. ssDNA-binding activity assay of full-length and truncated GstDnaB.](image-url)
Crystal structure of DnaB protein

SAXS analysis reveals an elongated shape for GstDnaB_{FL}

Because crystallization of GstDnaB_{FL} proved challenging due to its high flexibility, we performed solution scattering experiments to resolve the overall tetrameric structure of the full-length protein. SAXS analysis was conducted for both GstDnaB_{FL} and GstDnaB_{1–300}. The radius of gyration (R_g), maximum dimension (D_{max}), Guinier plots, and pair distance distribution plots for both GstDnaB_{FL} and GstDnaB_{1–300} were calculated using the SAXS profile (Fig. 6, A–D; supplemental Table S1). As expected, R_g values (derived from the Guinier plots using the program PRIMUS) for GstDnaB_{FL} and GstDnaB_{1–300} were significantly different; 57.45 ± 0.199 Å and 35.42 ± 0.036 Å, respectively (supplemental Table S1). This result clearly suggests that the overall dimensions of the SAXS shells and the structural architecture of GstDnaB_{FL} and GstDnaB_{1–300} are different. Indirect Fourier transformation of the curves calculated using the GNOM program and the experimental curves fitted with SAXS data for both GstDnaB_{FL} and GstDnaB_{1–300} are shown in Fig. 6, A and B. The observed linearity of the Guinier plots in the lower q-region indicates that the scattered intensities follow the Guinier law, suggesting that both protein samples were monodispersed and is indicative of good quality data (inner panel of Fig. 6, A and B). By using P(r) functions, we next determined D_{max} for both proteins and measured values of 200 Å for GstDnaB_{FL} and 112 Å for GstDnaB_{1–300} (Fig. 6, C and D). The significant difference in the D_{max} values suggests that GstDnaB_{FL} possesses an extended or elongated conformation compared with that of GstDnaB_{1–300}.

As shown in supplemental Figs. S6 and S7, we generated eight molecular envelopes for both GstDnaB_{FL} and GstDnaB_{1–300} from the scattering data using the ab initio modeling feature of the GASBOR program. These eight molecular envelopes were averaged by the program DAMAVER (41). The resulting “overall” envelope of GstDnaB_{1–300} is elliptical, with dimensions relatively consistent with those of our crystal structure in the tetrameric conformation. To compare our crystal structure of GstDnaB_{1–300} with the overall ab initio envelope derived from solution scattering, we performed docking using the SUPCOMB program (42). As expected, our crystal structure fitted well with the GstDnaB_{1–300} SAXS envelope (Fig. 6E). Furthermore, the observed experimental SAXS curve aligned well with the theoretical curve calculated from the crystal structure of GstDnaB_{1–300} using CRYSOL, with an acceptable Chi (χ) value of 3.46 (Fig. 6G). This suggests consistency of the

plays a key role in origin recognition by plasmids during replication (38). In contrast, bacterial SsTFE-NTD lacks DNA-binding activity (39), similar to GstDnaB-NTD. We calculated the surface potentials of these three proteins. As shown in Fig. 5B, SsRepA-NTD has a much more positively charged surface potential than GstDnaB-NTD and SsTFE-NTD. This finding may explain why we did not observe ssDNA-binding for GstDnaB_{1–300}. Although the N-terminal domains of GstDnaB and SsRepA exhibit structural similarities, their functional roles and their associations with other partner proteins differ.

We then performed the Dali homology search for the middle domain of GstDnaB (GstDnaB-MD) (residues 161–290). Our results showed that the N-terminal domain of DNA polymerase α-primerase B-subunit (p68N) (40) was the most structurally homologous to GstDnaB-MD. Despite the similarity in their architectures (Fig. 5C), an amino acid sequence alignment between the middle domain of GstDnaB and p68N revealed low sequence similarity, and the chemical nature of their surfaces differed considerably (Fig. 5D). The middle domain of GstDnaB is characterized by an electrostatic surface with both basic and acidic charges, whereas the N-terminal domain of p68N displays a predominantly acidic surface with a few small hydrophobic patches. As discussed above, the middle domain of GstDnaB plays a role in mediating interactions with PriA. However, the N-terminal domain of p68N physically interacts with the T antigen helicase domain. Despite the differences between the surfaces of these two domains of GstDnaB and p68N, they have a similar function in mediating protein-protein interactions.

Figure 5. Comparisons of structure and electrostatic surface potential. A, structural comparison of the N-terminal domain of GstDnaB with other proteins. The left panel is a ribbon diagram of GstDnaB-NTD. The middle and right panels are superimpositions of GstDnaB-NTD (colored in pink) with SsRepA-NTD (colored in green) and SsTFE-NTD (colored in blue), respectively. B, distribution of electrostatic surface potentials of GstDnaB-NTD (left), SsRep-NTD (middle), and SsTFE-NTD (right). Positively and negatively charged residues are colored in blue and red, respectively. C, superimposition between GstDnaB-MD (colored in pink) and p68N-NTD (colored in cyan). D, distribution of electrostatic surface potentials of GstDnaB-MD (left) and p68N (right).
Figure 6. Small angle X-ray scattering characterization of GstDnaB_{1-300} and GstDnaB_{FL}. A and B, experimental X-ray scattering curves (black lines) and theoretical fitting curves (red lines) of DnaB_{1-300} and DnaB_{FL} were generated with GNOM. The insets show the Guinier plots. C and D, the distance distributions of DnaB_{1-300} and DnaB_{FL}. E and F, ab initio models of DnaB_{1-300} and DnaB_{FL}. The models were obtained from GASBOR and superimposed with the crystal structure of DnaB_{1-300} using the SUPCOMB program. The color scheme is the same as that for Fig. 2A. G, experimental SAXS data (black line) and calculated scattering curves for the crystal structural of DnaB_{1-300} (red line) fitted by CRYSONL.
tetrameric conformation of GstDnaB<sub>1–300</sub> in both crystal form and in solution.

The overall GstDnaB<sub>FL</sub> envelope from the SAXS curve was elongated in shape, with an additional protruding density corresponding to the C-terminal region (residues 365–400). The protruding density was also found to be well-connected to the middle domain, so that the overall envelope resembled an “X-like” structure. To validate that the additional density could accommodate the C-terminal region of GstDnaB, we fitted the tetrameric conformation of the GstDnaB<sub>1–300</sub> crystal structure into the central region of the GstDnaB<sub>FL</sub> envelope using the SUPCOMB program. The docked crystal structure fit well into the SAXS envelope of GstDnaB<sub>FL</sub>, leaving additional density at the four corners that could adequately accommodate the C-terminal domain of GstDnaB (Fig. 6F).

Taken together, the results from our generated overall SAXS envelope and the corresponding docking studies support the unique structural conformation of the GstDnaB<sub>FL</sub> tetramer and the flexible nature of the C-terminal domain. This flexible C-terminal domain protruding from the stable tetramer points to the functional dynamics of GstDnaB in recognition and coiling of DNA substrate.

**Discussion**

Gram-positive bacterial DnaB is an essential protein required for both initiation and restart of parental DNA replication (19). Bacterial DnaB also associates with both DnaA and PriA loader proteins to initiate the cascade of DNA replication at oriC and stalled replication forks, respectively. Our structural and biochemical studies provide insights into the functional mechanics of this loader protein in primosome assembly. As shown in Fig. 3A, GstDnaB<sub>1–300</sub> forms a unique architecture of two basket-like structures in a domain-swap conformation. Our SAXS-derived envelope studies provide evidence that GstDnaB<sub>FL</sub> has an X-shaped conformation in solution. GstDnaB<sub>FL</sub> is an elongation of the crystal structure of GstDnaB<sub>1–300</sub> with a protruding C-terminal domain. Although previous studies concluded that BsuDnaB is a tetramer (consistent with our studies), the conformational architecture of GstDnaB differed from it greatly. We speculate that the previous low resolution EM tetrameric structure (29, 31) may be incorrect due to bias caused by choosing an inappropriate γ-complex clamp loader as an initial model, resulting in a different tetrameric arrangement. However, we cannot rule out the possibility that the different conformations might be due to the low sequence similarity of residues 148–205 (supplemental Fig. S1) from different bacterial species. As observed in our crystal structure, residues in this variable region (residues 148–205) also play some role in the formation of an interface between the monomers in the tetramer (Fig. 3).

Based on our crystal structure and SAXS data analysis, the X-shaped architecture of tetrameric GstDnaB<sub>FL</sub> has biological implications. The protruding GstDnaB C-terminal domains on both sides of the tetrameric conformation might be essential for its functional role in DNA binding, as these domains possess clusters of highly positively charged residues. Previous functional studies have shown that the BsuDnaB C-terminal region alone is sufficient to interact with both ssDNA and dsDNA substrate (34). The GstDnaB C-terminal region alone possesses 35 positively charged residues, the majority of which are clustered between residues 365 and 400 (see supplemental Fig. S1). Such a large number of highly positive residues clustering at one locus was not found for GstDnaB<sub>1–300</sub>. Although the overall tetrameric surface charge of GstDnaB<sub>1–300</sub> shows some exposed positively charged residues (supplemental Fig. S8), we did not detect noticeable DNA-binding activity even at protein concentrations of 240 μM. Instead, GstDnaB<sub>1–300</sub> exhibited interaction with GstPriA, and this association was relatively stable under ultracentrifugation. Association of DnaB with PriA is one of the key steps in the assembly of the primosome machinery during replication restart. BsuDnaB was not found to associate with PriA (22), which conflicts with our GstDnaB functional studies, but the non-association of DnaB with PriA in Bacillus species might be due to weak interactions under the in vitro conditions or low concentrations (10 μM) used in that study (22).

In addition, the lack of PriA-binding activity for GstDnaB<sub>1–200</sub> (see Fig. 1) suggests the importance of the middle domain in the PriA association. Our crystal structure and solution-scattering studies of GstDnaB<sub>1–300</sub> together with our in vitro functional studies of complex formation with PriA demonstrate the functional and structural roles of the N-terminal and middle domains of GstDnaB. Our ssDNA-binding studies also show that GstDnaB<sub>301–461</sub> (but not GstDnaB<sub>1–300</sub>) is involved in DNA binding.

In summary, we determined the crystal structure of a two-domain construct of DnaB from the Gram-positive G. stearothermophilus. The structure suggests a dimer-dimer tetrameric arrangement for DnaB and provides insights into the interdomain communication between the N-terminal and middle domains. Our biochemical assays also distinguished the PriA-binding and ssDNA-binding domains of GstDnaB, which extends our understanding of primosomal assembly and its mechanics.

**Experimental procedures**

**Plasmid construction**

The coding regions of full-length DnaB (DnaB<sub>FL</sub>) and truncated forms (DnaB<sub>1–300</sub>, DnaB<sub>301–461</sub>, DnaB<sub>1–200</sub>, and DnaB<sub>202–300</sub>) were individually generated by PCR amplification of genomic DNA isolated from G. stearothermophilus using Pfu DNA polymerase (Stratagene). NdeI and XhoI restriction sites were incorporated into forward and reverse primers, respectively, to permit insertion of amplified PCR products into the pET21b vector (Novagen) with a C-terminal His<sub>6</sub> tag for protein expression in E. coli. For N-terminal GST-tagged DnaB, the PCR-amplified coding region of DnaB<sub>FL</sub> or its truncated forms (DnaB<sub>1–300</sub> and DnaB<sub>301–461</sub>) were cloned into a pGEX-2TK vector using the BamHI and EcoRI sites. PCR amplification of the gene encoding full-length PriA (residues 1–801) was performed in a similar manner as that for DnaB, except for incorporation of NdeI and HindIII restriction sites. The resulting amplified fragment was cloned into pET21b (Novagen) with a C-terminal His<sub>6</sub> tag.
**Crystal structure of DnaB protein**

**Protein expression and purification**

Expression and purification procedures for all His<sub>6</sub>-tagged proteins (DnaB<sub>FL</sub>, DnaB<sub>1–300</sub>, DnaB<sub>301–461</sub>, DnaB<sub>1–200</sub>, DnaB<sub>201–300</sub> and PriA) were similar. Briefly, the individual plasmid was transformed and expressed in *E. coli* BL21 (DE3) cells. Transformed *E. coli* cells were cultured in LB medium containing 100 µg/ml ampicillin and grown at 37 °C until optical density (A<sub>600</sub>) reached 0.6. Overexpression of target protein was induced with 1 mM isopropyl-β-D-galactopyranoside and further incubated for 12 h at 20 °C. All purification procedures were performed at 4 °C. Cells were harvested by centrifugation and then suspended in an optimized buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5 mM imidazole (buffer A). The cell suspension was lysed by an M-110L Microfluidizer apparatus (Microfluidics) and then centrifuged at 30,000 × g for 30 min. The soluble cell extract was loaded onto a HisTrap HP (Ni<sup>2+</sup>-chelating) column equilibrated with buffer A. The column was washed with 10 column volumes of buffer A plus 50 mM imidazole to remove impurities. His<sub>6</sub>-tagged proteins were eluted with buffer A plus 200 mM imidazole. Eluted protein fractions were further purified by Superdex 200 gel filtration chromatography equilibrated with 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 5 mM β-mercaptoethanol (buffer B).

For the production of selenomethionine-substituted DnaB<sub>1–300</sub> protein (SeMet-DnaB<sub>1–300</sub>), cells were grown and expressed in Overnight Express Autoinduction System 2 medium (Novagen), and then cell suspensions were purified in a similar manner as that for DnaB<sub>1–300</sub>. All purified proteins were subjected to SDS-PAGE analysis, and purities of >95% were confirmed by peptide mass spectroscopy.

Recombinant GST-tagged proteins (GST-DnaB<sub>FL</sub>, GST-DnaB<sub>1–300</sub> and GST-DnaB<sub>301–461</sub>) were expressed as described above, and harvested cells were resuspended in 1 × phosphate-buffered saline (PBS) and then lysed. The lysate was clarified by centrifugation, and the resulting supernatant was loaded onto a GSTrap HP column equilibrated in buffer containing 1 × PBS buffer. The column was washed with 10 column volumes of 1 × PBS buffer. Bound GST-tagged protein was then eluted with an elution buffer containing 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 10 mM glutathione. Eluted protein fraction was further purified by gel filtration chromatography on a Superdex 200 column in 25 mM HEPES (pH 7.4), 500 mM NaCl, and 1 mM DTT before being subjected to SDS-PAGE analysis to confirm protein purity.

**Crystallization and X-ray data collection**

Crystallization trials were set up by using 15 mg/ml GstDnaB<sub>1–300</sub> in a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 5 mM β-mercaptoethanol. Initial screening was performed by using a Phoenix robot platform (Rigaku) with commercially available screening reagents (Hampton Research, Molecular Dimension, and Qiagen Ltd.) at 25 °C. Native crystals of GstDnaB<sub>1–300</sub> (0.15 mm × 0.15 mm × 0.02 mm) were obtained by the hanging-drop vapor diffusion method against a buffer containing 0.1 M Tris (pH 8.5) and 1.0 M ammonium sulfate. To solve the crystallographic phase problem, SeMet-GstDnaB<sub>1–300</sub> crystals were obtained against buffer containing 25% v/v ethylene glycol under hanging-drop vapor diffusion conditions and diffraction to 2.8 Å resolution. Both crystals belong to the space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, with cell dimensions of a = 110.04 Å, b = 117.12 Å, and c = 159.13 Å. Single-wavelength anomalous diffraction data were generated using synchrotron X-ray radiation at Beamline 15A1 of the National Synchrotron Radiation Research Center, Taiwan, and collected with a Rayonix MX300HE CCD Area Detector at 100 K. Selenium single-wavelength anomalous dispersion (Se-SAD) data acquired at a peak wavelength of 0.9792 Å was used to phase the SeMet-GstDnaB<sub>1–300</sub> crystal. The dataset was processed using the HKL2000 program suite (43), and statistics are shown in Table 1.

**Structural determination and refinement**

Identification of selenium positions and generation of the initial SAD phase of SeMet-GstDnaB<sub>1–300</sub> crystals at 2.8 Å resolution were performed using PHENIX (44, 45). After identifying 21 selenium positions, the initial phases were further refined using the maximum likelihood density modification algorithm in PHENIX. In the crystal there are four molecules in an asymmetric unit. Due to the limited resolution, automatic model building using PHENIX was not successful, so we used Coot (46) for model building. The final SeMet-GstDnaB<sub>1–300</sub> structure was obtained through iterative cycles of rebuilding and refinement using Coot (46) and PHENIX (44, 45), respectively. A total of 85 water molecules were found in the crystal structure by the general water molecule selection tool in PHENIX. The final structure was refined to an R factor of 22.8% and a R<sub>free</sub> value of 26.3% for the 1978 reflections (3.8%) of randomly chosen reflections. Due to the absence of an electron density for the region corresponding to residues 172–189, we could not trace and validate the secondary structure. The residues in the individual chains were identified as follows: chain A (residues 3–171 and 190–288), chain B (residues 7–171 and 190–288), chain C (residues 7–171 and 190–288), and chain D (residues 6–171 and 190–288). A PHENIX-generated Ramachandran plot and the ϕ-ψ angles for GstDnaB<sub>1–300</sub> show that 96.9% of residues are in the most favored regions, and 3.1% are in allowed regions. However, due to the unclear or discontinuous electron density map located in several loop regions, there are still some residues from different chains with poor fit to the density. Refinement statistics are summarized in Table 1. The figures were produced using PyMOL (53). Solvent-accessible and interface areas were calculated by PISA (36). The atomic coordinate and the structure factor for GstDnaB<sub>1–300</sub> have been deposited in the Protein Data Bank under accession code 5WTN.

**GST pulldown assays**

Purified GST (200 µg) or three GST-tagged DnaB proteins (200 µg: GST-DnaB<sub>FL</sub>, GST-DnaB<sub>1–300</sub>, GST-DnaB<sub>301–461</sub>) were individually mixed with 10 µl of glutathione agarose beads at room temperature for 30 min. The beads were pelleted by centrifugation and washed with 1 ml of a binding buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.05% Tween 20. The resulting immobilized indi-

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**Table 1**

| Realization | Resolution Range (Å) | R Factor (%) | R<sub>Free</sub> (%) |
|-------------|----------------------|-------------|-------------------|
| GSTDnaB<sub>1–300</sub> | 2.8 | 22.8 | 26.3 |

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**Table 2**

| Chain | Residues | r<sup>2</sup> (%) |
|-------|----------|-----------------|
| A | 3–171 and 190–288 | 96.9 |
| B | 7–171 and 190–288 | 96.9 |
| C | 7–171 and 190–288 | 96.9 |
| D | 6–171 and 190–288 | 96.9 |
individual proteins were then incubated with 700 μg of PriA monomer at room temperature for 30 min. Beads were pelleted and washed 5 times with 1 ml of a wash buffer containing 50 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 0.05% Tween 20. The bound proteins were eluted from the beads by boiling in SDS sample buffer before being subjected to SDS-PAGE. Western blotting was then performed to detect PriA interactions with GST alone or with the three GST-tagged DnaB proteins.

**Sedimentation-velocity analytical ultracentrifugation**

AUC experiments were performed with an XL-1 analytical ultracentrifuge (Beckman-Coulter, Brea, CA) equipped with a four-cell An60 Ti rotor to analyze the sedimentation velocity of PriA and DnaBFL (or its truncated variants: DnaB1–300, DnaB301–461, DnaB1–200, and DnaB202–300) alone or as complexes (DnaBFL/PriA, DnaB1–300/PriA, DnaB301–461/PriA, DnaB1–200/PriA, and DnaB202–300/PriA) at 20 °C. The molar ratio of different forms of DnaB and PriA was ~1:1, and the total A₂₈₀ was ~0.6. Buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT was used as the reference solution for all centrifugation experiments. The sedimentation velocity experiments for DnaBFL/PriA, DnaB1–300/PriA, DnaBFL/PriA, and DnaB1–300/PriA were performed at 40,000 rpm in a sample volume of 450 μl. Due to smaller molecular weights, DnaB1–200/DnaB202–300, DnaB1–200/PriA, and DnaB202–300/PriA samples were ultracentrifuged at a speed of 45,000 rpm. Absorbance scans were taken at 280 nm. Data were analyzed with the SEDFIT program (47) to fit a continuous distribution model, c(s).

** Isothermal titration calorimetry**

Experiments were carried out using the MicroCal iTC200 system (GE Healthcare). For all isotherms, the sample cell was filled with 0.1 mM GStPriA in 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl, and the syringe was loaded with 1 mM GStDnaB1–300 in the same buffer. The binding isotherm was obtained from 20 injections of GStDnaB1–300 into GStPriA at 25 °C. For the first titration, an injection volume of 1 μl was used; for subsequent titrations, 2 μl of GStDnaB1–300 was injected with an interval of 180 s. The stirring speed and reference power were 1000 rpm and 5 μcal/s, respectively. Binding isotherms were integrated and analyzed using Origin v7.0 software (MicroCal).

**Small angle X-ray scattering analyses**

Protein samples for SAXS analysis were collected by gel filtration. The SAXS experiments for DnaBFL and DnaB1–300 were performed at Beamline 23A at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan, equipped with a MAR 165 CCD detector at a sample-to-detector distance of 2.5 m. The X-ray wavelength was 0.8285 Å, with an energy of 15.0 keV, and the collection time was 300 s. Protein sample concentrations were 14 mg/ml and 20 mg/ml for DnaBFL and DnaB1–300, respectively. The gel filtration buffer (20 mM Tris (pH 8.0), 300 mM NaCl, and 5 mM β-mercaptoethanol) was used as the solvent blank. No sample aggregation was observed during the measurements. Background scattering from the buffer alone was subtracted from the sample, and data were scaled using PRIMUS (48). Primary data reduction was done with an NSRRC 23A SXAS data reduction program and further analyzed using the ATSAS package (49). The Rg was calculated from a Guinier plot using PRIMUS. The pair distance distribution function P(r) and the maximum distance Dmax were calculated using GNOM (50). The low-resolution shapes of protein samples were determined as ab initio models from the scattering data by GASBOR (51). The eight ab initio models from the eight individual GASBOR runs had a similar overall architecture. The eight ab initio models were aligned, averaged, and scored with a normalized structural difference (NSD) using DAMAVER (41). The eight ab initio models of DnaBFL and DnaB1–300 agreed well, yielding 1.071 ± 0.336 and 1.231 ± 0.088 (NSD ± S.D.), respectively. The SAXS data were fitted with the crystal structure of DnaB1–300 using CRYSOL (52). The SAXS envelopes and crystal structure of DnaB1–300 were superimposed using SUPCOMB (42). Data collection and other SAXS measurements together with the programs used are summarized in supplemental Table S1.

**Electrophoresis mobility shift assays**

To determine ssDNA-binding ability, interactions of purified recombinant proteins (GStDnaBFL, GStDnaB1–300, and GStDnaB301–461) with ssDNA (dT40) substrate were resolved on 8% polyacrylamide gels. All purified proteins were analysis by SDS-PAGE to confirm the purity (supplemental Fig. 59). Binding assays were carried out in a final volume of 20 μl of DNA-protein reaction mixture in buffer B for 30 min at 20 °C, with the proteins at the indicated concentrations against 20 nm dT40 ssDNA substrate labeled at the 5’ end with Cy5 (MDBio, Inc.). Post-reaction, the samples were mixed with 6X gel loading buffer (60 mM Tris-HCl (pH 7.6) and 30% glycerol) and the protein-ssDNA complexes were electrophoresed on 8% native polyacrylamide gels in 0.5X TBE buffer (45 mM Tris borate, 1 mM EDTA (pH 8.0)) at 4 °C for 160 min. The gels were immediately scanned for the florescence signals using the Cy5 channel with Typhoon FLA 9000 (GE Health) for visualization of DNA bands. The fluorescent signals in each DNA bands were quantified by using ImageJ software. Results of three independent repeats for ssDNA-protein affinity assays were measured. The Kᵣ values were determined using the one-site saturation equation by SigmaPlot 10.0 software.

**Author contributions**—Y.-C. L. and C.-D. H. conceived and designed the experiments. Y.-C. L., V. N., and M.-G. L. performed the experiments. Y.-C. L., V. N., M.-G. L., and C.-D. H. analyzed the data. Y.-C. L., V. N., and C.-D. H. wrote the paper. All authors reviewed the results, contributed to the data interpretation, and approved the final version of the manuscript.

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