966–974 Nucleic Acids Research, 2002, Vol. 30, No. 4

A functional interaction between the putative primosomal protein DnaI and the main replicative DNA helicase DnaB in *Bacillus*

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Received October 23, 2001; Revised and Accepted November 28, 2001

ABSTRACT

In Gram negative *Escherichia coli* there are two well-characterised primosomal assembly processes, the PriA- and DnaA-mediated cascades. The presence of PriA and DnaA proteins in Gram positive *Bacillus* spp. supports the assumption that both the PriA- and DnaA-mediated primosomal assembly cascades also operate in these organisms. However, the lack of sequence homology between the rest of the primosomal proteins indicates significant differences between these two bacterial species. Central to the process of primosomal assembly is the loading of the main hexameric replicative helicase (DnaB in *E.coli* and DnaC in *Bacillus subtilis*) on the DNA. This loading is achieved by specialised proteins known as 'helicase loaders'. In *E.coli* DnaT and DnaC are responsible for loading DnaB onto the DNA during primosome assembly, in the PriA- and DnaA-mediated cascades, respectively. In *Bacillus* the identity of the helicase loader is still not established unequivocally. In this paper we provide evidence for a functional interaction between the primosomal protein DnaI from *B.subtilis* and the main hexameric replicative helicase DnaB from *Bacillus stearothermophilus*. Our results are consistent with the putative role of DnaI as the 'helicase loader' in the Gram positive *Bacillus* spp.

INTRODUCTION

DNA replication is a complex process that can be separated into multiple stages. First is the initiation stage that involves the recognition of specific DNA sequences (origins of replication) by specialised initiator proteins. These proteins prepare the stage for the assembly of the primosomal complexes, in order to 'prime' the DNA replication process. Priming is an essential step during DNA replication and stems from the inability of DNA polymerases to synthesize DNA *de novo*. Primosomal assembly takes place in discrete steps and involves a multitude of different proteins that act in a sequential and coordinated manner (primosomal cascades) in order to assemble into large primosomal complexes. Two separate primosomal cascades have been identified and characterised in *Escherichia coli* (for reviews see 1,2). The PriA-mediated cascade involves PriA as the initiator protein. PriA binds to the DNA initiation site and recruits another protein, known as PriB, onto the site. DnaT then loads the hexameric helicase, DnaB, from a DnaB–DnaC complex in solution onto the PriA–PriB–DNA complex, forming the pre-primosome. Completion of the primosome requires addition of the primase, DnaG. The second cascade involves DnaA as the initiator protein that binds onto the DNA initiation site and then draws the DnaB–DnaC complex from solution onto the initiation site. In this case, DnaC acts as the helicase loader. Completion of the primosome then takes place with the addition of DnaG, as before.

PriA and DnaA homologues also exist in *Bacillus* spp., suggesting that at least the initial stages of primosomal assembly are similar in both species. Despite the additional presence of homologues of the helicase and primase in *Bacillus*, the rest of the primosomal proteins (PriB, PriC, DnaT and DnaC in *E.coli* and DnaB, DnaD and DnaI in *Bacillus subtilis*), with the exception of DnaC and DnaI, share no sequence homologies, implying that some aspects of primosomal assembly in the two species may be different. At this point, to avoid confusion that might be created by the nomenclature of these proteins, we should emphasize that DnaB in *B.subtilis* is a primosomal protein and not the main replicative helicase DnaB, as is the case in *Bacillus stearothermophilus*. Based upon weak sequence homology (Fig. 1), the *Bacillus* DnaI protein has been assigned as the *E.coli* DnaC homologue and is assumed to fulfil the role of the helicase loader. However, the sequence homology between the two proteins is confined largely to putative Walker A and B motifs characteristic of nucleotide triphosphate (NTP)-binding enzymes (3,4) and there is still no experimental evidence to support this assumption.

In an effort to elucidate the role of DnaI during primosomal assembly in *Bacillus* we have cloned the dnaI gene from *B.subtilis*, over-expressed it and purified the DnaI protein (36 kDa) to homogeneity and characterised its interaction with the main replicative DNA helicase, DnaB (50.5 kDa), from *B.stearothermophilus*. Our data show that DnaI interacts with DnaB, forming a stable complex that can be isolated by size exclusion chromatography. This interaction is mediated by the 33 kDa C-terminal domain (P33) of DnaB and results in an alteration of the highly cooperative ATPase profile of DnaB and a marked stimulation of its helicase activity. Furthermore, binding of DnaI to DnaB does not interfere with binding of the

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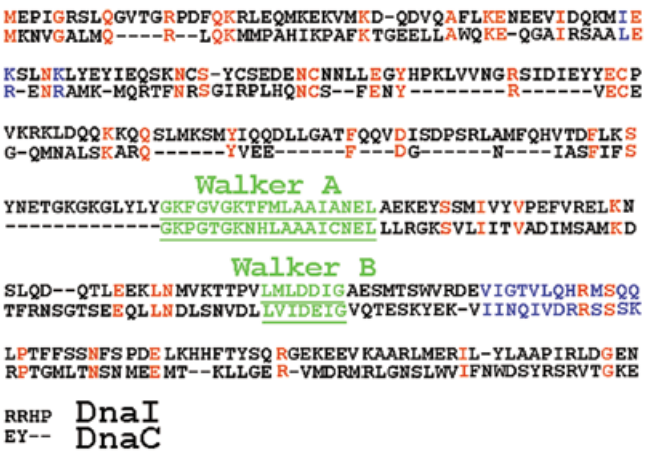

Figure 1. An amino acid sequence alignment of the DnaI (*B.subtilis*) and DnaC (*E.coli*) proteins. Identical amino acids are shown in red whereas sequences of extended similarity are shown in blue. The sequences that resemble Walker A and B motifs and are characteristic of NTP-binding/hydrolysing enzymes are underlined, labelled and coloured green.

primase, DnaG, to DnaB, as a stable triple complex of DnaI–DnaB–DnaG can also be isolated by size exclusion chromatography. However, binding of DnaI to the DnaB–DnaG complex results in inhibition of the stimulatory effect of DnaG on DnaB helicase activity, as the triple DnaI–DnaB–DnaG complex exhibits reduced helicase activity. A comparison with the previously characterised interaction of the *E.coli* homologue, DnaC, and the helicase, DnaB, reveals important differences, implying that loading of the ring helicase onto the DNA during primosomal assembly in *Bacillus* may be achieved by a different mechanism to that employed in *E.coli*. Our data are consistent with the putative role of DnaI as the helicase loader in *Bacillus* and show that the DnaI–DnaB interaction is functional.

MATERIALS AND METHODS

Cloning of dnaI from *Bacillus subtilis*

Genomic DNA was prepared from a 50 ml overnight culture of *B.subtilis* as follows. The cells were spun down at 5000 *g* and resuspended in 3 ml of 10 mM Tris–HCl, pH 7.5, 100 mM NaCl and 200 µl of 20 mg/ml lysozyme. Then 200 µl of 10% (w/v) SDS was added and the cells were incubated at 37°C for 1 h. The mixture was extracted twice with an equal volume of phenol and once with an equal volume of chloroform. The DNA was ethanol precipitated and redissolved in 50 µl of 10 mM Tris–HCl, pH 8.0. PCR primers for the dnaI gene were synthesized with restriction sites for *Nco*I and *Bam*HI incorporated into the forward and reverse primers, respectively. The forward primer had the sequence 5′-GCGACCATGGAACCAATCGGCCGTTCC-3′ and the reverse primer 5′-GCTCGGATCCTTATGGATGTCGGCGGTTTTCTC-3′. The PCR reaction was carried out in a total volume of 50 µl using 200 pmol each primer, 2 µg genomic DNA, 2 mM $MgCl_2$ and 250 µmol dNTPs. Thirty cycles of amplification were carried out using *Taq* polymerase (Promega). The PCR product was gel purified using a QiaexII kit (Qiagen), digested overnight with *Nco*I and *Bam*HI, then ligated into the *Nco*I and *Bam*H1 sites of a predigested pET28c vector (Novagen) to form the pET28c-dnaI expression vector.

Protein purification

Purification of DnaB, DnaG, P33 and PcrA was carried out as described elsewhere (5–8). The expression vector pET28c-dnaI, carrying the dnaI gene, was transformed into the B834 expression strain (Novagen). After growing the cells overnight on an agar plate in the presence of kanamycin (Kan), single colonies were isolated and used to inoculate 5 ml of LB/Kan mini-cultures that were also grown overnight. These mini-cultures were used to inoculate 1 l of LB/Kan cultures that were then cultured in a shaking incubator at 37°C until the OD_{600} reached 05–0.6. Following induction by IPTG (1 mM) the cells were cultured for a further 3 h under vigorous shaking. The cells were then harvested by centrifugation and redissolved in 10 ml of buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT) plus 10% (w/v) sucrose per litre of cultured cells. The cells were disrupted by sonication and the crude lysate was clarified by centrifugation at 17 500 r.p.m. for 30 min. The clarified supernatant was collected and the protein was precipitated by adding an equal volume of saturated ammonium sulphate solution. The protein pellet was collected by centrifugation at 17 500 r.p.m. for 30 min and redissolved in buffer A. The conductivity was adjusted to be equivalent to 100 mM NaCl. The protein was loaded onto a Fast Flow Sepharose Q column pre-equilibrated with buffer A. The conductivity was adjusted to be equivalent to 50 mM NaCl by mixing with buffer A during the loading process. The column was washed with buffer A and DnaI was eluted with a salt gradient (0–500 mM NaCl over 180 ml) in buffer A. The fractions containing DnaI were pooled and the protein was precipitated by adding an equal volume of saturated ammonium sulphate solution. The protein pellet was washed briefly with distilled water and then dissolved in buffer A until the conductivity was equivalent to 50 mM NaCl. The protein solution was loaded onto a pre-packed MonoQ HR 10/10 column (Amersham Pharmacia Biotech) pre-equilibrated with buffer A. The column was washed with buffer A and DnaI was eluted with a shallow salt gradient (0–150 mM NaCl over 60 ml). Fractions containing the DnaI protein were pooled and the conductivity was adjusted to be equivalent to 50 mM NaCl. The protein was then loaded onto a 5 ml Hi-Trap heparin column pre-equilibrated with buffer A. After washing with buffer A, DnaI was eluted with a shallow salt gradient (0–150 mM NaCl over 120 ml). Finally, DnaI was purified further by size exclusion chromatography through a Superdex S-75 column (Amersham Pharmacia Biotech) in buffer A plus 100 mM NaCl. Typically, 10–15 mg highly pure DnaI was obtained from 2 l of B834 cells. The purified DnaI was shown to be >99% pure as judged by SDS–PAGE (Fig. 2).

Size exclusion chromatography

The interactions between the DnaB, DnaI, DnaG and P33 proteins were studied using an AKTA purifier and an analytical Superdex S-200 10/30 size exclusion chromatography column (Amersham Pharmacia Biotech). Typically, size exclusion chromatography was carried out in low salt (buffer A + 100 mM NaCl) or high salt (buffer A + 500 mM NaCl) conditions using 0.1–0.5 mg protein(s). The flow rate was set at 0.5 ml/min and 0.5 ml fractions were collected. Samples from the peak fractions were then analysed by SDS–PAGE, as appropriate. Protein–protein complexes were pre-formed prior to loading onto

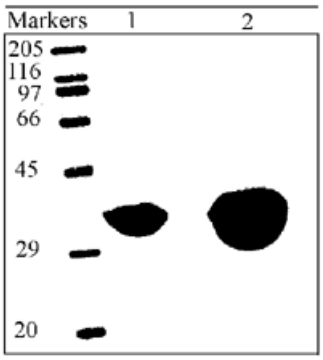

Figure 2. Assessing the purity of DnaI. Purified DnaI was analysed by SDS–PAGE through a 10% polyacrylamide gel. Aliquots of 20 and 250 μg DnaI were loaded in lanes 1 and 2, respectively. Molecular weight markers are shown in kDa.

the column by mixing the relevant proteins and incubating at room temperature for at least 10 min in the appropriate buffer.

ATPase assays

ATP hydrolysis was monitored using a coupled (ATP regeneration) assay by linking it to NADH oxidation (9). ATPase assays for DnaB were carried out at room temperature exactly as previously described (6). ATPase assays for DnaI were carried out under similar conditions using 0.3 μM DnaI, but no activity could be detected either in the presence or absence of ssDNA under our assay conditions (see Results). Typically, ATPase activity for DnaB was assayed using 24 nM DnaB (with reference to the monomer) in the reaction mixture in the presence or absence of 160 nM DnaI. The effect of DnaI on DnaG-mediated stimulation of ATPase activity was examined by adding DnaI to a DnaB–DnaG complex (pre-mixed) prior to addition to the ATPase assay reaction mixture. The final concentrations of DnaB, DnaG and DnaI in the reaction mixtures were 24, 160 and 160 nM, respectively. The ATPase activities of P33 and PcrA were assayed in a similar manner using 72 and 4.8 nM protein, respectively, and in the presence of 2.4 μM oligo(dT)$_{16}$. The effect of DnaI on the ATPase profiles of P33 and PcrA was examined by including increasing amounts (123 nM and 1.34 μM) of DnaI in the reaction mixture.

Helicase assays

The helicase activity of DnaB was assayed by monitoring the displacement of a short (68mer) radioactively labelled oligonucleotide annealed onto ssM13mp18 DNA, as described elsewhere (7). The annealed oligonucleotide produced a substrate with both 5′- and 3′-tails. Details of the oligonucleotide sequence and of the labelling and annealing procedures are given in Pan *et al*. (6). Helicase reactions were carried out at 0.12 μM DnaB (with reference to the monomer), 0.5 nM DNA substrate (annealings were done with a slight excess of ssM13mp18 DNA over labelled oligo, so the substrate concentration refers to that of the labelled oligo) and 3 mM ATP. These assay conditions correspond to one DnaB hexamer per 187 nt of ssM13mp18 DNA substrate. The effect of DnaI on DnaB helicase activity was examined by pre-incubating DnaB with a 5× molar excess of DnaI prior to adding the proteins to the reaction mixture. The final concentration of DnaI in the reaction mixture was 0.6 μM. The effect of DnaI on helicase activity of the DnaB–DnaG complex was examined as described above for DnaB alone, but in this case DnaG was also present in molar excess over DnaB. The final concentration of DnaG in the reaction mixture was 0.6 μM.

RESULTS

DnaI interacts with DnaB

Size exclusion chromatography was used to establish whether DnaI interacts with DnaB. Highly purified DnaI and DnaB proteins were mixed as described in Figure 3. DnaI (11.5 μM) was present in 5× molar excess over DnaB (2.3 μM). The proteins were allowed to form a complex by incubation at room temperature for 15 min and the complex was resolved by size exclusion chromatography. DnaB formed very stable hexamers (7) (Fig. 3A), whereas DnaI was eluted as a monomer (Fig. 3A). However, the DnaB–DnaI complex eluted slightly earlier than the DnaB hexamer because of its larger size (Fig. 3A). Formation of the DnaB–DnaI complex was verified by SDS–PAGE, as shown in Figure 3B. Furthermore, the stability of the complex was assessed by isolating the DnaB–DnaI complex and running it again through the gel filtration column. No dissociation of the complex was observed (data not shown). Therefore, DnaB and DnaI interact to form a very stable complex that can be isolated by size exclusion chromatography.

What is the stoichiometry of the DnaB–DnaI interaction?

An important question to answer is the stoichiometry of the DnaB–DnaI complex. Determining the molecular weight of the DnaB–DnaI complex by size exclusion chromatography is not accurate enough to reveal how many molecules of DnaI per DnaB hexamer interact to form the complex. Instead, we employed SDS–PAGE and comparative protein staining to try and answer this question. Figure 4 shows the results of such an experiment. By comparing the relative strength of protein staining in lanes 1 and 4 we can conclude that the DnaB–DnaI interaction is not equimolar. A comparison of the relative strength of protein staining in lane 4 suggests that either one or two molecules of DnaI interact with one hexamer of DnaB. However, this stoichiometry should be considered with caution because although we have used a 5× molar excess of DnaI over DnaB (with reference to the monomer), we can only assume that all of the DnaB is in a complex with DnaI. If some of the DnaB failed to form a complex with DnaI then the apparent stoichiometry would be altered somewhat. However, given the stability of the DnaB–DnaI complex (as described above) and the large molar excess of DnaI over DnaB, we believe that all of the DnaB is likely to be in complex with DnaI under our experimental conditions.

Is the DnaB–DnaI interaction functional?

Having detected a physical interaction between DnaB and DnaI by isolating the stable DnaB–DnaI complex, the next step was to establish whether this interaction has any functional relevance. Is it fortuitous or does it have any effect on the function of DnaB? Although DnaI by itself has no detectable helicase activity (data not shown), when complexed with DnaB it stimulates the activity of the helicase significantly (Fig. 5A).

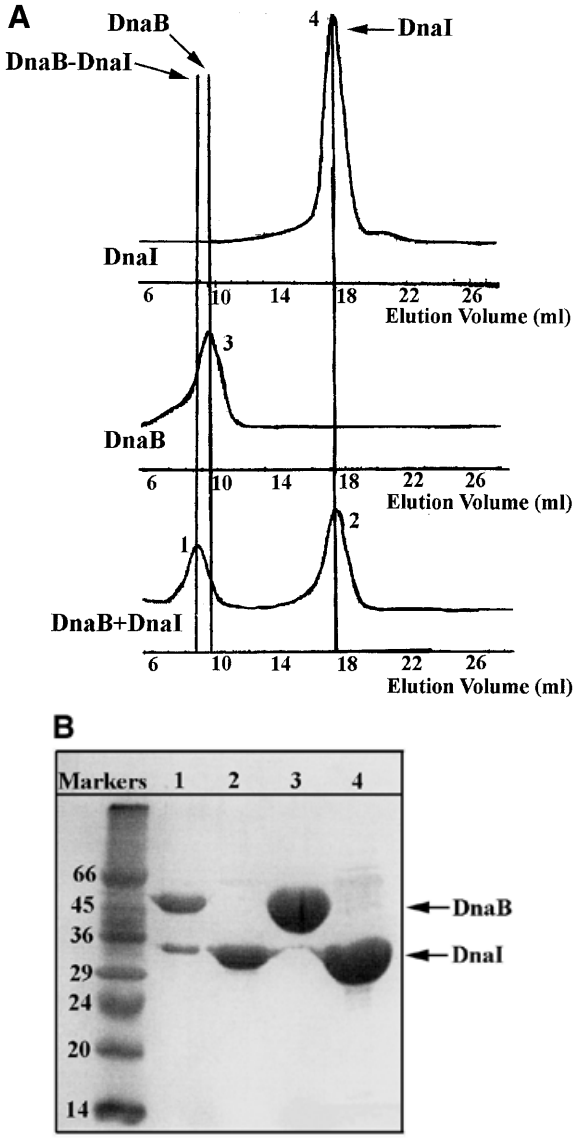

Figure 3. Investigating the DnaB–DnaI interaction by size exclusion chromatography. (**A**) Pure DnaI and DnaB proteins were concentrated to 31 and 4.6 μM, respectively, in 0.5 ml of buffer A, 200 mM NaCl, 1 mM DTT and then loaded separately onto a Superdex S-200 10/30 column pre-equilibrated in the same buffered solution. Size exclusion chromatography was carried out as described in Materials and Methods. DnaI and DnaB eluted as single peaks, corresponding to the monomer and hexamer, respectively. Pure DnaB (2.3 μM, with reference to the monomer) and DnaI (11.5 μM) proteins were pre-mixed in a total volume of 0.5 ml of the same buffered solution as above. The proteins were incubated at room temperature for 15 min to allow for formation of the complex. After incubation they were loaded onto the same column and resolved by size exclusion chromatography, as described above. Two well-separated peaks appeared in the elution profile. One of the peaks corresponded precisely to DnaI whereas the other peak corresponded to the DnaI–DnaB complex and was shifted slightly to the left relative to the peak corresponding to the DnaB hexamer. (**B**) Samples from all the peaks (numbered 1–4) were resolved by SDS–PAGE. The gel verified the presence of DnaI in peak 1, indicating the formation of a stable DnaB–DnaI complex. The sizes of appropriate molecular weight markers are shown in kDa units.

Does DnaI stimulate the ATPase activity of DnaB?

The stimulation of the helicase activity of DnaB could be the direct result of a concomitant stimulation of its ATPase activity by DnaI. Therefore, we compared the ATPase activity

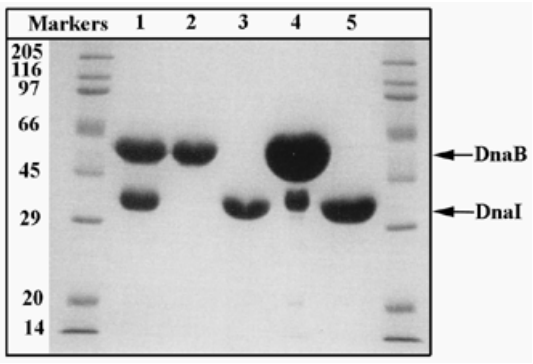

Figure 4. Estimation of the stoichiometry of the DnaB–DnaI interaction by SDS–PAGE and comparative Coomassie staining of proteins. Lane 1 shows staining of an equimolar mixture of DnaB and DnaI. Lanes 2 and 5 show purified DnaB and DnaI proteins, respectively. Lane 4 shows staining of the relative amounts of proteins in a purified DnaB–DnaI complex, whereas lane 3 shows excess DnaI that did not bind to DnaB during complex formation. Molecular weight markes are indicated in kDa and shown on both sides of the gel.

profiles of DnaB in the presence and absence of DnaI. The ATPase activity profile of hexameric DnaB is complicated because of cooperativity effects between the subunits of the hexamer (7). Such complications make it very difficult to explain in kinetic terms the details of ATP binding and hydrolysis. Instead, our analysis is somewhat superficial and confined to qualitative effects induced by DnaI on the ATPase activity profile of DnaB. Our data show that DnaI alters the ATPase activity profile of DnaB significantly (Fig. 5B). In the presence of DnaI the maximal ATPase activity was detected at 3.5 mM ATP, compared to 2.5 mM in the absence of DnaI. A comparison of the two profiles shows that in the presence of DnaI there is a shift to the right, towards higher concentrations of ATP (Fig. 5B). ATPase assays carried out in the presence of ssDNA co-factor also showed a similar effect in the presence of DnaI (data not shown). DnaI by itself has no detectable ATPase activity under our assay conditions (data not shown).

Mapping the DnaB–DnaI interaction

We have previously shown that *B.stearothermophilus* DnaB consists of two distinct domains, a 17 kDa (P17) N-terminal domain and a 33 kDa (P33) C-terminal domain, both of which can be purified in large quantities (7). P33 possesses all of the helicase signature motifs but has no detectable helicase activity despite the fact that it has good ATPase activity (7). Furthermore, its ATPase profile is drastically different to that of the full-length protein, probably reflecting the fact that it has lost the cooperative effects between the subunits, as it lacks the P17 N-terminal domain and does not form hexamers under low salt conditions (7). P33 hexamers will only form under high salt conditions, whereas under low salt conditions P33 exists as a mixture of lower oligomers (dimers and trimers). DnaI was found to interact with P33 only under high salt conditions (500 mM NaCl), forming a stable complex that was isolated by size exclusion chromatography (Fig. 6). A comparison of the relative strength of the protein staining in Figure 6A suggests that the stoichiometry of the P33–DnaI complex is the same as that observed for the full-length DnaB–DnaI complex.

The interaction of DnaI with P33 also has a functional effect. This effect is somewhat different to that observed for

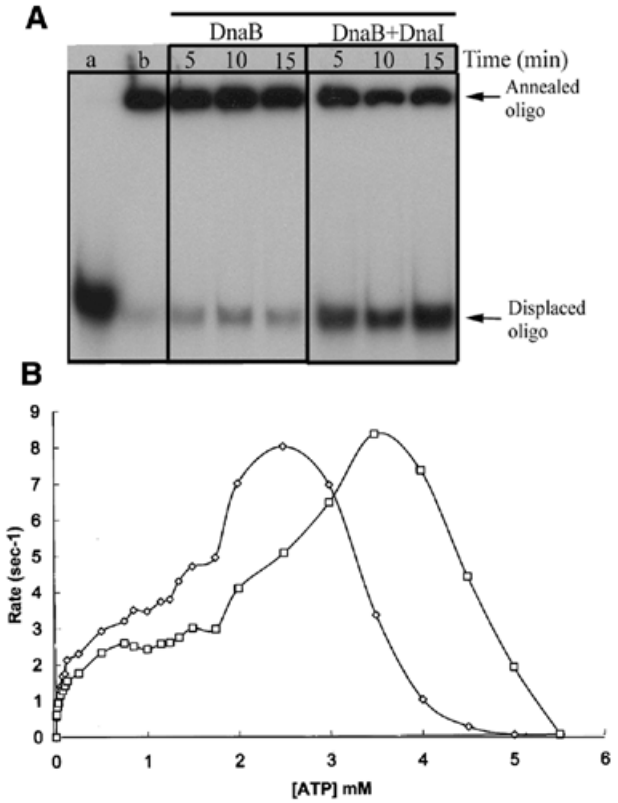

Figure 5. (**A**) Stimulation of the helicase activity of DnaB by DnaI. Lanes a and b show boiled and annealed substrate, respectively. Helicase assays were carried out as described in Materials and Methods either in the presence or absence of excess DnaI. A marked stimulation of DnaB helicase activity is observed in the presence of DnaI. (**B**) The effect of DnaI on the ATPase activity of DnaB. ATPase assays were carried out at different ATP concentrations, as described in Materials and Methods. All reactions were carried out with 24 nM DnaB (with reference to the monomer) in the absence of ssDNA. The effect of DnaI was examined by pre-incubating DnaB with a 6.7× molar excess of DnaI prior to adding the two proteins to the reaction mixture. The final concentration of DnaI in the reaction mixture was 160 nM. The ATPase profiles shown represent DnaB (diamonds) and DnaB–DnaI (squares).

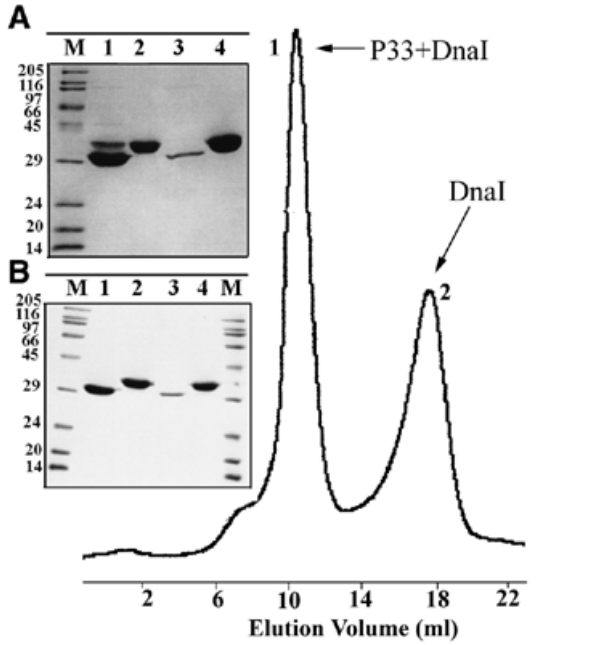

Figure 6. Investigating the P33–DnaI interaction by size exclusion chromatography. P33 and DnaI proteins were mixed in equimolar concentrations (30.3 μM, with reference to the monomers) in 0.5 ml of buffer A plus 500 mM NaCl and incubated at room temperature for 15 min. The proteins were then resolved by size exclusion chromatography as described in Materials and Methods. Samples from the peaks (numbered 1 and 2) were analysed by SDS–PAGE, as shown in (**A**). Control samples of pure P33 and DnaI proteins were also included in lanes 3 and 4, respectively. The same experiment was repeated using buffer A plus 100 mM NaCl. The results of the SDS–PAGE analysis from this experiment are shown in (**B**).

the full-length DnaB protein. We have shown previously that the ATPase profile of P33 is different to that of the full-length DnaB protein, probably attributable to the presence of the dimer under the low salt conditions employed in the ATPase assays, rather than the hexameric full-length protein (7). DnaI inhibits the ATPase activity of P33 in a concentration-dependent manner (Fig. 7A). Furthermore, this inhibition seems to be specific for P33, as DnaI fails to affect the ATPase activity of another DNA helicase protein, PcrA, under identical assay conditions (Fig. 7B). The fact that we cannot detect a stable P33–DnaI complex by gel filtration under low salt conditions (Fig. 6B) but can detect an inhibitory effect on P33 ATPase activity in the presence of DnaI suggests that the P33–DnaI interaction is transient under low salt conditions. From these data we can conclude that DnaI interacts with the C-terminal domain of DnaB.

The DnaG–DnaB–DnaI triple complex

We previously reported a stable complex between DnaB and DnaG (7) and were thus interested to find out whether DnaI affects the biochemical properties of the DnaB–DnaG complex *in vitro*. Since DnaG greatly stimulates the ATPase and helicase activities of DnaB (7), we proceeded to investigate the effect of DnaI on helicase activity of the DnaB–DnaG complex. DnaI was found to inhibit DnaG-mediated stimulation of the helicase activity of DnaB (Fig. 8A). DnaG stimulation of the helicase activity of DnaB is the result of a concomitant stimulation of its ATPase activity (7). Is, therefore, the observed reduced helicase activity of the DnaB–DnaG complex the direct result of inhibition of its ATPase activity in the presence of DnaI or is it the result of direct interference of DnaI with formation of the DnaB–DnaG complex? An investigation of the effect of DnaI on the ATPase profile of the DnaB–DnaG complex revealed that there is no inhibition of the ATPase activity, instead there is a shift in the reaction profile (Fig. 8B) similar to that observed for the effect of DnaI on the ATPase activity of DnaB (Fig. 5B). The reactions shown in Figure 9 were carried out in the absence of ssDNA, but equivalent reactions carried out in the presence of ssDNA gave qualitatively similar results, i.e. there was a similar shift in the ATPase activity profile (data not shown). Given the complexity of the DnaB ATPase kinetics and the lack of a kinetic mechanism it is not possible to interpret these data kinetically. Instead, we again limit ourselves to a superficial qualitative description of the DnaI-induced effects. A stable triple complex DnaG–DnaB–DnaI can also be isolated by size exclusion chromatography (Fig. 9), eliminating the suggestion that DnaI might somehow interfere with formation of the DnaB–DnaG complex. In the experiments shown in Figure 9, DnaG was added to a pre-formed and

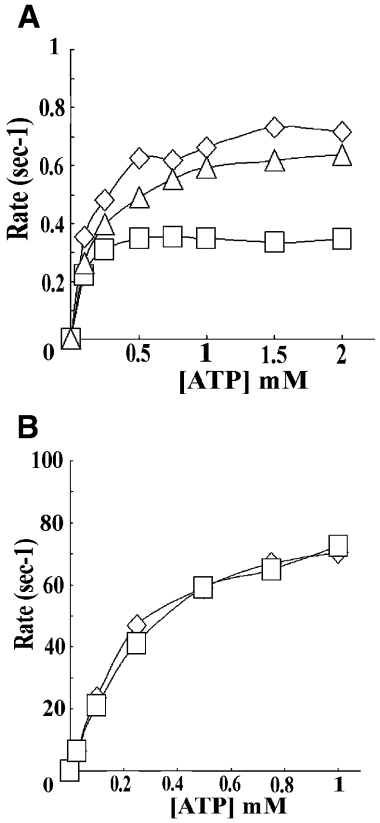

Figure 7. The effect of DnaI on the ATPase activity of P33 (**A**) and PcrA (**B**). ATPase assays were carried out at 72 nM P33 (with reference to the monomer) or 4.8 nM PcrA and in the presence of 2.4 μM oligo(dT)$_{16}$ at varying ATP concentrations, as described in Materials and Methods. The effect of DnaI was examined by pre-incubating P33 or PcrA with a molar excess of DnaI prior to addition of the proteins to the reaction mixture. In (A) the reactions were carried out in the presence of 123 nM (triangles), 1.34 μM (squares) and no (diamonds) DnaI. The reactions shown in (B) were carried out in the presence of 1.34 μM (squares) or no (diamonds) DnaI.

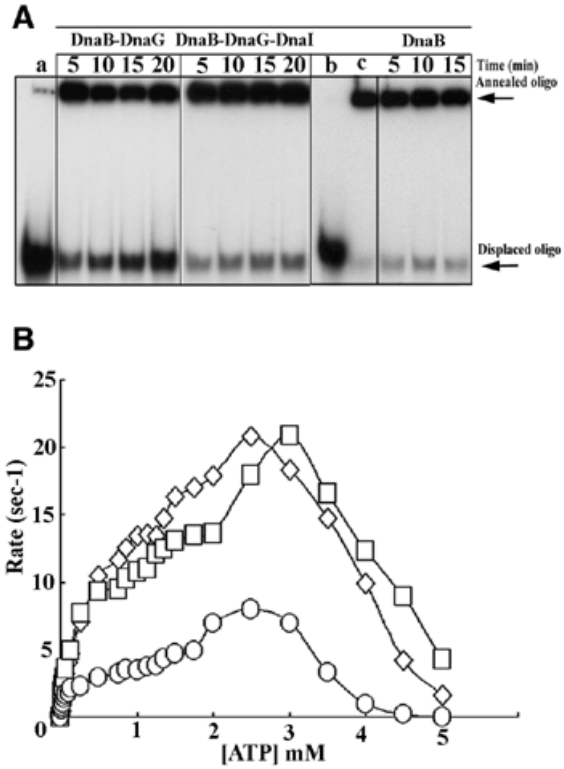

Figure 8. (**A**) The effect of DnaI on helicase activity of the DnaB–DnaG complex. Lanes a and b represent boiled controls to show full displacement of the annealed oligonucleotide, whereas lane c represents the fully annealed oligonucleotide prior to boiling. Experimental details are given in Materials and Methods. (**B**) The ATPase activity profile of DnaB in the presence of both DnaG and DnaI. The reaction profiles shown represent reactions carried out in the presence of DnaB–DnaG (diamonds), DnaB–DnaG–DnaI (squares) and DnaB (circles). Experimental details are explained in Materials and Methods. The reactions shown were carried out in the absence of ssDNA co-factor.

purified DnaB–DnaI complex. Under our experimental conditions DnaG interacted with the DnaB–DnaI complex forming a stable DnaG–DnaB–DnaI triple complex. No dissociation of DnaI from the complex was observed, indicating that both DnaG and DnaI can interact simultaneously with DnaB at different sites.

DISCUSSION

Primosomal assembly is a complex process involving as many as eight proteins in *E.coli* that interact in a coordinated and sequential manner to form the primosome at a specific site on the DNA known as the 'primosome assembly site' (PAS) (2). In other systems, like phage ϕX174, primosomal assembly is equally complex, with many proteins participating in the process and with their functions known (or unknown) to various degrees (10–12). One of these proteins, DnaC, forms a complex with DnaB that facilitates loading of DnaB onto DNA (13,14). The ratio of DnaB to DnaC in this complex is crucial for DnaB binding to DNA. DnaB binds to a PAS site only when the ratio is ≤1 and in the presence of ATP (10). ATP hydrolysis is thought to be essential, as AMP-PNP (a non-hydrolysable analogue of ATP) does not enhance binding of DnaB to the PAS site, although another slowly hydrolysable analogue of ATP, ATPγS, can substitute for ATP in stabilising DnaB–DnaC complex formation (13). Consequently, DnaC acts as the helicase loader, facilitating the transfer of DnaB onto the DNA in an ATPase-dependent reaction that is also accompanied by release of DnaC from the complex (15). The evidence for a cryptic ATPase activity of DnaC that mediates release of DnaC from the primosomal complex after loading of the helicase is indirect (10,13,16). There is still no convincing direct evidence for a DnaC-specific ATPase activity.

In contrast to *E.coli*, primosome assembly in *Bacillus* sp. is poorly understood, despite the availability of the entire *B.subtilis* genome sequence. The main proteins appear to be similar, with homologues of the helicases (DnaB and PriA), primase (DnaG) and replication initiation protein (DnaA) also found in *Bacillus*, but with other apparently unrelated primosomal proteins. Based upon amino acid sequence homology DnaI has been identified as the *Bacillus* homologue of the *E.coli* DnaC helicase loader. Both proteins possess putative Walker A and B motifs (Fig. 1) characteristic of NTP-utilising enzymes (3). However, the sequence homology is confined within these motifs and is rather weak in the rest of the sequence. Recent evidence has implicated DnaI, together with

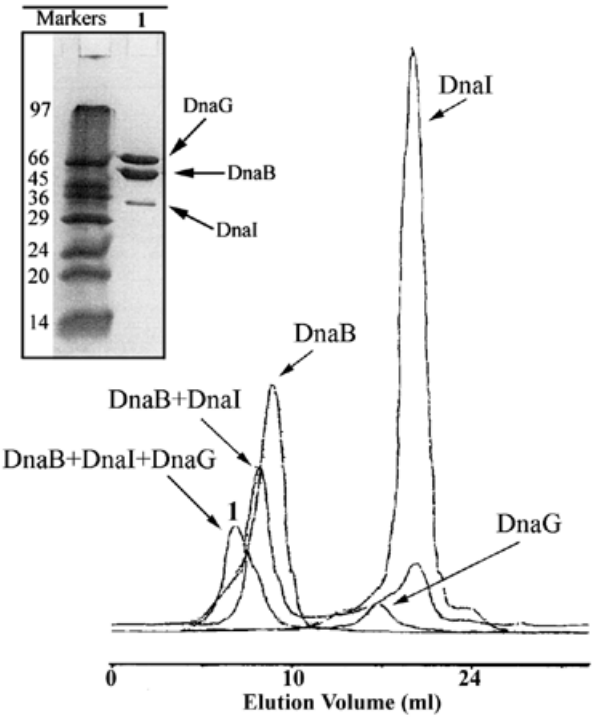

Figure 9. Investigation of the DnaG–DnaB–DnaI interaction by size exclusion chromatography. Superimposed gel filtration profiles for the DnaB and DnaI proteins as well as the DnaB–DnaI and DnaB–DnaI–DnaG complexes are shown. All protein–protein complexes were pre-formed in buffer containing 100 mM NaCl using a 5× molar excess of DnaI and/or DnaG over DnaB (concentrations refer to monomers), prior to loading onto a Superdex S-200 10/30 column. The DnaB–DnaI–DnaG triple complex was formed by first forming and purifying the DnaB–DnaI complex (see relevant profile above), which was then incubated with DnaG in order to form the triple complex. Elution profiles for DnaB and DnaI alone are also shown as controls. A sample from peak 1 was analysed by SDS–PAGE as shown.

the *B.subtilis* primosomal proteins DnaB and DnaD, in a cascade that loads the hexameric replicative helicase onto non-specific ssDNA (17). So is DnaI the true functional homologue of DnaC in *Bacillus*? If it is, then does it operate via a similar or different mechanism to that proposed for DnaC? In an attempt to elucidate the functional significance of DnaI in *Bacillus* we examined whether DnaI interacts with the main replicative hexameric helicase DnaB. We then proceeded to carry out a preliminary structural and biochemical characterisation of this interaction in order to examine its functional significance.

DnaI interacts physically with DnaB and modulates its activity

If DnaI plays the role of the helicase loader in *Bacillus* then it should be able to interact with the main replicative hexameric helicase. Although an interaction between DnaI and the replicative helicase was detected previously in *B.subtilis* by yeast two-hybrid technology (18), no formation of a stable complex between the two proteins has been directly demonstrated. Here we show, for the first time, that highly purified DnaI from *B.subtilis* interacts with DnaB from *B.stearothermophilus in vitro*, forming a stable complex that can be isolated by size exclusion chromatography (Figs 3 and 4). Although formation of the DnaB–DnaI complex does not require ATP or AMP-PNP (a non-hydrolysable analogue of ATP), we cannot rule out, at this stage, the possibility of modulation of the stability of the DnaB–DnaI complex by ATP binding and/or hydrolysis. Mixing the two proteins in the presence of ATP (or AMP-PNP) and Mg^{2+} prior to chromatography did not appear to alter the stoichiometry and/or stability of the complex (data not shown). The DnaB–DnaI interaction has a functional significance, at least *in vitro*, since the helicase activity of DnaB is stimulated when in complex with DnaI (Fig. 5A). At present it is not clear how this stimulation of DnaB helicase activity takes place. The obvious possibility of stimulation of DnaB ATPase activity resulting in concomitant stimulation of helicase activity can be ruled out, as DnaI fails to stimulate the ATPase activity of DnaB to any significant degree, although the ATPase profile of DnaB appears somewhat different in the presence of DnaI than in its absence (Fig. 5B). In fact, at 3 mM ATP, which is the concentration at which the helicase assays were carried out, the ATPase activity of DnaB is approximately the same in the presence or absence of DnaI (Fig. 5B). Clearly, more work is required, first to understand the complex ATPase kinetic profile of DnaB in order to explain the effect of DnaI. However, at this stage it is clear that DnaI is somewhat different than DnaC, which has been previously reported to inhibit the ATPase activity of DnaB (14). From our present data one could speculate that DnaI recognises some structural feature of the DnaB hexamer and upon binding modulates its conformation, thus affecting the cooperativity between the individual monomers. This notion is consistent with the fact that DnaI interacts stably with the hexameric form of P33 under high salt conditions rather than with lower oligomeric complexes of P33 under low salt conditions (Fig. 6). However, DnaI must also interact with lower oligomeric forms of P33 under low salt conditions (perhaps transiently or less stably), as manifested by the observed inhibition of P33 ATPase activity in the presence of increasing amounts of DnaI (Fig. 7). The fact that the interaction of DnaI with lower oligomeric forms of P33 inhibits the ATPase activity whereas it fails to inhibit the ATPase activity of the intact hexamer suggests that there are important functional differences between these two types of interaction. What these differences are is at present unclear. Another possibility is that DnaI, in the presence of ATP, facilitates efficient loading of the hexameric helicase onto the circular ssM13 DNA substrate and then dissociates from the complex, allowing the tethered helicase to translocate along the DNA and displace the annealed oligonucleotide.

DnaI interacts physically with the DnaB–DnaG complex and modulates its activity

Our data show that the highly purified proteins DnaB, DnaG and DnaI interact to form a stable 'triple complex' *in vitro*, which can be isolated by size exclusion chromatography (Fig. 9). The stoichiometries of the interacting proteins in the triple complex appear to be the same as those observed for the separate DnaB–DnaG and DnaB–DnaI complexes (compare Figs 3B, 4 and 9), implying that DnaG and DnaI bind to the DnaB hexamer at different and non-overlapping sites. However, the functional properties of the triple complex are very different to those of the DnaB–DnaG and DnaB–DnaI complexes. Whereas the latter exhibit stimulated helicase activity, the triple complex in contrast has depressed activity (compare Figs 5A and 8A) (7). Again, this inhibition of the helicase

activity cannot be attributed to inhibition of the ATPase activity, since the presence of DnaI does not inhibit the ATPase activity of the DnaB–DnaG complex (Fig. 8B). In contrast, at 3 mM ATP, which is the concentration in the helicase assays, the ATPase activity of the DnaB–DnaG complex was slightly higher in the presence of DnaI (21.5/s) than in its absence (18/s). Instead, the effect of DnaI on the ATPase profile of the DnaB–DnaG complex appears to be similar to that observed for DnaB alone (Figs 5B and 8B). It is plausible from the complexity of these different effects that DnaG and DnaI may be able to 'fine tune' the activity of the helicase by somehow altering the cooperative interactions between the monomers of the DnaB hexamer. We have suggested before that the DnaG–DnaB interaction may not be distributive and that the primase may remain bound to the translocating helicase during DNA replication on the lagging strand (7). In addition, it is believed that the release of DnaC from the DnaB–DnaC complex after loading is mediated by DnaC-specific ATP hydrolysis and this is a necessary step to activate the helicase activity of DnaB, since when DnaC is bound to the helicase its activity is inhibited (13). If DnaI acts in a similar manner then the inhibitory effect on the helicase activity of the DnaG–DnaB complex may be explained by a relative 'slowing down' of DnaI release from the triple complex. Such a scenario may be problematic because it will slow down DNA replication in the lagging strand, but after the initial priming event and release of DnaI at the initiation site, subsequent reassociation of DnaI may be prevented by interactions of the helicase with other components of the assembled replisome. Genetic evidence showed that certain dnaC alleles are defective in the elongation process during DNA replication (19–21). Allen and Kornberg pointed out that 'it seems paradoxical that DnaC inhibits the function of a complex whose formation it promotes' and they proposed that this dual action reflects a feedback control mechanism, slowing down DNA replication under conditions that are unfavourable for rapid DNA synthesis (22). Alternatively, DnaI may be unable to interact with the helicase bound to DNA. Variable affinity of DnaC for different conformational states of the DnaB hexamers has been suggested before (22).

The architecture of the DnaI–DnaB–DnaG triple complex

Our results show that DnaI interacts with the P33 C-terminal domain of DnaB (Fig. 6). The stoichiometry of the DnaB–DnaI interaction appears to be one or two DnaI molecules per DnaB hexamer (Figs 4 and 6). We have previously reported that the DnaB–DnaG interaction involves two or three DnaG molecules per DnaB hexamer and that this interaction is likely to involve the 'hinge region' that connects the P17 N-terminal and P33 C-terminal domains of DnaB (7). Since the DnaB–DnaG and DnaB–DnaI interactions appear to be 'structurally' separate, we can start picturing the architecture of the triple complex (Fig. 10A). Low resolution electron microscopy (EM) studies revealed details of the DnaB–DnaC interactions (23). The complex appears to involve six monomers of DnaC per hexamer of DnaB, each DnaC monomer interacting with two monomers of DnaB and vice versa in a ring-like configuration. Biochemical evidence also supports the $DnaC_6$–$DnaB_6$ stoichiometry (24–26) in a complex that is stabilised by ATP bound to DnaC (13,16). Mutational analysis of DnaC revealed that an N-terminal domain is required for functional binding to DnaB, since mutants that were defective in DnaB binding were also inactive in an *in vitro* DNA replication assay and failed to complement a temperature-sensitive dnaC28 mutant *in vivo* (27). As shown by EM studies, these interactions also appear to involve the C-terminal domains of the DnaB monomers (23). Therefore, both DnaC and DnaI proteins interact with the C-terminal domains of the monomers within the DnaB hexamer. However, our data suggest that only one or two monomers of DnaI interact with the DnaB hexamer, at least *in vitro*. Although we cannot rule out the possibility that this stoichiometry might be different *in vivo* (under the influence of ATP or ssDNA or other modulator proteins), assuming that it is correct we can suggest two different possibilities for the architecture of the triple complex, shown in Figure 10B and C. If we assume that the DnaB hexamer is a functional trimer of dimers, then DnaI can interact either with one dimer (Fig. 10B) or with two adjacent dimers (Fig. 10C). These relative architectural arrangements may also be important when considering opening of the ring during loading of the helicase onto the DNA. Ring opening during loading may take place either within a dimer or between adjacent dimers of the hexameric ring. Important questions still remain unanswered. Do the DnaG and DnaI proteins interact with the same or different monomers of the hexameric DnaB? How do the different proteins in the complex interact with the DNA? Is the conformation of the DnaB hexamer different in the DnaB–DnaG, DnaB–DnaI and DnaG–DnaB–DnaI complexes? How is cooperativity between the DnaB monomers modulated by DnaG and/or DnaI? Clearly, more work is required to answer these crucial questions.

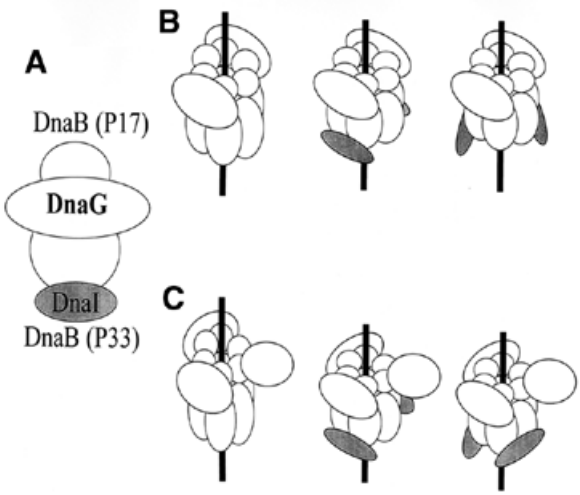

Figure 10. (**A**) A diagrammatic representation mapping the DnaG and DnaI interactions with DnaB. (**B** and **C**) Diagrams outlining the possible architectures of the triple complex. In (B) we assume that two DnaG molecules interact with the DnaB hexamer, whereas in (C) we assume that three DnaG molecules interact with the DnaB hexamer. In all cases we have assumed that two DnaI molecules interact with the DnaB hexamer, but equivalent complexes can be drawn with only one DnaI molecule and/or two DnaG molecules interacting with the DnaB hexamers. In all cases DNA is represented as a solid bar running through the hexameric ring formed by DnaB.

ACKNOWLEDGEMENTS

I would like to thank Drs Dale B. Wigley and Martin R. Singleton for useful discussions and critical reading of the

manuscript. My special gratitude goes to Dr Dale B. Wigley for his invaluable support. This work was supported by a BBSRC grant (42/B15519) to P.S.

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