Importance of the N-terminal Region of the Phage GA-1 Single-stranded DNA-binding Protein for Its Self-interaction Ability and Functionality*

The single-stranded DNA-binding protein (SSB) of phage GA-1 displays higher efficiency than the SSBS of the related phages φ29 and Nf. In this work, the self-interaction ability of GA-1 SSB has been analyzed by visualization of the purified protein by electron microscopy, glycerol gradient sedimentation, and in vivo cross-linking of bacterial cultures infected with phage GA-1. GA-1 SSB contains an insert at its N-terminal region that is not present in the SSBS of φ29 and Nf. Three deletion mutant proteins have been characterized, ΔN19, ΔN26, and ΔN33, which lack the 19, 26 or 33 amino acids, respectively, that follow the initial methionine of GA-1 SSB. Mutant protein ΔN19 retains the structural and functional behavior of GA-1 SSB, whereas mutant proteins ΔN26 and ΔN33 no longer stimulate viral DNA replication or display helix-destabilizing activity. Analysis of the mutant proteins by ultracentrifugation in glycerol gradients and electron microscopy indicates that deletion of 26 or 33 but not of 19 amino acids of the N-terminal region of GA-1 SSB results in the loss of the oligomerization ability of this protein. Our data support the importance of the N-terminal region of GA-1 SSB for the differential self-interaction ability and functional behavior of this protein.

Single-stranded DNA-binding proteins (SSBs)† contribute to DNA metabolism, playing essential roles in different processes such as DNA replication, repair, and recombination (1–3). SSBS bind single-stranded DNA (ssDNA) in a selective, cooperative, and non-sequence-specific way, protecting it from nuclease attack and preventing the formation of secondary structures on it. SSBS are ubiquitous. They have been isolated from bacteria and their phages, eukarya and their viruses, and archaea (1–6).

Different oligomerization states have been reported for SSBS. Thus, monomeric (T4gp32 from bacteriophage T4 or AdDBP from adenovirus (5)), dimeric (SSBS from filamentous phages M13 (1) and P3 (7)), homotetrameric (EcoSSB from Escherichia coli (2, 8) or human mitochondrial SSB (9)), and heterotrimeric (hRPA, human RPA (3)) SSBS have been described. Important differences have been also reported mainly concerning the amino acid sequence and ssDNA binding properties of the proteins of this family.

The function of the SSB is particularly important in the case of organisms that replicate their genetic material via a protein-priming mechanism followed by strand displacement, as large amounts of ssDNA are generated in the process (10–12). This is the case of adenovirus and of the φ29 family of Bacillus phages. The genome of the latter consists of a linear dsDNA molecule of about 20 kb that contains a phage-encoded terminal protein (TP) covalently linked to each 5’ end. Replication of the viral genome starts at either DNA end non-simultaneously by a protein-priming mechanism. After a sliding-back step (13), the viral DNA polymerase elongates the initiation product proceeding by strand displacement toward the other DNA end. The displaced strand is cooperatively bound by the viral SSB. When two growing chains, running from opposite ends, collide and separate, two DNA molecules are generated where strand displacement is no longer required (10–12) and where the SSB must be eliminated from the ssDNA by the advancing DNA polymerase.

The φ29 family of phages has been classified into three evolutionary branches according to the comparison of nucleotide and amino acid sequences of selected DNA regions and proteins (14, 15). One branch is composed of phages φ29, PZA, φ15, and BS32; the second branch consists of phages Nf, B103, and M2Y; and the third branch has phage GA-1 as its sole member (15).

Initial studies carried out with the SSB of φ29 indicated that it has ability to protect ssDNA against nuclease degradation (16) and to bind the ssDNA generated during viral DNA replication in vitro, as analyzed by electron microscopy (17). Furthermore, it was determined that φ29 SSB has helix-destabilizing ability (18), and the parameters of the complex it forms with ssDNA were defined (19).

Recent comparison of φ29 SSB with the SSBS of phages Nf and GA-1, representative examples, respectively, of the second and third branches in which the φ29 family of phages is divided, indicate that, despite some global similarities, GA-1 SSB displays significant functional and structural differences with respect to the other two SSBS. Thus, it binds ssDNA with higher affinity and displays helix-destabilizing ability with lower protein concentrations than the other two SSBS (20, 21). The complex formed by GA-1 SSB with ssDNA is clearly dif-

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The abbreviations used are: SSB, ssDNA-binding protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; TP, terminal protein; TP-DNA, viral TP-DNA template; DBP, double-stranded DNA-binding protein; β-ME, β-mercaptoethanol; DTT, dithiothreitol; BS3, bis(sulfosuccinimidyl)suberate; BSA, bovine serum albumin; wt, wild-type.
ferent from the φ29-like nucleoprotein complex, as determined by electron microscopy in the presence of DNA (19, 20). Thus, GA-1 SSB produces an average 6-fold reduction in the length of the ssDNA in contrast to the 2-fold reduction factor obtained upon binding of φ29 SSB to ssDNA. Besides, the nucleoprotein complex formed by GA-1 SSB shows a compact beaded appearance (20), very different from the simple array of protein monomers of φ29 SSB/ssDNA fibers (19). Further approaches oriented to the structural comparison of the three SSBs indicate that φ29 and Nf SSBs behave as monomers in solution in glycerol gradients, whereas GA-1 SSB self-interacts sedimenting at a position corresponding to an average hexameric complex. This differential self-interaction ability correlates with a higher efficiency of GA-1 SSB in functional assays.

Amino acid sequence comparison of the three SSBs highlights the high degree of homology between φ29 and Nf SSBs, as well as the higher divergence of GA-1 SSB. The latter contains an insert at its N-terminal region that is lacking in the other two SSBs (21).

In the present report, further approaches to the characterization of GA-1 SSB have been carried out. Thus, the self-interaction ability of GA-1 SSB has been analyzed by visualization of the purified protein by electron microscopy in the absence of DNA, by glycerol gradient sedimentation, and by in vivo cross-linking of bacterial cultures infected with phage GA-1. Furthermore, the effect of the partial or complete deletion of the N-terminal insert of GA-1 SSB on the self-interaction ability of the protein as well as on its functional activity has been also examined. We show that a mutant protein lacking the 19 N-terminal amino acids retains the structural and functional behavior of GA-1 SSB, whereas mutants lacking 26 or 33 amino acids from the N-terminal end are greatly affected.

The influence of the N-terminal region of GA-1 SSB on the self-interaction ability of the protein and the requirement of this ability for an efficient functional behavior are also discussed.

**EXPERIMENTAL PROCEDURES**

**Nucleotides and DNA—**Phage φ29 TP-DNA was isolated as described (22). Oligonucleotides were obtained from Isogen, and plasmid pALTER-Ex2 from Promega. [α-32P]dATP (3000 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol) were obtained from Amersham Biosciences. Unlabeled nucleotides and M13mp18 ssDNA were from Amersham Biosciences. DNA sequencing was carried out in the Servicio Interdepartamental de Investigación of the Universidad Autónoma de Madrid. Purification of the Mutant Proteins—φ29 DNA polymerase, T4 DNA ligase, and restriction enzymes BamHI and EcoRI were purchased from New England Biolabs. The φ29 DNA polymerase, TP, and DBP were overproduced in E. coli and purified as described (23–25). GA-1 SSB was purified from infected Bacillus sp. G1R cells (26) as previously described (21).

**Construction of Plasmids—**Using GA-1 DNA as template and the appropriate oligonucleotide primers, DNA fragments corresponding to the mutant proteins ΔN19, ΔN26, and ΔN33 were amplified by PCR. Vent DNA polymerase, which contains a proofreading function, was used. The primers corresponding to the N-terminal region of each protein contained a recognition site for BamHI, whereas the oligonucleotide corresponding to the C-terminal region, common to all of them, contained a recognition site for EcoRI.

PCR-generated DNA fragments were digested with BamHI and EcoRI, and then cloned into the BamHI and EcoRI sites of plasmid pALTER-Ex2. All the clones generated from the PCR-amplified DNA were sequenced and found to be correct.

**Purification of the Mutant Proteins—**E. coli JM109 cells (endA recA rgaA96 thi hsdR17 (r P m t g) relA1 supE44 Δ(lac-proAB) (P truD98 proA2 lacZAM15)) (27) carrying the plasmids of interest were grown at 37 °C in LB broth (28) supplemented with 10 mg/ml tetracycline. At a cell density corresponding to $A_{600}$ = 0.5, isopropyl-β-D-thiogalactopyranoside (Sigma) was added to a final concentration of 1 mM. Further incubation was at 37 °C for 3 h. Bacteria were harvested by centrifugation and after cell disruption by grinding with alumina (2:1, w/v), resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 7 mM β-ME, and 5% glycerol) in the presence of 0.4 mM NaCl.

All purification steps were carried out at 4 °C. The supernatant of a 1500 × g centrifugation step of 5 min was further centrifuged for 20 min at 22,000 × g. Mutant proteins ΔN26 and ΔN33 were recovered in the supernatant of this centrifugation step, whereas ΔN19 was mainly contained in the sediment. DNA was removed from the supernatant of proteins ΔN26 and ΔN33 by addition of polyethyleneimine to 0.3% after adjusting the absorbance at $A_{260}$ nm, to 120 units/ml and centrifugation for 20 min at 20,000 × g. The supernatant was made 0.1 M NaCl with buffer A, and the proteins were recovered in the supernatant after centrifugation as above. The samples were precipitated with 65% ammonium sulfate, dissolved in buffer A, and, after dialysis against the same buffer, applied to the following chromatography column in the case of ΔN26, phosphocellulose, heparin-agarose, Mono-Q, and hydroxyapatite; in the case of ΔN33, phosphocellulose, heparin-agarose, DEAE-cellulose, and Mono-Q. Mutant protein ΔN19 was recovered from the sediment with denaturation buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25% glycerol (v/v), 10 mM DTT, and 4 mM guanidinium chloride). After centrifugation for 20 min at 20,000 × g, the supernatant was dialyzed against renaturation buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25% glycerol (v/v), 10 mM DTT, and 0.1 M NaCl) and centrifuged under the same conditions. The supernatant containing the native protein was applied to the following columns: phosphocellulose, heparin-agarose, and Mono-Q. In all three cases, protein samples eluted from the last column were precipitated with ammonium sulfate up to 35% dissolved in buffer A, and dialyzed against the same buffer containing 50% glycerol (v/v). All purification steps were followed by SDS-PAGE (10–20% acrylamide gradients). Protein concentration was determined both by the Lowry method and by comparison with a known amount of BSA in polyacrylamide gel electrophoresis.

**In Vivo Cross-linking of GA-1 SSB—**Both noninfected and GA-1-infected Bacillus sp. G1R cell cultures were harvested after 15 min of infection at 37 °C. Aliquots of 1.5 ml were centrifuged at 4 °C, and the corresponding pellets were incubated with increasing amounts of the cross-linking agent BS3 (Pierce) in a final volume of 200 μl. After 30 min at room temperature, 40 μl of 1× Tris-HCl, pH 7.5, were added to each reaction to quench it. After centrifugation, pellets were resuspended in 300 μl of loading buffer, samples sonicated, and aliquots of 10 μl loaded on to 10–20% SDS-PAGE. Proteins were electrothermally transferred for 70 min at 100 mA and 4 °C to PVDF membranes using a transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Membranes were incubated with 1/1000 diluted rabbit antibodies against GA-1 SSB. Antigen-antibody complexes were detected with anti-rabbit horseradish peroxidase-linked antibody and ECL Western blotting detection reagent (both from Amersham Biosciences). Protein molecular weight markers were loaded in the gel as molecular weight controls.

TP-DNA Amplification Assay—The incubation mixture contained, in 25 μl, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 20 mM ammonium sulfate, 10 mM MgCl2, 50 μM each dCTP, dGTP, dTTP, and [α-32P]dATP (2 μCi), 3.4 ng of φ29 TP-DNA, 7 ng of DNA polymerase, 25 ng of TP, and 10 μg of DBP, all from φ29. The indicated amount of mutant protein or the corresponding buffer were added. After incubation for 1 h at 30 °C, reactions were stopped by adding EDTA up to 10 mM and SDS up to 0.1% (w/v), and the samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The excluded volume was subjected to alkaline agarose gel electrophoresis as described (29), followed by autoradiography and ethidium bromide staining.

**Helix-stabilizing Assay—**The incubation mixture contained, in 12.5 μl, 62.5 ng of primed M13mp18 ssDNA, 50 mM Tris-HCl, pH 7.5, 4% glycerol, 0.1 mg/ml BSA, and the indicated amounts of GA-1 SSB, the mutant proteins or the corresponding buffer. After 30 min at 37 °C, reactions were stopped with 1.25 μl of 0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol, and 0.5% SDS. Samples were subjected to electrophoresis at 4 °C in an 8% polyacrylamide gel containing 0.1% SDS. The gel was dried and autoradiographed.

**Cell Mobility-shift Assays—**The incubation mixture contained, in a final volume of 20 μl, a heat-denatured α-32P-labeled φ29 DNA HindIII fragment and the indicated amounts of φ29, Nf, or GA-1 SSB in 50 mM Tris-HCl, pH 7.5, and 4% glycerol. After incubation for 5 min at 4 °C, the samples were subjected to electrophoresis in a 0.4% agarose gel containing 12 mM Tris-acetate, pH 7.5, and 1 mM EDTA, and run at 4 °C in the same buffer at 8 V/cm essentially as described (30). After drying, the gels were autoradiographed and the SSB/ssDNA complexes detected as mobility of the labeled DNA.

**Glycerol Gradient Assays—**Glycerol gradients (15–30%), containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 7 mM β-ME, and 25 mM NaCl (loading buffer), were formed in 5-mL Beckman polyelemulose centrifuge
is not present in the SSBs of the related phages.

Electron Microscopy—GA-1 SSB or the mutant proteins (10 nl of a 55

μm solution) were applied to carbon-coated copper grids for 2 min. Grids

were then washed, with a few drops of water and stained for 30 s with

2% uranyl acetate. Electron micrographs were taken in a Jeol 1010
electron microscope at 80 kV.

RESULTS

Analysis of the Ability of GA-1 SSB to Self-interact by Elec-
tron Microscopy and in Vivo Cross-linking—The ability of GA-1
SSB to self-interact was analyzed both by visualization of the
purified protein by electron microscopy and by in vivo cross-
linking of bacterial cultures infected with phage GA-1. After
negative staining, GA-1 SSB was visualized in the absence of
DNA and chemical cross-linking reagents by electron micro-
scopy as described under “Experimental Procedures.” Under
these conditions purified GA-1 SSB oligomerized in vitro, as
can be observed in Fig. 1A, which shows a representative
example of the most abundant structures formed by wt GA-1
SSB. They consist of protein filaments of different lengths
and a diameter of ~10 nm. Along with these filaments, protein
structures where regions of different compacting levels coexist
were detected, like the one displayed in Fig. 1B.

The ability of GA-1 SSB to self-interact was also analyzed in
vivo by treatment of GA-1-infected *Bacillus* sp. G1R cell cul-
tures with the cross-linking agent BS², followed by Western
blot analysis with antibodies raised against GA-1 SSB (for
details, see “Experimental Procedures”). Fig. 2 shows both non-
infected (−I) and GA-1-infected (+I) cell cultures after treat-
ment with increasing amounts of the cross-linking agent. Pos-
itons corresponding to the mono-, di-, tri-, and tetrameric forms
of the protein are indicated. Oligomers of higher molec-
ular mass were also detected. Additional bands appear that
could correspond to interaction of GA-1 SSB with other pro-
enins.

Deletion of the N-terminal Region of GA-1 SSB Affects Its
Stimulatory Effect on Viral DNA Replication—Previous studies
indicate that GA-1 SSB displays differential structural and
functional behavior to those of the SSBs of the related phages
ϕ29 and Nf (20, 21). GA-1 SSB contains an insert on its N-
terminal region, rich in polar and aromatic amino acids, which
is not present in the SSBs of the related phages ϕ29 and Nf (see
Fig. 3). To analyze the effect of the partial or complete deletion
of this insert on the global activity of GA-1 SSB, the three
mutant proteins ∆N19, ∆N26, and ∆N33 indicated in Fig. 3

were expressed from the corresponding plasmids in *E. coli*
JM109 cells and purified as previously described. ∆N19, ∆N26,
and ∆N33 lack the 19, 26, or 33 amino acids, respectively, that
follow the initial methionine residue in the ∆N29, ∆N26, and ∆N33 mutant proteins of
GA-1 SSB are indicated with arrows. The polar and aromatic residues
within the N-terminal region are also highlighted.

FIG. 1. Visualization of GA-1 SSB by electron microscopy. A, field showing filaments of different lengths and ~10-nm diameter. B, coexistence of different compacting levels within the same structure.

FIG. 2. Ability of GA-1 SSB to self-interact in vivo. Noninfected (−I) and GA-1-infected (+I) *Bacillus* sp. G1R cultures were harvested, treated with the indicated amounts of BS², and subjected to 10–20% SDS-PAGE. Western blot analysis of the gel was then carried out. Positions corresponding to the mono-, di-, tri-, and tetrameric forms of the protein are indicated at the right. Increasing amounts of GA-1 SSB were used as control.

FIG. 3. Sequence alignment of ϕ29, Nf, and GA-1 SSBs. Identical residues in the three SSBs are white on a black background. Similar residues of the ϕ29 and/or NF SSBs with respect to GA-1 SSB, the least related of them, are black on a gray background. Similarities between the ϕ29 and NF SSBs are not highlighted. Numbers indicate the amino acid position. The first amino acids that follow the initial methionine residue in the ∆N29, ∆N26, and ∆N33 mutant proteins of GA-1 SSB are indicated with arrows. The polar and aromatic residues within the N-terminal region are also highlighted.
55 μM for the mutant proteins ΔN26 and ΔN33 did dNTP incorporation reach the levels obtained with 3.5 μM wt protein. Furthermore, addition of concentrations as high as 250 μM for the mutant proteins ΔN26 and ΔN33 did not significantly improve the levels of dNTP incorporation (data not shown). The lack of global functional efficiency of mutant proteins ΔN26 and ΔN33 is discussed below, and a comparison with the SSBs of φ29 and Nf is also established.

The N-terminal Region of GA-1 SSB Is Essential for Its Helix-destabilizing Activity—The helix-destabilizing ability of GA-1 SSB mutant proteins ΔN19, ΔN26, and ΔN33 was examined on a substrate consisting of M13 ssDNA to which a 5' radioactively labeled 17-mer oligonucleotide had been hybridized. This substrate was incubated with increasing amounts of GA-1 SSB or the mutant proteins, and the reaction products analyzed by native polyacrylamide gel electrophoresis. As can be seen in Fig. 5, ΔN19 mutant protein displayed the same helix-destabilizing ability as the wt GA-1 SSB, displacing all the 17-mer oligonucleotide from the M13 ssDNA with a 10 μM protein concentration. As also shown in Fig. 5, neither ΔN26 nor ΔN33 mutant proteins were able to displace the oligonucleotide, even at protein concentrations as high as 160 μM.

Deletion of the N-terminal Region of GA-1 SSB Does Not Fully Abolish Its Ability to Bind ssDNA—To determine whether the lack of functional efficiency displayed by the mutant proteins ΔN26 and ΔN33 on TP-DNA amplification and dsDNA unwinding was a consequence of their inability to bind ssDNA, the affinity of these mutant proteins for ssDNA was tested in a gel-mobility shift assay. For this purpose, a radioactively labeled 273-nt-long ssDNA fragment was incubated with increasing amounts of either the mutant proteins or the wt GA-1 SSB. As can be seen in Fig. 6, both ΔN26 and ΔN33 displayed a lower affinity for ssDNA than wt GA-1 SSB. Thus, although GA-1 SSB shifted the band corresponding to free ssDNA with a concentration of 1 μM, a 5-fold higher concentration of ΔN26 was required to produce the same effect. This protein concentration did not suffice in the case of ΔN33 to shift all the DNA present in the assay. Instead, complete binding of all the DNA present in the assay was achieved in the case of ΔN33 with a concentration of 8 μM (data not shown). Mutant protein ΔN19 displayed affinity for ssDNA similar to that of the wt GA-1 SSB (data not shown). These results indi-

![Fig. 4. Stimulatory effect of GA-1 SSB or the mutant proteins ΔN19, ΔN26, and ΔN33 on viral DNA replication using the φ29 DNA amplification system. The indicated amounts of each protein were added to the reaction mixture. Electrophoresis mobility of full-length φ29 TP-DNA is also indicated.](image1)

![Fig. 5. Helix-destabilizing activity of GA-1 SSB and of the mutant proteins ΔN19, ΔN26, and ΔN33. M13 ssDNA to which 5’ radioactively labeled 17-mer oligonucleotide was hybridized was incubated with increasing amounts of each protein. Positions of the hybrid substrate and the displaced oligonucleotide are indicated. C is the control of the heat-denatured substrate.](image2)

![Fig. 6. Gel mobility shift assay of wt GA-1 SSB, ΔN26, and ΔN33 mutant proteins. A radioactively labeled 273-nt-long ssDNA fragment was incubated with the indicated amounts of GA-1 SSB, ΔN26, or ΔN33 at 4°C and subjected to electrophoresis in a 4% polyacrylamide gel as described under “Experimental Procedures.” The band corresponding to the protein-free ssDNA is indicated (D).](image3)
that ΔN19 (17.0 kDa/monomer) sediments in an average position of 106 kDa that would correspond to an hexamer, as wt GA-1 SSB (20). By contrast, the protein peak in the case of ΔN26 mutant protein (16.1 kDa/monomer) is ~51 kDa. This position would correspond to a trimeric complex of ΔN26. Aggregation ability of ΔN33 mutant protein (15.2 kDa) is even more affected, with a sedimentation peak at ~29 kDa that would correspond to a dimeric complex. Thus, deletion of 26 or 33 but not of 19 amino acids of the N-terminal region of GA-1 SSB results in loss of the oligomerization ability of the protein.

Visualization of the Deletion Mutant Proteins by Electron Microscopy—Analysis of the deletion mutant proteins by negative-stain electron microscopy was carried out in the absence of DNA as already described for wt GA-1 SSB. The appearance of protein ΔN19 in solution was identical to that of the filaments formed by GA-1 SSB (data not shown). In contrast, in the case of the mutant proteins ΔN26 and ΔN33, the ability to form protein filaments is abolished. Instead, smaller, ring-shaped structures like the ones shown in Fig. 8A for ΔN26, were detected. ΔN33 formed the same kind of structures (data not shown). A closer view of these structures is displayed in Fig. 8B. A correlation between the inability of these mutant proteins to form filaments and their loss of functionality is suggested under “Discussion.”

DISCUSSION

When compared with the SSBs of related phages, the SSB of phage GA-1 displays some noticeable features, one of them being its self-association ability. Thus, on the one hand, even in the absence of ssDNA, GA-1 is able to form filamentous and helicoidal structures that can be visualized by electron microscopy. Under these experimental conditions, neither of the SSBs of the related phages φ29 and Nf are able to form these high order aggregates.2 On the other hand, when subjected to ultracentrifugation in glycerol gradients, φ29 and Nf SSBs behave as monomers in solution, whereas GA-1 SSB sediments in the average position of an hexameric complex (20). It must be taken into account that protein-assembly processes are strictly dependent of protein concentration. Thus, the fact that a 10-fold lower protein concentration was used in glycerol gradient centrifugation than in electron microscopy may explain why no material was detected at the bottom of the tube of the GA-1 SSB gradient that would correspond to the filament-forming protein population. However, filament disruption because of the more drastic experimental conditions of the ultracentrifugation with respect to the electron microscopy experiments cannot be ruled out. In vivo cross-linking of Bacillus cultures infected with phage GA-1 confirms the ability of GA-1 SSB to

2 I. Gascón, unpublished data.
self-interact, although under our experimental conditions monomers and low molecular weight oligomers were seen in addition to high order aggregates that could correspond to the protein filaments. Anyway, whether the filaments formed by GA-1 SSB are the active form of the protein or not, at least they are representative of the differential ability of the protein to self-interact, which is lacking in the SSBs of the related phages φ29 and Nf.

The importance of the self-interaction ability of GA-1 SSB is further demonstrated by the results obtained with the deletion mutants ΔN19, ΔN26, and ΔN33 of the N-terminal region of the protein. Deletion of the 26 and 33, but not of the 19, amino acids that follow the initial methionine residue of GA-1 SSB results in the abolishment of the helix-destabilizing ability of the wt protein, as well as in a drastic reduction of its functional efficiency in DNA replication assays. These activities are much more affected in GA-1 SSB deletion mutant proteins than in φ29 and Nf SSBs, proteins that also lack the N-terminal region present in GA-1 SSB. Thus, both φ29 and Nf SSBs are able to displace the oligonucleotide hybridized to the M13 ssDNA molecule with a protein concentration of 40 μM versus 10 μM GA-1 SSB (21), whereas no destabilization of the oligonucleotide was obtained with a concentration of the mutant proteins ΔN26 and ΔN33 as high as 160 μM. Furthermore, in the φ29 DNA amplification system, equivalent stimulatory effects of DNA replication to those of GA-1 SSB were obtained with 5-fold higher concentrations of φ29 SSB (20), whereas dNTP incorporation levels equivalent to those obtained with 3.5 μM GA-1 SSB were not reached with ΔN26 and ΔN33 GA-1 SSB deletion mutants, even with 250 μM protein concentration. These effects are not just a consequence of the inability of the mutant proteins to bind ssDNA, as they conserve this ability, although somewhat affected with respect to that of GA-1 SSB. Besides, as inferred from the data presented in this paper, deletion of the 26 or 33 but not of the 19 amino acids of the N-terminal region of GA-1 SSB results in a loss of the oligomerization ability of this protein, as observed by ultracentrifugation in glycerol gradients. Furthermore, mutant proteins ΔN26 and ΔN33 are no longer able to form filaments, as visualized under the electron microscope. All these experimental evidences indicate that, in contrast with φ29 and Nf SSBs, GA-1 SSB requires its differential self-interaction ability for an efficient functional behavior, and that the N-terminal region of GA-1 SSB comprised between amino acids 19 and 26 plays an essential role on it. Structural defects in GA-1 SSB that affect the self-interaction ability correlate well with a drastic reduction of its functional efficiency. At the present time mutant phages containing GA-1 SSB proteins ΔN19, ΔN26, and ΔN33 are not available. Therefore, no experiments in vivo have been done to determine whether oligomerization of GA-1 SSB is required for its biological activity.

The fact that, in contrast with ΔN26 and ΔN33, the mutant protein ΔN19 behaves like the wt GA-1 SSB draws our attention to the amino acid residues of this region, the main distinguishable feature of which is the abundance of polar and aromatic residues. Thus, 2 of 7 of the amino acids that are present in ΔN19 but not in ΔN26 are positively charged, 1 has negative charge, and 2 more are aromatic (see Fig. 3). Furthermore, 3 of 7 amino acids present in ΔN26 but not in ΔN33 have positive charge, 1 is negatively charged, and 1 is aromatic. All these data together suggest that the residues of the N-terminal region of GA-1 SSB could be establishing electrostatic interactions important for the differential self-association ability of this protein. An additional piece of evidence that would point in this direction is the fact that, when GA-1 SSB is subjected to ultracentrifugation in glycerol gradient in the presence of 0.2 M NaCl, it behaves like a monomer. In addition, no protein filaments are visible by electron microscopy when GA-1 SSB is in the presence of 1 M NaCl. This kind of behavior has already been detected in proteins that bind ssDNA and that play important roles in recombination events, such as T4 uvsY or RecA. Thus, T4 uvsY, essential in the formation of the presynaptic filament of phage T4 because of its noncooperative binding to ssDNA and establishment of specific interactions with other proteins of the T4 recombination machinery, forms heteropolymers capable of reversible association into higher aggregates in a manner dependent on both salt and protein concentration (32). Additionally, RecA, which promotes homologous pairing and exchange of DNA strands ubiquitously in eubacteria, has a monomer-hexamer-higher aggregate self-association state strongly dependent on the kind and concentration of the salt as well as on the protein concentration (33). Nevertheless, RecA is active only as a helical filament of indefinite length polymerized on DNA, and even in the absence of DNA it can self-assemble into a variety of multimeric forms (34). Resolution of the crystal structure of RecA reveals that the N-terminal region of the molecule (resides 1–30) is formed by an α-helix followed by a β-strand, and protrudes from the rest of the molecule stabilizing polymer formation (35). Interestingly, secondary structure predictions of the N-terminal region of GA-1 SSB point to the existence of a similar structure to that of the N-terminal region of RecA. These evidences support the importance of an α-β domain in these proteins for promoting filament formation.

The only examples of SSBs in which the effect of the deletion of a part of the protein resembles that of GA-1 SSB and with similar self-interaction ability to that of GA-1 SSB are the SSBs of herpes simplex virus type I (ICP8) and the SSB of adenovirus (AdDBP). ICP8 is a 128-kDa protein able to form protein filaments in the absence of DNA, although it sediments as a monomer in glycerol gradients (36). Deletion of its 60-amino acid C-terminal region does not affect the intrinsic DNA-binding ability of the protein, but results in a total loss of cooperativity on long ssDNA stretches (37). It has been demonstrated that ICP8 interacts with other components of the replication machinery, like UL9 protein helicase (38–40) or the herpes simplex virus-1 DNA polymerase and helicase-primase (41–45). On the other hand, AdDBP is able to form small aggregates visible under the electron microscope. Resolution of the crystal structure of AdDBP suggested that its C-terminal extension could hook on to an adjacent monomer (46). As a matter of fact, deletion of this C-terminal arm results in a greatly reduced affinity for ssDNA and unwinding activity of the protein (47). As already mentioned, adenovirus replicates its genome via a protein-priming mechanism like the one displayed by the phages of the φ29 family. Like the SSBs of these phages, AdDBP cooperatively binds to the displaced DNA strand. However, AdDBP has additional roles during adenovirus DNA replication, like the indirect stimulation of the initiation step by increasing the binding of NFI to the replication origin (48, 49), and a direct stimulation by lowering the $K_{m}$ for the first dNTP (50). Taking together these data with the differences displayed by GA-1 SSB with respect to the SSBs of the related phages φ29 and Nf, it is possible that GA-1 SSB might be implicated in additional roles to those common to the replication systems of the three phages. Future studies concerning phage GA-1 SSB will be oriented to shed some light into possible additional roles of this protein.

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Importance of the N-terminal Region of the Phage GA-1 Single-stranded DNA-binding Protein for Its Self-interaction Ability and Functionality

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