The binding of α/β-type small, acid-soluble spore proteins (SASP) to DNA of spores of Bacillus species is the primary mechanism by which spore DNA is protected from the damaging effects of heat, peroxides, and UV radiation. The α/β-type SASP are rapidly degraded by a sequence-specific endoproteinase, termed the germination protease (GPR), which recognizes and cleaves within a pentapeptide sequence found within all α/β-type SASP (4).

The interaction between α/β-type SASP and DNA has been studied in detail, and several features of α/β-type SASP–DNA binding have been characterized (5, 6, 8–10). DNA structure changes from B-DNA to an A-like conformation upon binding to α/β-type SASP (7, 8), whereas α/β-type SASP undergo a transition from random coil to a largely α-helical conformation upon binding to DNA (6). The binding interaction is significantly cooperative, with α/β-type SASP having a 50- to ~600-fold greater affinity for contiguous DNA binding sites than for noncontiguous sites depending upon the bound polynucleotide and the salt concentration (5, 6). The binding cooperativity is thought to be due at least in part to protein–protein interactions between adjacent, DNA-bound α/β-type SASP (8, 11). Contacts between the α-amino group and the carboxylate side chains of three acidic residues found within the N-terminal 40–50% of a variety of α/β-type SASP have been identified previously using a zero-length cross-linking reagent (11). The N-terminal third of α/β-type SASP varies both in length and amino acid sequence, and its sequence is generally less well conserved than the C-terminal two-thirds of these proteins (Fig. 1) (4). Because individual α/β-type SASP have significantly different affinities for DNA, it seems reasonable to speculate that these differences in binding affinity could be due to differences in the length and sequence of the N-terminal third of these proteins. Consequently, we have investigated the effects of modifications of the N-terminal sequence upon the DNA binding properties of α/β-type SASP. This analysis has further
led to a model for α/β-type SASP protein-protein interaction, which involves an electrostatic interaction between the positively charged N-terminal region of one protein and an acidic region on an adjacent DNA-bound protein.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The Escherichia coli strains used were JM38 (ara laci-proAB) pRSL 680 lacZAM15 (12) and BL21 (DE3) (T7 RNA polymerase under control of the lac promoter) (13). The *B. subtilis* strains used were all derivatives of strain 168. *E. coli* strains were routinely grown in Terrific broth (24 g of yeast extract, 12 g of tryptone, and a line of glycerol per liter of water) at 37 °C. *B. subtilis* strains were grown at 30 °C.

**Construction of Gene Encoding SspC N-terminal Variants**—The genes encoding all SspC deletion variants were generated by the polymerase chain reaction (PCR) using plasmid pSF708 (10) as the template and the following oligonucleotide primers. The upstream primers were: SSCP (5′-CGATGGCTCAACAA AGTATGA-3′), SSPCΔ5 (5′-CCATGGCTAGATCAAACAATAATG-3′), SSPCΔ15 (5′-CCATGGCAGGCGAACATGAC-3′), SSPCΔ31 (5′-CCATGGCGAATTCTGGTCATCAAC-3′), SSPCΔ11-D31N (5′-CCATGGGATAATCTAACTTCTCAAGGC-3′), SSPCΔ11-D13K (5′-CGATGGCTAAATTTACTAATTCCTCGGACCTCGGG-3′); the downstream primer was SSPC2 (5′-AGCTGGATCCGAAATTCTCTTCCTAGCAG-3′). All PCR products were ligated to plasmid pET11d vectors (14), the medium was supplemented with 200 μg/ml ampicillin and 0.5% glucose. *B. subtilis* was transformed to kanamycin resistance with pUB110 derivatives as described previously (14).

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**Purification of α/β-type SASP and Polynucleotides**—The α/β-type SASP was chemically deaminated as follows; 120 µl of purified protein (~1 mg/ml in 10 mM sodium phosphate (pH 7.5)) was slowly added to 350 µl of 2.5 M NaOAc (pH 5.2), 50 mM sodium glyoxylate, 5 mM CuSO4 (20, 21). The resulting solution was incubated at 22 °C for 20 min, followed by dialysis in Spectra/Por 3 tubing (molecular mass cut-off 3500 Da) against 10 mM sodium phosphate (pH 7.5).

**Binding Conditions**—For α/β-type SASP—α/β-type SASP were pooled, concentrated by lyophilization, and dialyzed exhaustively against 10 mM sodium phosphate (pH 7.5) at 4 °C. This protein was used for in vitro DNA binding assays.

**Analysis of SspC Variants in Vivo and in Vito**—DNA binding was assayed in vitro using DNAse I protection assays (5) and by circular dichromism (22). The presence of DNA binding by affinity chromatography on Q-Sepharose was determined by incubation of a cell suspension containing 10 mM Tris-HCl (pH 8.0) at 4 °C. Proteins were eluted by a linear salt gradient from 0 to 200 mM NaCl in 10 mM Tris-HCl (pH 8.0) at 4 °C. Column fractions containing purified α/β-type SASP were pooled, concentrated by lyophilization, and dialyzed exhaustively against 10 mM sodium phosphate (pH 7.5). All proteins were >95% pure, as judged by SDS-PAGE and staining with Coomassie Blue. The concentrations of protein stock solutions were determined by quantitative amino acid analysis.

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**Purification of α/β-type SASP**—α/β-type SASP were pooled, concentrated by lyophilization, and dialyzed exhaustively against 10 mM sodium phosphate (pH 7.5) at an enzyme to substrate ratio of 1:200 (w/w) at 22 °C for 6 h. SspC (1 mg/ml) was digested with the protease (recombinant GPR from Bacillus megaterium) (22) at an enzyme to substrate ratio of 1:200 (w/w) in 10 mM Tris-HCl (pH 7.4), 2 mM CaCl2 at 22 °C for 3 h. The C-terminal proteolytic fragments from both digests were purified by reverse phase-HPLC as described (11).

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**Protein-Protein Interactions in α/β-Type SASP**

**Results**

**SspC N-terminal Variants Bind to DNA in Vitro**—Amino acid sequence alignment of all identified α/β-type SASP from *Bacillus, Sporosarcina*, and *Thermoactinomyces* species show that these proteins are most highly conserved in the C-terminal two-thirds of the protein (Fig. 1) (4), and part of this region is thought to be involved in directly contacting the DNA (30). In contrast, the sequence of the N-terminal one-third of α/β-type SASP is less well conserved with variations in both length and amino acid sequence (4). The identification of protein-protein contacts between amino acid residues found within the N-terminal 40–50% of α/β-type SASP in the DNA-bound state (11) suggests that the N termini of α/β-type SASP mediate protein-protein interactions which may be important for binding cooperativity, and therefore the apparent binding affinity of the DNA binding interaction. To explore this question in more detail, a number of N-terminal variants of SspC, a minor α/β-type SASP from *B. subtilis*, were produced and their DNA binding properties analyzed. SspC was chosen for this study because it is easily overexpressed and purified from both *B. subtilis* spores and *E. coli*, and its interaction with and effects upon DNA have been studied extensively both in vitro and in vivo (5, 9, 10). Furthermore, SspC binds to DNA with a higher affinity than many other α/β-type SASP, and also has one of the longest N termini of all identified α/β-type SASP (4, 15).

Variants were designed in which blocks of approximately five amino acid residues were progressively removed from the N terminus of SspC (Fig. 1). In addition, SspC111 was constructed to assess the effect of an N-terminal deletion that removes an asparagine residue conserved in all α/β-type SASP from *Bacillus* species and their close relatives (Fig. 1). Each deletion variant was designed such that the protein would contain an amino acid residue marked by an asterisk (*). Amino acid residues marked by an asterisk (*) are completely conserved in all α/β-type SASP identified from *Bacillus, Sporosarcina*, and *Thermoactinomyces* species (4, 31); underlined acidic residues have been identified as sites of intermolecular EDC protein cross-linking (11). The downward-pointing arrow indicates the peptide bond cleaved by GPR.

**Fig. 1.** Amino acid sequences of SspC N-terminal deletion variants. The amino acid sequences of SspC N-terminal deletion variants are given in one-letter code. Sequences are as follows: SspC (SspC), SspC11 (Δ5), SspC10 (Δ10), SspC11 (Δ11), SspC111 (Δ14). Asp-N digest C-terminal peptide (Asp-N frag), and GPR digest C-terminal peptide (GPR C-frag). Amino acid residues marked by an asterisk (*) are completely conserved in all α/β-type SASP identified from *Bacillus, Sporosarcina*, and *Thermoactinomyces* species (4, 31); underlined acidic residues have been identified as sites of intermolecular EDC protein cross-linking (11). The downward-pointing arrow indicates the peptide bond cleaved by GPR.
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Far UV difference spectra of SspC N-terminal variants bound to poly(dG)−poly(dC). For UV difference spectra of SspC N-terminal deletion variants bound to poly(dG)−poly(dC), the CD spectrum of poly(dG)−poly(dC) was subtracted from the CD spectrum of complexes of SspC N-terminal deletion variant (25 μM) and poly(dG)−poly(dC) (115 μM in bp), as described under “Experimental Procedures” to obtain the presented difference spectra.

Total residues) may be removed without abolishing DNA binding.

All α/β-type SASP are essentially random coils in the absence of double stranded-DNA, but become α-helical upon binding to DNA (6). These changes in protein conformation are conveniently monitored by CD spectroscopy, which was used to confirm the DNA binding properties of the SspC N-terminal variants (Fig. 2). The far UV CD spectra of all N-terminal SspC variants were indicative of unstructured polypeptides (Fig. 2 and data not shown), but upon addition of poly(dG)−poly(dC) each variant except the GPR C-terminal fragment of SspC became significantly α-helical (Fig. 2 and data not shown). The α/β-type SASP bind to poly(dG)−poly(dC) very tightly (5, 6), and the vast majority of the α/β-type SASP (−98%) is bound to DNA under these conditions (25 μM α/β-type SASP and 115 μM in bp poly(dG)−poly(dC)). Therefore, the secondary structure content of the DNA-bound protein could be estimated using [θ] values from difference spectra in which the spectrum of free poly(dG)−poly(dC) had been subtracted from the spectrum of the complex (6, 25). The estimates of secondary structure content suggest that the great majority of the removed residues are not part of regular secondary structure in the full-length protein as the calculated number of α-helical residues is similar (44–50 residues) in each deletion variant (data not shown).

Thermal Stability and Equilibrium Binding Affinity of SspC Variant-DNA Complexes—The DNase I protection assays mentioned above indicate that all SspC N-terminal variants (with the exception of the C-terminal GPR fragment) are able to bind to plasmid DNA. However, these experiments are performed at protein concentrations in excess of the dissociation constants for the complexes, and thus it is difficult to detect differences in α/β-type SASP-DNA binding affinities using this assay. Therefore, we conducted more sensitive and quantitative studies of SspC N-terminal variant-DNA binding, which were monitored by CD spectroscopy. It has been demonstrated previously that corrected [θ] values from α/β-type SASP-DNA mixtures can be used to determine the relative concentrations of DNA-bound and unbound α/β-type SASP in solution (6). Thus, [θ] values are an index of α/β-type SASP-DNA binding, which can be used to determine the thermal stabilities (Tm) and equilibrium binding constants (Kd) for α/β-type SASP-DNA interactions (6).

The thermal stability of SspC N-terminal variant complexes with poly(dG)−poly(dC) was determined by monitoring the CD at 222 nm for each protein-DNA complex as a function of temperature. Each α/β-type SASP-poly(dG)−poly(dC) complex underwent a sharp thermal dissociation transition over a characteristic temperature range, and the midpoint of each transition (defined as Tm) was determined (Table I) (6). The thermal stability hierarchy was as follows: SspC > SspC14 > SspC11 > SspC11-D13K > Asp-N C-fragment > SspC11 (Table I). This result was unexpected in that the deletion of additional residues from SspC11 to SspC14 actually increased the stability of the α/β-type SASP-poly(dG)−poly(dC) complex.

We next wanted to determine the apparent equilibrium binding constants (Kd) for the interaction between SspC N-terminal deletion variants and DNA to confirm the relative binding affinities suggested by the thermal stability studies. Intrinsic binding constants (K) and cooperativity factors (ω) may be extracted from equilibrium binding titrations of homogeneous synthetic polynucleotides by fitting the McGhee-von Hippel model of cooperative, nonspecific protein-nucleic acid binding to the experimental data (6, 33). The cooperativity factor (ω) is a dimensionless thermodynamic parameter, which describes the relative affinity of a protein ligand for a ligand-contiguous binding site versus an isolated binding site (33). Therefore, differences between SspC variants in both their intrinsic binding constants and binding cooperativity may be detected and quantitated; this is potentially important as the N termini of α/β-type SASP are thought to be involved in DNA-dependent protein-protein interactions (11). Initial equilibrium binding studies were conducted with the N-terminal variants and poly(dG)−poly(dC); however, because the binding cooperativity is low with this polynucleotide and the interactions are very tight (6), it was impossible to accurately determine K and ω values. The interaction between α/β-type SASP and poly(dA-dT)poly(dA-dT) is much more cooperative than with poly(dG)−poly(dC) (6). Unfortunately, most of the SspC N-terminal variants did not bind to poly(dA-dT)poly(dA-dT) at concentrations that could be accurately measured by CD spectroscopy. However, we were able to conduct titrations of poly(dA-dT)poly(dA-dT) with SspC and SspC14, and determined a significant decrease in both K and ω for the interaction with SspC14 compared with that with SspC (Table II). To obtain binding constants for all the N-terminal variants, additional equilibrium titrations were conducted using linear pUC19 plasmid DNA, which tends to bind to α/β-type SASP more tightly.
than poly(dA-dT)-poly(dA-dT). Apparent equilibrium binding constants (Kω) from these titrations confirmed the hierarchy of binding affinities as determined by thermal stability assays, although individual fits of K and ω were not possible because the McGhee-von Hippel model was not designed to describe binding to polynucleotides of heterogeneous sequence (Table II).

Protein-Protein Contacts between DNA-bound SspC N-terminal Variants—Previously, we used chemical cross-linking to identify amino acid residues that form close contacts between adjacent DNA-bound α/β-type SASP (11). These residues are located within the N-terminal 40–50% of the proteins, and all identified cross-links occurred between the α-amino group of the protein and either of the two glutamate residues of the GPR recognition sequence, or a relatively nonconserved acidic residue (aspartate 13 in SspC) found closer to the N terminus of α/β-type SASP (11) (Fig. 1). Therefore, the N terminus of a DNA-bound protein appears to be interacting with negatively charged residues on an adjacent DNA-bound protein. This proposed protein-protein interaction could partially explain the unexpected increase in binding affinity seen in SspC N-terminal variants as additional amino acid residues are removed from SspC<sup>11</sup> to SspC<sup>34</sup> (Table II). SspC<sup>10</sup>, SspC<sup>31</sup>, and the Asp-N C-fragment all contain a negatively charged aspartate residue near the N terminus (Fig. 1), which could interfere with the postulated DNA-dependent protein-protein interactions through electrostatic repulsion. This N-terminal aspartate residue is absent in SspC<sup>34</sup> and may therefore account for the increase in DNA binding affinity over SspC<sup>34</sup>, SspC<sup>31</sup>, and the Asp-N C-fragment. To determine if the SspC N-terminal variants made the same type of protein-protein contacts when bound to DNA, these proteins were cross-linked in the presence of DNA and the cross-linking sites identified.

All N-terminal variants except the GPR C-fragment were cross-linked by EDC in a DNA-dependent fashion, although a very small amount of cross-linking was also seen in the absence of DNA (Fig. 3). DNA-independent cross-linking is probably nonspecific and occasionally occurs during lyophilization of the samples if EDC is not completely removed by prior dialysis. The smaller SspC N-terminal variants tended to cross-link with somewhat lower efficiency than wild-type SspC, whereas the cross-linking of SspC<sup>34</sup> was significantly reduced compared with the larger variants (Fig. 3). To determine whether cross-linking was occurring between the same amino acid residues in the SspC N-terminal variants as in wild-type SspC, EDC cross-linked and monomeric proteins were purified by SDS-PAGE, digested with trypsin, and the total digests analyzed by MALDI-TOF mass spectrometry. MALDI maps of tryptic digests of wild-type SspC EDC dimer showed three additional peptides not seen in tryptic digest mass maps of EDC-treated SspC monomer (Fig. 4, A and B). The mass of one of these peptides (Fig. 4B, labeled 2745.12 Da) corresponded to that of a previously characterized EDC cross-linked peptide in SspC (Ala<sup>1</sup>–Arg<sup>8</sup> × Ser<sup>28</sup>–Lys<sup>27</sup>), which contains an isopeptide cross-link between the α-amino group of alanine 1 and the β-carboxyl of aspartate 13 (Table III) (11). The mass of a second unique peptide (Fig. 4, labeled 2490.75 Da) corresponded to that predicted for a peptide containing a cross-link between the α-amino group of alanine 1 and the γ-carboxyl of either glutamate 29 or 33 (Ala<sup>1</sup>–Arg<sup>8</sup> × Leu<sup>28</sup>–Arg<sup>66</sup>) (Table III); these glutamate residues are located within the GPR recognition and cleavage site (Fig. 1). This type of cross-link has previously been identified in other α/β-type SASP (SASP-A and SASP-C from <i>B. megaterium</i>, and Bee1<sup>610K</sup> from <i>Bacillus cereus</i>) treated with EDC (11), and therefore it was presumed that this peptide has the same structure. The mass of the third unique peptide was consistent with a peptide containing a cross-link between the α-amino group of alanine 1 and the C-terminal carboxyl group (Fig. 4, labeled 1802.55 Da). This third cross-linked species is probably a minor cross-linking product, as it has never been detected in reverse phase-HPLC tryptic maps of cross-linked SspC (11); therefore, we have not been able to purify this peptide nor confirm its structure. MALDI tryptic digest maps of EDC-cross-linked SspC<sup>34</sup> and SspC<sup>31</sup> indicated that the same amino acid residues were cross-linked as in wild-type SspC (Table III and data not shown). The MALDI tryptic map of SspC<sup>31</sup> dimer only showed two cross-linked peptides corresponding to cross-links between the α-amino group and aspartate 13 and the C terminus (Table III). It is possible that the other expected cross-linked peptide from SspC<sup>31</sup> failed to ionize efficiently and therefore was not detected by mass spectrometry. Only two cross-linked peptide species were expected in SspC<sup>34</sup> dimer trypsin digests because the aspartate residue involved in cross-linking in the other proteins (aspartate 13 in wild-type SspC) has been deleted in this variant (Fig. 1). We were only able to detect one cross-linked peptide, corresponding to a cross-link between the α-amino group and the C terminus, in the trypsin digest of SspC<sup>34</sup> dimer. However, we hypothesize that additional cross-linking occurs between the α-amino group and the glutamates.
of the GPR sequence, and that this cross-linked peptide probably does not ionize efficiently during mass spectrometry. These data, in conjunction with the estimates of DNA-bound N-terminal variant secondary structure content, suggest that the nature of the protein-protein interactions formed by the N-terminal variants while bound to DNA is very similar to those formed by wild-type SspC.

**The α-Amino Group and N-terminal Charge α/β-type SASP Are Important for DNA Binding**—Amino acid residues within the N-terminal half of α/β-type SASP have been shown to be in close contact with one another while in the DNA-bound state by cross-linking and all identified cross-links involved the α-amino group of the N terminus (11). The data presented thus far in this report also suggest that residues in the N-terminal region of α/β-type SASP are important in determining the strength of protein-protein interactions and therefore DNA binding affinity. Therefore, we sought to determine whether the α-amino group of α/β-type SASP is important for binding of these proteins to DNA. The N-terminal α-amino group of proteins can be specifically converted to an α-keto group by a nonenzymatic transamination reaction with glyoxylate and copper(II) ions, resulting in an oxidative deamination of the α-amino group (20, 21). Several α/β-type SASP were deaminated quantitatively as determined by polyacrylamide gel electrophoresis at acid pH (34), which demonstrated a net loss of positive charge (data not shown). The transamination reaction was specific as no other modifications were detected by reverse phase-HPLC peptide mapping of trypsin and endoproteinase.
of a DNA with significantly higher affinity than SspC found at this position in other because asparagine (or glutamine) and lysine residues are asparagine or a lysine residue. These changes were chosen was even more dramatic for another ing constant (Table III). The effect of N-terminal deamination of poly(dA-dT) more tightly than even wild-type SspC for pUC19 with a N-terminal deamination significantly reduced the affinity of deaminated SspC conferred somewhat less protection to plas-(data not shown). DNase I protection assays showed that (Fig. 5). N-terminal deamination of SspC 11 had the same effect upon the ability to provide DNase I protection to plasmid DNA as was seen with SspC-A (Fig. 5 and data not shown), whereas the effect was less dramatic comparing SspC and deaminated SspC (Fig. 5).

To further confirm that charged residues at the N terminus of α/β-type SASP are important for high affinity DNA binding, the aspartate 13 residue of SspC 11 was changed to either an asparagine or a lysine residue. These changes were chosen because asparagine (or glutamine) and lysine residues are found at this position in other α/β-type SASP (4, 31). For UV CD spectroscopy of the resulting proteins, SspC 11-D13N and SspC 11-D13K, bound to an excess of poly(dG)poly(dC) indicated that these proteins contain essentially the same amount of secondary structure as SspC 11 in the DNA-bound state (data not shown). As predicted, SspC 11-D13N and SspC 11-D13K, poly(dG)poly(dC) complexes were significantly more thermostable than the SspC 11-poly(dG)poly(dC) complex (Table I). Accordingly, SspC 11-D13N and SspC 11-D13K also bound to DNA with significantly higher affinity than SspC 11 (Table II). SspC 11-D13K had 10-fold greater affinity for pUC19 plasmid DNA than did SspC 11, whereas SspC 11-D13N bound to pUC19 and poly(dA-dT)poly(dA-dT) more tightly than even wild-type SspC (Table III). EDC cross-linking of SspC 11-D13K and subsequent MALDI mass spectrometry analysis confirmed that this protein (and presumably also SspC 11-D13N) makes the same DNA dependent protein-protein contacts as the other SspC N-terminal variants (Table III).

Analysis of the Effects of SspC N-terminal Variants on Spore Properties—The in vitro analysis of SspC N-terminal variants described above demonstrates that up to 14 amino acid resid-

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**Table III**

Identification of cross-linked tryptic peptides by MALDI-TOF mass spectroscopy

| Protein | Predicted peptide | Observed mass | Predicted mass |
|---------|-------------------|---------------|---------------|
| SspC    | Ala1–Arg5 × Ser2–Lys27 | 2745.12       | 2744.0        |
|         | Ala1–Arg5 × Leu6–Arg45 | 2480.75       | 2480.7        |
|         | Ala1–Arg5 × Leu4–His71 | 1802.55       | 1803.0        |
| SspC 11-D13K | Ala1–Arg5 × Ser2–Lys27 | 2398.96       | 2399.7        |
| SspC 11-D13N | Ala1–Arg5 × Leu4–Arg45 | 2134.86       | 2136.4        |
| SspC 11-D13K | Ala1–Arg5 × Leu4–His71 | 1457.51       | 1458.7        |
| SspC 11-D13N | Ala1–Lys27 × Leu61–His71 | 3604.8h | 3610.2        |
| SspC 11-D13K | Ala1–Lys27 × Leu61–His71 | 3699.2h | 3705.2        |
| SspC 11-D13N | Ala1–Lys27 × Leu61–His71 | 3024.7h | 3027.5        |
| SspC 11-D13K | Ala1–Lys27 × Leu61–His71 | 3378.9h | 3382.0        |
| SspC 11-D13N | Ala1–Lys27 × Leu61–His71 | 2910.2h | 2913.4        |
| SspC 11-D13K | Ala1–Lys27 × Leu61–His71 | 2571.1h | 2572.0        |
| SspC 11-D13N | Ala1–Lys27 × Leu61–His71 | 2108.5h | 2108.4        |
| SspC 11-D13K | Ala1–Lys27 × Leu61–His71 | 1430.7h | 1431.7        |

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* Amino acid residues are numbered according to their position in wild-type SspC. These peptides were detected only within EDC-treated cross-linked proteins and were absent in tryptic digests of EDC-treated monomeric proteins.

* These masses were determined using the negative ion mode, which resulted in underestimation of all peptide masses.

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Glu-C digests of unmodified and deaminated α/β-type SASP (data not shown). DNase I protection assays showed that deaminated SspC conferred somewhat less protection to plasmid DNA than did untreated SspC (Fig. 5, lanes 3 and 6). CD-based equilibrium binding studies also demonstrated that N-terminal deamination significantly reduced the affinity of SspC for pUC19 with a >6-fold decrease in the apparent binding constant (Table III). The effect of N-terminal deamination was even more dramatic for another α/β-type SASP, SASP-A, a major α/β-type SASP from *B. megaterium*, which binds less tightly to DNA than does SspC; deaminated SASP-A provided much less DNase I protection to plasmid DNA than untreated SASP-A (Fig. 5). N-terminal deamination of SspC 11 and SspC 11-D13 had the same effect upon the ability to provide DNase I protection to plasmid DNA as was seen with SASP-A (Fig. 5 and data not shown), whereas the effect was less dramatic comparing SspC and deaminated SspC 11-D13N (Fig. 5).

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**Fig. 5.** The α-amino groups of α/β-type SASP are important for DNA binding. α/β-type SASP were chemically deaminated to the α-κeto form and used in DNase I protection assays as described under “Experimental Procedures.” The arrows labeled a and b indicate the positions of 2.3- and 0.56-kilobase pair DNA size markers, respectively. Each reaction contained 0.12 mg/ml pUC19 plasmid DNA with unmodified or α-keto protein at 0.15 mg/ml (lane 1), 0.3 mg/ml (lane 2), or 0.6 mg/ml (lane 3). Some lanes contain bands at ~2.7 kilobase pairs, which probably arise from microdrops of plasmid DNA, which escaped exposure to DNase I.
DNA, suggest that proteins smaller than SspC cannot function as activators. Overexpression of shorter SspC N-terminal deletion variants, and significant to the SspC-DNA binding interaction. Our inability to measure DNA binding of the deleted residues contributes significantly to this phenomenon. These residues are therefore probably not directly involved in contacting the DNA, consistent with the low level of sequence conservation in this region of SspC.

N-terminal charge are due to the removal of arginines 5 and 7, and the increasing proximity of aspartate 13 to the N terminus, which may be deleted without destroying DNA binding function. In particular, the presence of the negatively charged aspartate 13 residue at the N terminus (in SspC) is essential for DNA binding. However, the net charge of the N terminus of wild-type SspC at pH 7.0 is +2, and is conferred by arginine 5 and the α-amino group (Fig. 1). By the same criteria, most of the N-terminal deletion variants of SspC have a less positively charged N terminus: SspCΔ5 is +2, SspCΔ10 is 0, SspCΔ11 is 0, Asp-N C-fragment is 0, and SspCΔ14 is +1 (Fig. 1). These changes in N-terminal charge are due to the removal of arginines 5 and 7, and the increasing proximity of aspartate 13 to the N terminus as amino acid residues are deleted from wild-type SspC (Fig. 1). In particular, the presence of the negatively charged aspartate 13 residue at the N terminus (in SspC) appears to interfere with protein-DNA interaction in SspCΔ14, the DNA binding affinity is significantly increased. Therefore, we hypothesize that electrostatic repulsion between the now N-terminal aspartate 13 and the acidic

Protein-Protein Interactions in α/β-Type SASP

DISCUSSION

The results reported here indicate that a substantial portion of the N terminus of SspC (~20% of total amino acid residues) may be deleted without destroying DNA binding function. These residues are therefore probably not directly involved in contacting the DNA, consistent with the low level of sequence conservation in this region of SspC. However, SspC N-terminal deletion variants do show reduced binding affinity for DNA, indicating that the deleted residues contribute significantly to the SspC-DNA binding interaction. Our inability to overexpress shorter SspC N-terminal deletion variants, and the failure of the GPR C-terminal fragment of SspC to bind DNA, suggest that proteins smaller than SspCΔ14 do not interact with DNA very strongly, and are presumably rapidly degraded in vivo. Additionally, it appears that the N terminus of wild-type SspC is considerably flexible because protein cross-linking demonstrated that the N termini of the SspC N-terminal variants all make similar close contacts with acidic residues on adjacent DNA-bound proteins. Indeed, heterotypic cross-links have also been identified between SASP-A and SASP-C from B. megaterium, whose N termini vary greatly in length (4, 11). These conclusions are consistent with the variability in N-terminal length and sequence seen in the amino acid alignment of all identified α/β-type SASPs; in fact, the first significantly conserved amino acid residue in wild-type SspC is asparagine 12 (Fig. 1). However, although not conserved or required for DNA binding, residues glutamate 2 through asparagine 11 in wild-type SspC are important in that they significantly increase the affinity of this α/β-type SASP for DNA.

Thermal stability and equilibrium binding analysis of the SspC N-terminal variant-DNA complexes indicate that SspCΔ14 binds to DNA with higher affinity than SspCΔ10, SspCΔ11, and the large Asp-N fragment of SspC. The DNA binding affinity of these proteins seems to be related to the net charge in the N-terminal regions. These findings, in conjunction with the EDC cross-linking data showing that the N terminus of each protein is in close contact with negatively charged residues on adjacent DNA-bound protein (11), may be explained by a model of DNA-dependent protein-protein interaction which involves a significant electrostatic interaction. According to this model, a flexible and positively charged N terminus from each DNA-bound protein interacts with a proposed acidic patch (formed by aspartate 13 and glutamates 29 and 33) found on a neighboring DNA-bound protein (Fig. 7). If the N terminus is arbitrarily defined as the first five amino acid residues, the net charge of the N terminus of wild-type SspC at pH 7.0 is +2, and is conferred by arginine 5 and the α-amino group (Fig. 1). By the same criteria, most of the N-terminal deletion variants of SspC have a less positively charged N terminus: SspCΔ5 is +2, SspCΔ10 is 0, SspCΔ11 is 0, Asp-N C-fragment is 0, and SspCΔ14 is +1 (Fig. 1). These changes in N-terminal charge are due to the removal of arginines 5 and 7, and the increasing proximity of aspartate 13 to the N terminus as amino acid residues are deleted from wild-type SspC (Fig. 1). In particular, the presence of the negatively charged aspartate 13 residue at the N terminus (in SspC) appears to interfere with protein-DNA interaction and therefore with DNA binding affinity, because when this residue is removed (along with leucines 14 and 15) in SspCΔ14, the DNA binding affinity is significantly increased.

Furthermore, we hypothesize that electrostatic repulsion between the now N-terminal aspartate 13 and the acidic

FIG. 6. Heat and UV radiation resistance of spores expressing SspC N-terminal deletion variants. Spores of various strains were purified, and their resistances to UV radiation at 254 nm (panel A) and heat at 85 °C (panel B) were determined as described under "Experimental Procedures." The following spores were tested: αβ-SspC (■), αβ-SspCΔ5 (○), αβ-SspCΔ10 (△), αβ-SspCΔ11 (□), αβ-SspCΔ14 (▲), αβ-SspCΔ11/pUB110 (no αβ-type SASP, •).

FIG. 7. Model of SspC protein-protein contacts in the DNA-bound state. Unbound SspC are depicted as random coil peptide chains that interact with one another and the DNA binding site for productive binding to occur (6). Significant structural rearrangements occur during binding (DNA-bound SspC are represented as ovals), and protein-protein interactions between the positively charged N-terminal region (labeled + and N+) and a proposed negatively charged patch are shown.

The results reported here indicate that a substantial portion of the N terminus of SspC (~20% of total amino acid residues) may be deleted without destroying DNA binding function. These residues are therefore probably not directly involved in contacting the DNA, consistent with the low level of sequence conservation in this region of SspC. However, SspC N-terminal deletion variants do show reduced binding affinity for DNA, indicating that the deleted residues contribute significantly to the SspC-DNA binding interaction. Our inability to overexpress shorter SspC N-terminal deletion variants, and the failure of the GPR C-terminal fragment of SspC to bind DNA, suggest that proteins smaller than SspCΔ14 do not interact with DNA very strongly, and are presumably rapidly degraded in vivo. Additionally, it appears that the N terminus of wild-type SspC is considerably flexible because protein cross-linking demonstrated that the N termini of the SspC N-terminal variants all make similar close contacts with acidic residues on adjacent DNA-bound proteins. Indeed, heterotypic cross-links have also been identified between SASP-A and SASP-C from B. megaterium, whose N termini vary greatly in length (4, 11). These conclusions are consistent with the variability in N-terminal length and sequence seen in the amino acid alignment of all identified α/β-type SASPs; in fact, the first significantly conserved amino acid residue in wild-type SspC is asparagine 12 (Fig. 1). However, although not conserved or required for DNA binding, residues glutamate 2 through asparagine 11 in wild-type SspC are important in that they significantly increase the affinity of this α/β-type SASP for DNA.

Thermal stability and equilibrium binding analysis of the SspC N-terminal variant-DNA complexes indicate that SspCΔ14 binds to DNA with higher affinity than SspCΔ10, SspCΔ11, and the large Asp-N fragment of SspC. The DNA binding affinity of these proteins seems to be related to the net charge in the N-terminal regions. These findings, in conjunction with the EDC cross-linking data showing that the N terminus of each protein is in close contact with negatively charged residues on adjacent DNA-bound protein (11), may be explained by a model of DNA-dependent protein-protein interaction which involves a significant electrostatic interaction. According to this model, a flexible and positively charged N terminus from each DNA-bound protein interacts with a proposed acidic patch (formed by aspartate 13 and glutamates 29 and 33) found on a neighboring DNA-bound protein (Fig. 7). If the N terminus is arbitrarily defined as the first five amino acid residues, the net charge of the N terminus of wild-type SspC at pH 7.0 is +2, and is conferred by arginine 5 and the α-amino group (Fig. 1). By the same criteria, most of the N-terminal deletion variants of SspC have a less positively charged N terminus: SspCΔ5 is +2, SspCΔ10 is 0, SspCΔ11 is 0, Asp-N C-fragment is 0, and SspCΔ14 is +1 (Fig. 1). These changes in N-terminal charge are due to the removal of arginines 5 and 7, and the increasing proximity of aspartate 13 to the N terminus as amino acid residues are deleted from wild-type SspC (Fig. 1). In particular, the presence of the negatively charged aspartate 13 residue at the N terminus (in SspC) appears to interfere with protein-DNA interaction and therefore with DNA binding affinity, because when this residue is removed (along with leucines 14 and 15) in SspCΔ14, the DNA binding affinity is significantly increased.

Therefore, we hypothesize that electrostatic repulsion between the now N-terminal aspartate 13 and the acidic
patch on an adjacent DNA-bound SspC\(^{11}\) (or SspC\(^{10}\)) contributes to the lower DNA binding affinity. Consistent with this model, derivatives of SspC\(^{11}\) that contain either an asparagine or a lysine residue in place of aspartate 13 have significantly higher affinity for DNA than SspC\(^{11}\).

The model presented above (Fig. 7) is probably applicable to all \(\alpha/\beta\)-type SASP; the evidence for this is as follows. First, DNA-dependent EDC cross-links identical to those found in SspC have been identified in three other \(\alpha/\beta\)-type SASP from different species (11). Second, higher \(\alpha/\beta\)-type SASP-DNA binding affinity roughly corresponds to a net positively charged N terminus (5, 6, 11). Third, deamination of SASP-\(\alpha\) and SspC and its variants reduces the N-terminal net positive charge and results in significantly lower DNA binding affinity. Of course, we cannot exclude the possibility that the N-terminal \(\alpha\)-keto group in deaminated \(\alpha/\beta\)-type SASP actually disrupts binding interactions. However, the electrostatic component of \(\alpha/\beta\)-type SASP protein-protein interactions has been shown to be thermodynamically significant because the cooperativity parameter (\(\omega\)) of the \(\alpha/\beta\)-type SASP-DNA interaction decreases with increasing salt concentration (6), suggesting that DNA dependent protein-protein interactions are destabilized by salt.

Although the deletion of 10 amino acid residues from the N terminus of SspC resulted in a protein (SspC\(^{10}\)) that showed significantly lower affinity for DNA in vitro, it was still able to confer almost full resistance to UV radiation and significant protection from heat to \(\alpha/\beta\) spores. This is not particularly surprising because SspC-\(\alpha\), the \(\alpha/\beta\)-type SASP in \(B. subtilis\) most responsible for spore UV resistance, has a much lower affinity for DNA than does wild-type SspC (5, 7). In fact, it is thought that high affinity for DNA is not necessary for \(\alpha/\beta\)-type SASP function because the major \(\alpha/\beta\)-type SASP are present at very high concentrations (\(\sim 1-2 \text{ mm}\)) within the spore core (4). At these concentrations, \(80-90\%\) of \(\alpha/\beta\)-type SASP would be DNA-bound at binding constants as low as \(1.0-2.0 \times 10^{-4} \text{ M}\). In addition, the spore core is very dehydrated compared with the corresponding cell cytoplasm, and most of the divalent cations are probably chelated by the enormous level of dipicolinate in the spore core (1); both of these spore core environmental factors should tend to favor \(\alpha/\beta\)-type SASP-DNA interaction. However, although SspC\(^{11}\) has a similar or even higher affinity for DNA in vitro compared with SspC\(^{10}\), it was less able to confer UV and heat resistance to \(\alpha/\beta\) spores. The reason for this is not clear, but may be due to the removal of the very highly conserved asparagine 12 residue, which is found in all \(\alpha/\beta\)-type SASP identified from \(Bacillus\) species (4, 31). Perhaps this conserved asparagine residue plays an significant structural role in vivo.

It appears that the function of amino acid residues glutamine 2 through asparagine 11 in wild-type SspC is to increase the DNA binding affinity of the protein. Although this increased affinity is not necessary for \(\alpha/\beta\)-type SASP function in vivo, it may reflect the actual role SspC plays in wild-type (\(\alpha/\beta\) ) spores. SspC is a minor \(\alpha/\beta\)-type SASP and is only present as \(\sim 10\%\) of total \(\alpha/\beta\)-type SASP within wild-type \(B. subtilis\) spores (4), with SASP-\(\alpha\) and SASP-\(\beta\) comprising most (\(\sim 80\%)\) of the remaining \(\alpha/\beta\)-type SASP (18). SspC has a higher affinity for DNA than both SASP-\(\alpha\) and SASP-\(\beta\), and therefore SspC may have been selected to bind regions of the spore chromosome that are not bound efficiently by SASP-\(\alpha\) or SASP-\(\beta\). Although there is no significant decrease in spore heat or UV radiation resistance in the laboratory when the sspC gene is inactivated, the role for SspC proposed above could confer a selective advantage to spores. This proposed function for SspC may be generalizable because all \(Bacillus\) examined to date contain a number of minor \(\alpha/\beta\)-type SASP (4, 31).

The results in this report also have practical value in aiding in the designing of a minimal high affinity \(\alpha/\beta\)-type SASP for biophysical studies. Because all \(\alpha/\beta\)-type SASP are largely unstructured in the absence of double-stranded DNA (6), a well defined \(\alpha/\beta\)-type SASP-oligonucleotide complex will be required to obtain a high resolution structure. This complex should be small to avoid potential problems with degeneracy arising from the nonspecific nature of \(\alpha/\beta\)-type SASP-DNA binding. In addition, only small complexes (<25 kDa) are routinely tractable by multidimensional NMR methods. For these reasons, the identification of minimal \(\alpha/\beta\)-type SASP that maintain high affinity for DNA is desirable. This study indicates that \(\sim 1.2 \text{ kDa}\) of SspC may be removed while retaining DNA binding, although there is an attendant loss of binding affinity. However, we have been able to produce a truncated \(\alpha/\beta\)-type SASP, SspC\(^{11-13}\), which binds to DNA with higher affinity than even wild-type SspC. Small, double-stranded oligonucleotides that bind to \(\alpha/\beta\)-type SASP have previously been identified by CD spectroscopic studies (6), and these oligonucleotides are currently being tested with SspC\(^{11-13}\) in an attempt to determine the solution structure of an \(\alpha/\beta\)-type SASP-DNA complex by multidimensional NMR studies.

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