DNA Binding by the Meningococcal RdgC Protein, Associated with Pilin Antigenic Variation

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The RdgC protein of *Neisseria gonorrhoeae* is required for efficient pilin antigenic variation, although its precise role has yet to be established. We demonstrate that the nearly identical RdgC from *Neisseria meningitidis* binds DNA with little specificity for sequence or structure, like the *Escherichia coli* protein. We also show that neither protein is able to constrain torsional tension in relaxed DNA. These data exclude several possible roles for RdgC in pilin antigenic variation and suggest that RdgC performs a similar function in both *E. coli* and the *Neisseria* spp.

Orthologues of the *rdgC* gene are found only in the beta and gamma subdivisions of the *Proteobacteria*, including *Escherichia coli* and the obligate human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The *E. coli* RdgC gene encodes a DNA binding protein of 34 kDa (14). Deletion of this alone causes no obvious phenotype but is highly deleterious in strains lacking certain enzymes involved in recombination and replication restart (14, 18). The explanation(s) for these effects is uncertain but could imply that RdgC aids replication processivity.

In *N. gonorrhoeae* (the gonococcus) *rdgC* is required for efficient pilin antigenic variation and plays some role in cell growth (12). Pilin antigenic variation allows *N. gonorrhoeae* and *N. meningitidis* (the meningococcus) to alter the sequence of the main structural component of the type IV pilus, PilE (5, 15). It has been proposed that the expression of variant type IV pili through pilin antigenic variation promotes adhesion to different tissue types (9, 17) as well as contributing to evasion of the host immune response (2). Unfortunately, the molecular mechanisms and enzymology underlying pilin antigenic variation are poorly understood (7). It is known that sequence from one of numerous silent loci (*pilS*) is copied to the expression locus, *pilE* (5). Several conserved sequences present in *pilS* and *pilE* are important, including the coding *cys1* and *cys2* elements and the Sma/Cla repeat located in the 3' untranslated region (6, 8, 24). In terms of proteins involved, genetic studies have shown a strong dependence on *recA* as well as the *recO*, *recQ*, and *recJ* genes. Hence, pilin antigenic variation appears to utilize a RecF-like pathway for recombination, suggesting functions targeted at DNA single-strand gaps or at replication forks (10, 13, 19). Recently identified protein RecX also participates in these reactions and is likely to regulate RecA activity (20, 22). As noted, RdgC is also important, but its role is unknown (12).

A current working hypothesis for pilin antigenic variation utilizes the common occurrence of circular DNAs, including hybrid *pilE-pilS* molecules, in the gonococcus (1, 7). Recombination directed by pilin-antigenic-variation-specific factors, independent of the RecFOR pathway, initiates an exchange between *pilE* and *pilS* loci on one chromosome. Resolution of the junction(s) formed creates a circular molecule with a hybrid *pilE-pilS* locus. This intermediate is then utilized in a second, RecFOR-dependent, recombination reaction with the *pilE* locus on an intact chromosome.

We report here that purified meningococcal RdgC binds DNA in a sequence- and structure-independent manner and does not introduce torsional tension in DNA, arguing against a structural role in pilin antigenic variation.

Purification of meningococcal RdgC. The meningococcal *rdgC* gene was amplified from strain B16B6 with primers introducing restriction sites (5'-ACAGGAAACCATATGTGGTTCAAGC-3' and 5'-ATTGGATCCTGGCTGACGGTATAAA-3'; *NdeI* and *BamHI* sites underlined). These sites were used to insert the product into pT7-7, yielding pDIM008. Nucleotide sequencing of *rdgC* revealed that the predicted protein sequence differed from that of the protein of the sequenced serogroup B strain, MC58, by a single substitution (T288I) but was identical to that of the protein of the serogroup A strain Z2491. Compared to gonococcal RdgC there are two changes (T288I and R231Q); neither is highly conserved (Fig. 1A). The biochemical activities of meningococcal and gonococcal RdgC proteins are likely to be identical, and therefore the proteins are likely to perform the same function in both *Neisseria* species.

Meningococcal RdgC was overexpressed in a $\Delta rdgC::Tm^r$ (14) derivative of *E. coli* B strain BL21(DE3) (DIM026) carrying pLysS and pDIM008, following addition of IPTG (isopropyl- β -D-thiogalactopyranoside). Induced cells were lysed on ice by sonication in buffer A (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol [DTT]) before the addition of NaCl to 1 M. RdgC was precipitated from the cleared lysate with a 50 to 80% ammonium sulfate cut, resuspended in buffer A plus 0.1 M NaCl, applied to a 10-ml heparin-Sepharose 6 column, and eluted on a linear gradient of buffer A plus 0.1 to 2 M NaCl. Fractions eluting between 1.0 and 1.5 M NaCl were pooled, dialyzed into buffer B (50 mM Tris-HCl [pH 8.0], 1

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FIG. 1. Production of meningococcal RdgC. (A) Alignment of RdgC C termini highlighting the two amino acid substitutions between gonococcus and meningococcus (asterisks). The position in each protein relative to the first residue is indicated at the start of each sequence. Residues identical or functionally similar between the proteins are shaded. *Eco*, *E. coli*; *Hin*, *Haemophilus influenzae*; *Pae*, *Pseudomonas aeruginosa*; *Ngo*, *N. gonorrhoeae*; *Nme*, *N. meningitidis* B16B6. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis involving Coomassie blue staining summarizing purification of meningococcal RdgC. Lane i, molecular weight markers; lane ii, crude cell lysate; lane iii, pooled 50 to 80% $(\text{NH}_4)_2\text{SO}_4$ cut; lane iv, pooled fractions from heparin column; lane v, pooled fractions from Q Sepharose column. The RdgC band is indicated. (C) Gel filtration of purified meningococcal RdgC protein. Elution profiles of molecular weight standards (upper profile) and meningococcal RdgC (lower profile) are shown. The molecular mass of each standard is indicated next to its peak; interpolation between the elution points of these was used to estimate the mass of RdgC.

mM EDTA, 1 mM DTT) plus 0.1 M NaCl, and applied to a 4-ml Q-Sepharose fast-flow column, and bound proteins were eluted with a linear gradient of buffer B plus 0.2 to 1 M NaCl. RdGc eluted at approximately 0.5 M NaCl. Peak fractions were pooled, dialyzed into buffer A plus 0.2 M NaCl and 50% glycerol, and stored as aliquots at -80°C . The protein concentration was estimated by a modified Bradford assay (Bio-Rad) with bovine serum albumin as the standard.

Gel filtration on a Superose-12 column with buffer A plus 0.1

M NaCl revealed that, like *E. coli* RdgC, meningococcal RdgC is a dimer in solution (Fig. 1C). We have therefore expressed concentrations of the protein as moles of dimer.

Meningococcal RdgC binds to DNA. Using band shift assays performed as previously described (14) we tested whether meningococcal RdgC binds DNA (Fig. 2). Details of the substrates used are given in Table 1. Stable complexes were formed with linear single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) and with a variety of branched mol-

FIG. 2. Band shift assays showing binding of meningococcal RdgC to different DNA substrates. Binding reaction mixtures contained 0.1 nM DNA and increasing RdgC concentrations: 0, 0.5, 5, 50, and 500 nM. The substrates are illustrated above each panel. Half arrows, 3' ends. (A) Fifty-nucleotide ssDNA; (B) 50-bp dsDNA; (C) 5'-tailed duplex; (D) 3'-tailed duplex; (E) hairpin; (F) replication fork with leading strand; (G) replication fork with lagging strand; (H) replication fork with both strands; (I) Holliday junction. Details of the substrates used are given in Table 1.

TABLE 1. Substrates used in DNA binding assays^a

Oligonucleotide	Sequence (5'-3')	Length (nucleotides)
a	CAACGTCATAGACGATTACATTGCTACATGGAGCTGTCTAGAGGATCCGA	50
b	TAGCAATGTAATCGTCTATGACGTT	25
c	GTCGGATCCTCTAGACAGCTCCATG	25
d	TCGGATCCTCTAGACAGCTCCATGTAGCAATGTAATCGTCTATGACGTTG	50
e	GTCGGATCCTCTAGACAGCTCCATGATCACTGGCACTGGTAGAATTCGGC	50
f	TGCCGAATTCTACCAGTGCCAGTGAT	26
g	AACGTCATAGACGATTACATTGCTA	25
h	TAGCAATGTAATCGTCTATGACGTT	25

^a DNA substrates were assembled with the oligonucleotides listed or described in the references cited as follows: 50-nucleotide ssDNA (a), 5'-tailed duplex (a plus b), 3'-tailed duplex (a plus c), 50-bp dsDNA (a plus d), hairpin (HP56-TTTT [4] has a 26-bp dsDNA stem with a 4-nucleotide TTTT loop), replication fork with leading strand (a plus b plus e), replication fork with lagging strand (a plus e plus f), replication fork with both strands (a plus b plus e plus f), Holliday junction (J12 [11] is composed of 50-nucleotide oligonucleotides and has a 12-nucleotide homologous core), 25-nucleotide ssDNA (g), 25-bp dsDNA (g plus h).

ecules designed to mimic intermediates in recombination and replication. The affinities for these substrates appear broadly similar, suggesting that meningococcal RdgC, like *E. coli* RdgC (14), does not target branch points in DNA (Fig. 2). With the 50-nucleotide ssDNA only a single stable complex was formed (Fig. 2A), whereas with dsDNA, tailed duplexes, and the hairpin, two stable complexes were formed (Fig. 2B to E). With the branched fork and junction structures, three or more complexes were formed (Fig. 2F to I). The differences can be explained in terms of the number of arms available for protein binding, or potentially binding to different numbers of DNA ends. However, since meningococcal RdgC is also able to bind to circular plasmid DNA (see below), the latter possibility is unlikely.

Binding studies with 25-nucleotide ssDNA and a 25-bp duplex revealed that, like *E. coli* RdgC, meningococcal RdgC has a preference for binding dsDNA over ssDNA (Fig. 3). However, the difference in affinity for ss- versus dsDNA on these substrates was less obvious with than with the *E. coli* protein. We also note that the affinity of meningococcal RdgC for both substrates was slightly higher than that of *E. coli* RdgC under the conditions used.

We failed to observe substantial differences in affinities for substrates of different nucleotide sequences (Fig. 2 and 3; data not shown), suggesting that meningococcal RdgC does not

target specific sequences in DNA. In addition, multiple rounds of selective enrichment for dsDNA sequences preferentially bound by meningococcal RdgC according to the procedure of Pollock and Treisman (16) did not generate any enhanced affinity for the duplex DNA (data not shown).

Effect of RdgC on topology of DNA. The binding of proteins to DNA can cause distortion at the binding site, increasing or decreasing the DNA twist. As a consequence, positive or negative torsional tension is constrained. To probe the ability of RdgC to twist DNA, pUC19 was relaxed by topoisomerase in the absence of RdgC and then the relaxation was continued after RdgC addition. First, a pool of pUC19 was relaxed with vaccinia virus topoisomerase I (Sigma; 5 U/17.5 ng of pUC19) in VTBI (50 mM Tris-HCl [pH 8.0], 100 µg of bovine serum albumin/ml, 2 mM MgCl₂, 1 mM DTT) for 3 h at 30°C. One aliquot of the relaxed DNA was deproteinized by standard phenol-chloroform-indoleacetic acid (25:24:1) treatment. The remainder of the relaxed DNA was aliquoted to tubes containing additional topoisomerase (10 U/17.5 ng of pUC19) and various concentrations of protein. These were incubated for a further 3 h at 30°C. After secondary incubation, samples were deproteinized as before. Plasmids were then resolved by electrophoresis in a 0.9% agarose gel with Tris-borate-EDTA (TBE) buffer (Bio-Rad; Mini Sub Cell GT; 7 by 10 cm) for 2 h at 45 V. DNA was visualized under UV light, after being stained in TBE with SYBR gold.

Both meningococcal and *E. coli* RdgC induced at most one extra turn in the plasmid DNA before inhibiting topoisomerase activity (Fig. 4A and B, compare lanes with 0 and 0.8 µM RdgC). We assume that the inhibition of topoisomerase activity by 1.6 µM RdgC correlates with high occupation (75 to 100%) of pUC19 DNA by the protein. Thus, at 0.8 µM RdgC, a significant proportion of the plasmid must have been coated, suggesting that each RdgC molecule introduced a small degree of twist. Additional assays involving resolution in gels containing chloroquine revealed that a positive turn had been introduced (data not shown), indicating that RdgC "overwinds" DNA. In contrast, a plasmid relaxed in the presence of HU, which is known to constrain negative supercoils in DNA (3), gained approximately five negative turns prior to inhibition of topoisomerase activity (Fig. 4C, compare lanes containing 0 and 0.2 µM HU; data not shown). The results suggest that the ability of RdgC to twist DNA was about five times less potent than that of HU, which has previously been estimated at 1 turn

FIG. 3. Comparison of ss- and dsDNA binding by meningococcal (diamonds) and *E. coli* (triangles) RdgC (14). Binding curves for RdgC with 25-nucleotide ssDNA (open symbols) and 25-bp dsDNA (shaded symbols) are shown. A 1 nM DNA substrate was used in each reaction, with the indicated concentration of RdgC. Data are means of two experiments.

FIG. 4. Topoisomerase-mediated relaxation of plasmid DNA bound by RdgC. Shown is relaxation in the presence of meningococcal RdgC (A), *E. coli* RdgC (14) (B), and *E. coli* HU (C). For all reactions the pUC19 DNA concentration was 1 nM; U, untreated DNA; Rel, DNA from relaxed pool. Concentrations of protein added to the reaction after initial relaxation are indicated. Arrows indicate increasing supercoiling of plasmid DNA.

per 12 or 13 dimers bound (23). We conclude that RdgC does not introduce considerable torsional tension into DNA upon binding and is therefore unlikely to constrain supercoils to a significant degree in vivo.

Concluding remarks. The data presented suggest that meningococcus RdgC does not target specific sequences or structures in DNA to promote pilin antigenic variation. It also introduces little twist in DNA, arguing against an architectural role similar the kind seen in many site-specific recombination reactions, although the possibility that it bends DNA without inducing torsional tension cannot be excluded.

Given the similarities between meningococcal and *E. coli* RdgC proteins now established, it is reasonable to envisage that they perform similar functions in both species. Since *E. coli* lacks an equivalent of pilin antigenic variation, this also argues against a role unique to that system. An association between RdgC and RecFOR recombination proteins in *E. coli* was uncovered by Moore et al. (14). In this case, the *recFOR* genes are deleterious in the absence of *rdgC* in a strain which also carries *priA* and *dnaC212* mutations. This suggests that RdgC acts to limit a toxic effect of the RecFOR complex, perhaps during reinitiation of a stalled replication fork. Since multiple RecF pathway products participate in pilin antigenic variation, a role for the DNA binding activity of RdgC in these processes is likely. The gonococcal RecX gene, which regulates

RecA activity, is also required for these reactions (21, 22), indicating that control of recombinational exchanges involving the loading and unloading of RecA, RecFOR, and SSB is a critical feature for generating the recombinants that ultimately lead to pilin antigenic variation.

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