Characterization of Single-stranded DNA-binding Proteins from Mycobacteria

THE CARBOXYL-TERMINAL DOMAIN OF SSB IS ESSENTIAL FOR STABLE ASSOCIATION WITH ITS COGNATE RecA PROTEIN*

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Single-stranded DNA-binding proteins (SSB) play an important role in most aspects of DNA metabolism including DNA replication, repair, and recombination. We report here the identification and characterization of SSB proteins of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. Sequence comparison of *M. smegmatis* SSB revealed that it is homologous to *M. tuberculosis* SSB, except for a small spacer connecting the larger amino-terminal domain with the extreme carboxyl-terminal tail. The purified SSB proteins of mycobacteria bound single-stranded DNA with high affinity, and the association and dissociation constants were similar to that of the prototype SSB. The proteolytic signatures of free and bound forms of SSB proteins disclosed that DNA binding was associated with structural changes at the carboxyl-terminal domain. Significantly, SSB proteins from mycobacteria displayed high affinity for cognate RecA, whereas *Escherichia coli* SSB did not under comparable experimental conditions. Accordingly, SSB and RecA were coimmunoprecipitated from cell lysates, further supporting an interaction between these proteins *in vivo*. The carboxyl-terminal domain of *M. smegmatis* SSB, which is not essential for interaction with ssDNA, is the site of binding of its cognate RecA. These studies provide the first evidence for stable association of eubacterial SSB proteins with their cognate RecA, suggesting that these two proteins might function together during DNA repair and/or recombination.

Single-stranded DNA-binding proteins (SSB)[1] play essential roles in many processes related to DNA metabolism such as DNA replication, repair, and homologous genetic recombination (reviewed in Refs. 1 and 2). *Escherichia coli* SSB, a proto- typical member of this super family, binds single-stranded DNA as a homotetramer with high affinity and generally with positive cooperativity (1, 2). SSB binds to single-stranded DNA in at least three distinct binding modes as $(SSB)_n$, where $n = 35$, 56, and 65 nucleotides/SSB tetramer (reviewed in Ref. 1). The binding site size of *E. coli* SSB is influenced by solution variables such as pH, temperature, monovalent and divalent cations, anions, and binding density (3–7). Electron microscopic analysis of SSB-ssDNA complexes suggests that the binding modes of SSB correspond to different morphologies of DNA-protein lattice (8). The $(SSB)_{56}/(SSB)_{65}$ binding mode formed in solutions containing high salt and lower binding densities display "limited" cooperativity and a "beaded" appearance to ssDNA. The $(SSB)_{35}$ mode formed in solutions containing low salt and higher binding density confers "unlimited" cooperativity and "smooth" appearance to ssDNA (1, 8). The transition between the two binding modes is reversible and modified by the solution variables (1, 2). In this regard, different binding modes may be used selectively for different processes such as DNA replication, recombination, and repair.

Both genetic and biochemical studies indicate that *E. coli* SSB influences the activities of its cognate RecA and RecBCD proteins (reviewed in Refs. 9–11). The most extensively investigated homologous recombination process is the 3-strand exchange reaction between circular single-stranded DNA and homologous linear duplex DNA. The reaction proceeds in three sequential phases: (i) the *presynaptic* polymerization of RecA protein on single-stranded DNA forming a helical nucleoprotein filament; (ii) *synapsis*, the homologous alignment of nucleoprotein filament with naked duplex DNA; and (iii) unidirectional *strand exchange*, which creates long heteroduplex DNA (9, 10). *In vitro*, SSB is involved in both early and later steps of homologous recombination promoted by RecA (9, 10). During the early phase, SSB abets the formation of nucleoprotein filaments of RecA-ssDNA. This activation depends on the ability of SSB to remove secondary structure in ssDNA and mediate coordinated assembly of RecA across structural barriers (12–15), and remains bound to the nucleoprotein filament (15–17). However, the latter point is controversial and is the subject of ongoing debate. In the subsequent steps of three-strand exchange reaction, SSB helps in sequestering the 5′ end of the displaced ssDNA and, consequently, inhibits its participation in additional pairing reactions (18, 19).

Mycobacterium tuberculosis has emerged as a successful obligate human pathogen and a leading cause of mortality worldwide. Combined with the increasing number of people infected with both *M. tuberculosis* and human immunodeficiency virus, it presents an imminent hazard to public health. Although much research has focused on immunology, biochemistry, and microbiology of this pathogen, investigations into molecular

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[1] The abbreviations used are: SSB, single-stranded binding protein; EcRecA, RecA protein of *E. coli*; EcSSB, single-stranded binding protein of *E. coli*; MsSSB, single-stranded binding protein of *M. smegmatis*; MsSSBᶜ, chymotryptic fragment of *M. smegmatis* single-stranded binding protein; MtRecA, RecA protein of *M. tuberculosis*, MtSSB, single-stranded binding protein of *M. tuberculosis*; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SPR, surface plasmon resonance; ssDNA, single-stranded DNA; RU, response unit(s).

interactions between specific gene products has not been possible because of the lack of defined mutants with specific phenotypes. Therefore, understanding of the mechanistic aspects of homologous recombination may help molecular genetic manipulation of mycobacteria. We previously reported the biochemical characterization and x-ray structure of *M. tuberculosis* RecA protein (20–22). To explore the molecular aspects of homologous recombination promoted by RecA and its cognate SSB, we have isolated and characterized *ssb* genes and developed expression systems and purification protocols for both *M. tuberculosis* and *Mycobacterium smegmatis* SSB proteins. We observed that DNA binding by SSB proteins of mycobacteria was associated with enhanced sensitivity to proteases at the carboxyl-terminal domain, which is also the site of interaction of its cognate RecA. Accordingly, SSB and RecA co-immunoprecipitated from *M. smegmatis* cell lysates, further supporting an interaction between these proteins *in vivo*. These results provide the first evidence for stable association of an eubacterial SSB with its cognate RecA suggesting that these two proteins might function together *in vivo* during DNA repair and/or recombination.

MATERIALS AND METHODS

Reagents, DNA, and Proteins—All chemicals were of reagent grade. Buffers were prepared using deionized water. Phage T4 polynucleotide kinase was obtained from New England Biolabs. Chymotrypsin was purchased from Sigma-Aldrich. *E. coli* RecA protein (23), SSB (24), *M. tuberculosis* RecA (20), and *M. smegmatis* RecA[2] were purified, and their concentrations were determined as described (25). RecA proteins were purified as described (23). Briefly, cell lysate was treated with polymin P, the pellet was extracted with NaCl, the supernatants were combined, and the proteins were precipitated with ammonium sulfate. The dialyzed fraction was chromatographed on DEAE-cellulose, and active RecA protein was obtained from ssDNA-cellulose column as described (23). Single-stranded oligonucleotides were purchased from Life Technologies, Inc. Oligonucleotides were purified on 15% polyacrylamide gels containing 8 M urea, eluted from acrylamide gels with 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, and their concentration was estimated using ϵ_{260} provided by the manufacturer. Purified oligonucleotides were stored in the same buffer at −20 °C. Oligonucleotides were labeled at the 5′ end using [γ-^{32}P]ATP and phage T4 polynucleotide kinase (26). Free ATP was removed from the labeling reaction by gel filtration on a Sephadex G50 column (26). The sequence of 40-mer DNA used in mobility shift assays is as follows: 5′-ACTATGT-ATGTACTATGACTATAATACTATGATGAGCTAC-3′.

Isolation of M. smegmatis and M. tuberculosis ssb Genes—The structural gene coding for SSB of *M. smegmatis* mc[2]155 and *M. tuberculosis* were amplified by polymerase chain reaction (PCR) using *Taq* DNA polymerase from genomic and cosmid DNA, respectively. Two PCR primers designed from the reported *M. tuberculosis ssb* gene sequence (27) were used to obtain both *M. tuberculosis* and *M. smegmatis* PCR products of *ssb*. The 5′ primer contained a restriction site for *Nde*I (underlined) and 3′ primer for *Kpn*I (underlined). The nucleotide sequence is as follows: 5′ primer, 5′-GAATTCC<u>CATATG</u>GCTGGTGACAC-CACCATC-3′; 3′ primer, 5′-GG<u>GGTACC</u>TCAGAATGGCGGTTCGTC-AT-3′. The PCR product (~0.5 kilobase pair) was purified from agarose gel, digested with both *Nde*I and *Kpn*I to remove the flanking 15 base pairs. The PCR products were phenol-extracted, followed by precipitation with ethanol. The 498-base pair fragment was ligated into the expression vector, pET17b (Novagen Inc., Madison, WI) between restriction sites *Nde*I and *Kpn*I downstream of the phage T7 promoter. Similarly, the structural gene coding for *M. tuberculosis* SSB was amplified from cosmid MTCY21D4 (27) and inserted into the expression vector, pET17b, as described above. The resultant recombinant plasmids were designated pSRMS and pSRMT, which harbor *M. smegmatis* and *M. tuberculosis ssb* genes, respectively. The recombinant plasmids were transformed into *E. coli* DH5α cells. Plasmids prepared from this host were subsequently transformed into *E. coli* BL21(DE3)pLysS strain (Novagen) for protein expression. The nucleotide sequence of the cloned *M. smegmatis ssb* gene is the same as reported for its genomic sequence (preliminary sequence data were obtained from The Institute

for Genomic Research web site at www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism=m_smegmatis).

DNA and Protein Amino Terminus Sequence Determination—The DNA sequence of *ssb* genes was determined by dideoxy DNA sequencing of both strands using insert-specific fluorescent primers in an ABI Prism 377 automated sequenator and with the Sequenase DNA sequencing kit (28). The nucleotide sequence of *M. smegmatis ssb* has been deposited with GenBank® (accession number AF349434). The native and chymotryptic peptides of SSB were subjected to 10% SDS-PAGE and then electroblotted onto a poly(vinylidene difluoride) membrane (Bio-Rad). The bands were excised from the membrane, and the amino-terminal sequencing of native and tryptic peptides was carried out on a PerkinElmer Life Sciences protein sequencer.

Computer Analysis—Nucleic acid and protein sequence analysis was carried out using programs in GCG (29) and ClustalW 1.81 (30) and displayed using BOXSHADE version 3.31 (31). *M. tuberculosis* and *E. coli ssb* sequences were obtained from the Wellcome Trust Pathogen Genome Unit (Sanger Center, Hinxton Hall, United Kingdom) (http://www.sanger.ac.uk/Projects/M.tuberculosis/blast-server.shtml) and the NCBI genome data base, respectively. The multiple alignments were performed to maximize regions of overlap using PILEUP.

Purification of SSB Proteins from M. smegmatis and M. tuberculosis—*E. coli* strains harboring plasmids pSRMS and pSRMT were cultured in 1 liter of Luria-Bertani broth containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. At mid-exponential phase (A_{600} = 0.4), gene expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Cells were further incubated for 5 h and collected by centrifugation at 5000 × g for 10 min. All subsequent steps were performed at 4 °C unless indicated otherwise. The cells were washed with 30 ml of buffer containing 10 mM Tris-HCl (pH 8), 1 mM EDTA, and 0.1 M NaCl and resuspended in ice-cold lysis buffer (50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 0.2 M NaCl, 10% (w/v) sucrose, and 0.1 mM PMSF). Lysozyme was added to a final concentration of 200 μg/ml, and the suspension was incubated at 4 °C for 30 min. The cells were disrupted by sonication (Vibra Cell Sonicator, Sonics and Materials Inc, Danbury, CT) in a pulse mode (50% duty cycle) and a power setting of 8 for 10 min. The cell lysate was clarified by centrifugation at 25,000 rpm in a Beckman SW 28 rotor for 90 min. SSB protein was precipitated from supernatant by the addition of polymin P (pH 6.9) to a final concentration of 0.4% over 15 min. The pellet was collected by centrifugation at 10,000 rpm for 20 min in a Beckman JA20 rotor. The pellet was resuspended in 30 ml of ice-cold buffer containing 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 0.4 M NaCl, and 20% (v/v) glycerol and stirred for 15 min. The suspension was centrifuged at 10,000 rpm for 20 min. SSB protein was precipitated from supernatant by the addition of $(NH_4)_2SO_4$ (0.2 g/ml) over 30 min. The pellet obtained by centrifugation at 15,000 rpm for 30 min was resuspended in 25 ml of buffer containing 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 0.3 M NaCl, and 20% (v/v) glycerol. This procedure was repeated, and the pellet thus obtained was resuspended in 5 ml of buffer containing 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 0.3 M NaCl, and 20% (v/v) glycerol. The resuspended solution was dialyzed against 500 ml of buffer containing 20 mM Tris-HCl (pH 8.3), 1 mM EDTA, 0.5 M NaCl, 1 mM 2-mercaptoethanol, and 10% (v/v) glycerol. The dialyzed protein solution was applied onto a DEAE-cellulose column, which had been equilibrated with a buffer containing 0.02 M Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM 2-mercaptoethanol, and 10% glycerol. The column was washed with the same buffer containing 0.1 M NaCl until the eluate contained no material that absorbed light at 280 nm. The bound proteins were eluted with the equilibration buffer containing 0.3 M NaCl. The fractions containing SSB were combined and dialyzed against 500 ml of buffer containing 20 mM Tris-HCl (pH 8.3), 1 mM EDTA, 500 mM NaCl, 1 mM 2-mercaptoethanol, and 50% (v/v) glycerol. Aliquots of the dialyzed SSB preparation were stored at −20 °C. The yield of SSB was in the range of 1.5 mg/g cell paste. Protein purity was assessed by 10% SDS-PAGE/Coomassie Blue staining and was generally >98% pure (Fig. 2). The presence of both ATP-dependent and ATP-independent exo- and endonucleases in purified SSB preparation was assayed by incubating M13 circular single-stranded, linear, or negatively supercoiled DNA for 1 h at 37 °C. Samples were subjected to agarose gel electrophoresis, and the identity of DNA was visualized by UV illumination of agarose gel stained with ethidium bromide. Protein concentration was determined by the dye-binding method (32) using bovine serum albumin as the internal standard.

Electrophoretic Mobility Shift Assays—Standard reaction mixtures (20 μl) containing 20 mM Tris-HCl (pH 7.5) and 3 μM ^{32}P-labeled 40-mer DNA was incubated with the indicated concentrations of SSB. After incubation at 37 °C for 10 min, the reactions were terminated by the

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addition of 3 μl of loading buffer (20% glycerol containing 0.12% (w/v) each of bromophenol blue and xylene cyanol) to each reaction mixture. The individual samples were separated on a 8% polyacrylamide gel by electrophoresis in 13.2 mM Tris acetate buffer (pH 7.4) at 12 V/cm for 4 h at 4 °C. The gel was dried at 80 °C on a Whatman no. 3MM filter paper, and DNA-SSB complexes were visualized by autoradiography.

Limited Proteolysis of SSB Proteins with Chymotrypsin—Reaction mixtures (40 μl) contained 10 mM Tris-HCl (pH 7.5), 25 mM NaCl, 300–600 pmol of SSB, 2.4–4.8 nmol of M13 ssDNA (where mentioned), and 0.2 μg of chymotrypsin. After incubation for indicated periods of time at 37 °C, reaction was terminated by the addition of SDS-PAGE loading buffer and PMSF to a final concentration of 0.2 mM to each reaction mixture. Samples were separated on a 10% SDS-polyacryl-amide gel (33). The gel was stained with Coomassie Blue R-250.

The chymotryptic peptide of *M. smegmatis* SSB lacking the 35 amino acid residues at the carboxyl terminus was generated by limited chymotryptic digestion. Reaction mixtures like those described in the preceding section were scaled up with increase in the amount of SSB to 3 mg. After digestion the resulting mixture was subjected to fast protein liquid chromatography on a Superdex 75 column. The second peak of the chromatographic profile contained the chymotryptic peptide and appeared to be homogeneous, as ascertained by 12% SDS-polyacryl-amide gel electrophoresis.

Surface Plasmon Resonance (SPR) Measurements—SPR studies were performed using a BIAcore 2000 instrument (Amersham Pharmacia Biotech Asia Pacific Pvt. Ltd.) at 25 °C. RecA protein in buffer A (10 mM Hepes-HCl (pH 7.5), 0.1 mM EDTA, 5% glycerol, and 0.2 mM 2-mer-captoethanol) was diluted to 100 μg/ml in 10 mM sodium citrate buffer (pH 5.5). RecA protein from the indicated source was immobilized on a CM5 sensor chip, which had been activated previously using the standard coupling chemistry as recommended by the manufacturer, by passing protein solutions at a flow rate of 2 μl/min. Coupling of RecA protein on CM 5 chip produced response signals of 2000 response units (RU). (Immobilization of SSB resulted in loss of its ability to interact with RecA, perhaps because of masking of sites.) A control empty surface was created in a similar fashion. SSB from *E. coli*, *M. smegmatis*, or *M. tuberculosis* was injected at varying concentrations at a flow rate of 5 μl/min for 10 min. The flow buffer was 20 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 0.1 mM EDTA, and 1 mM 2-mercaptoethanol. In addition, where mentioned, the flow buffer contained 1 mM ATP plus 12 mM MgCl₂. Surfaces were regenerated using a solution containing 10 mM NaOH.

To prepare biosensor surface with DNA, 3′-biotinylated 60-mer DNA (5′-AATTCTGGGTGTGTGGGTGTGTGGGTGTGTGGGTGTGGTCAA-GTTGACTACGTATACATC-biotin-3′) was immobilized in the flow cells of a streptavidin-coated chip to ~400 RU, with flow cell number 1 serving as control. The interaction of native forms of SSB or the amino-terminal domain of *M. smegmatis* SSB was monitored using a flow rate of 5 μl/min for 5 min. The flow buffer contained 30 mM Tris-HCl (pH 7.5), 12 mM MgCl₂, 1.4 mM dithiothreitol, 0.1 mM EDTA, and 50 mM NaCl. The surface was regenerated using solutions containing 0.1% SDS, followed by 100 mM NaCl. Affinity and kinetic analysis was performed using BIAevaluation software, version 3.0. Each experiment was repeated at least two times to ensure reproducibility of results.

Immunoprecipitation and Immunoblotting—*M. smegmatis* mc²155 wild-type or *M. smegmatis* ΔrecA (HS42) (34) cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5) and 0.2 M NaCl by sonication in a pulse mode (50% duty cycle) and power setting of 5 for 2 min. The supernatant was collected by centrifugation at 14,000 rpm in a Beckman JA 20 rotor for 15 min at 4 °C, and stored at −70 °C. For nuclease treatment, cell lysates (200 μg) were incubated with 10 μg of DNase I at 37 °C for 15 min, before coimmunoprecipitation. Under conditions of DNase I treatment, 5 ng of M13 DNA was completely degraded to acid-soluble nucleotide residues. Coimmunoprecipitation and immuno-blotting experiments were performed as described (35). Briefly, an aliquot (50 μl) of cell-free lysate was incubated with preimmune serum, anti-RecA or anti-SSB antibodies in the presence of 20 mM Tris-HCl (pH 7.5), 0. 2 M NaCl, and 1 mM ATP at 4 °C for 6 h, and was then combined with a 20-μl slurry of protein A-Sepharose beads and incubated for an additional 2 h. Immunoprecipitates were washed six times with 500 μl of 20 mM Tris-HCl (pH 7.5), 0.2 M NaCl, and 1 mM ATP, suspended in SDS sample buffer (33), and separated by 10% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membrane using a submerged transfer apparatus filled with a solution containing 25 mM Tris and 192 mM glycine. After blocking with NET buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 0.5% bovine serum albumin, and 0.05% Triton X-100) for 1 h, the membrane was incubated with appropriate primary antibody for 6 h. The membranes were washed extensively with phosphate-buffered saline containing 0.1%

FIG. 1. **Sequence alignment of SSB proteins of *M. smegmatis*, *M. tuberculosis*, and *E. coli*.** Protein sequence deduced from the nucleotide sequences were aligned using the ClustalW algorithm and displayed by BOXSHADE. Identical amino acids are shown in *black*, and similar residues are highlighted in *gray*.

Nonidet P-40 and incubated with horseradish peroxidase-linked secondary antibody. Protein-antibody complexes were visualized by chemi-luminescence (36).

RESULTS

Cloning and Structural Comparison of M. smegmatis and M. tuberculosis SSB Proteins—*ssb* structural genes of *M. smegmatis* and *M. tuberculosis* were amplified using a set of primers by PCR from genomic and cosmid DNA, respectively. Each primer contained 2 or 6 nucleotide residues of unrelated sequence, followed by a site for restriction enzyme, and then nucleotide residues corresponding to the amino acid sequence of *M. tuber-culosis* or *M. smegmatis ssb* genes. PCR amplification of *ssb* genes of *M. smegmatis* and *M. tuberculosis* yielded products of predicted length and nucleotide sequence (data not shown). The products purified from agarose gels were ligated into the expression vector, pET17b. Recombinant plasmids were amplified in *E. coli* DH5α strain. Fig. 1 depicts the amino acid sequence of SSB proteins deduced from the nucleotide sequence. Pairwise comparison of amino acid sequence suggested that *M. smegmatis* SSB displayed 84% identity and 89% similarity to *M. tuberculosis* SSB. A similar comparison of the amino acid sequence of *E. coli* SSB with those of mycobacteria suggest that the carboxyl-terminal tail is extended by 14 amino acid residues in the former. The identity of amino acid sequence between *E. coli* and mycobacterial SSBs is ~36% (61% similarity), when compared over their entire length. There are two highly conserved portions between the mycobacterial SSB proteins. The first stretch includes the amino-terminal amino acid residues from Met[1] through Gly[125] corresponding to the DNA-binding and tetramerization domains (1, 2). The second conserved region, situated at the carboxyl-terminal domain comprises a short stretch of amino acid residues from Asp[145] through Phe[165] except at Ser[157] and Ala[159], which are replaced by Gly at both the positions in MtSSB. Strikingly, sequence comparison between mycobacterial SSBs encompassing amino acid residues from Gly[124] through Gly[136] revealed that MsSSB contains a significantly high amount of glycine (11 of 13), and substantial sequence divergence between residues Phe[130] and Lys[143].

Purification of SSB Proteins from Mycobacteria—To ascertain whether *M. smegmatis* and *M. tuberculosis* open reading frames amplified truly represents their SSBs, we purified them for biochemical characterization. A source for large scale preparation of SSB was developed by allowing expression of recombinant plasmids in *E. coli* strain, BL21(DE3)pLysS. SSBs that
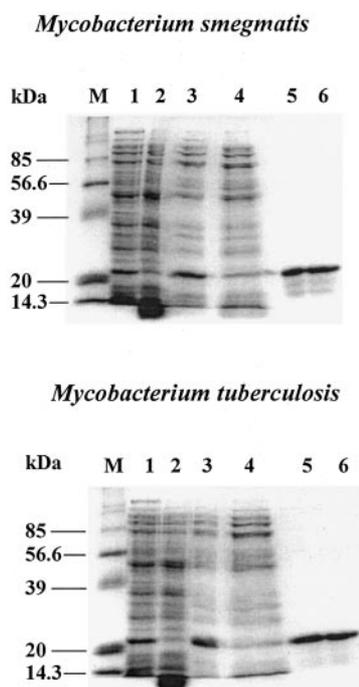
Mycobacterium smegmatis

Mycobacterium tuberculosis

FIG. 2. **Purification of *M. smegmatis* and *M. tuberculosis* SSB.** SSB proteins were produced in *E. coli* and were purified as described under "Materials and Methods." Approximately 5–10 μg of protein was separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. *Upper panel*, purification of *M. smegmatis* SSB. *Lane M*, molecular size markers; *lane 1*, cell lysate; *lane 2*, supernatant from polymin P precipitation; *lane 3*, 0.4 M NaCl extract from polymin P precipitate; *lane 4*, supernatant from $(NH_4)_2SO_4$ precipitation; *lane 5*, 0.3 M NaCl extract of $(NH_4)_2SO_4$ precipitate; *lane 6*, purified SSB from chromatography over DEAE-cellulose column. *Lower panel*, purification of *M. tuberculosis* SSB. *Lane M*, molecular size markers; *lane 1*, cell lysate; *lane 2*, 0.4 M NaCl extract of polymin P precipitate; *lane 3*, 0.4 M NaCl extract from polymin P precipitate; *lane 4*, supernatant from $(NH_4)_2SO_4$ precipitation; *lane 5*, 0.3 M NaCl extract of $(NH_4)_2SO_4$ precipitate; *lane 6*, purified SSB from chromatography over DEAE-cellulose column. *Numbers* on the *left* correspond to the size (kDa) of molecular mass markers.

accumulated as soluble proteins were detected on SDS-poly-acrylamide gel and Coomassie Blue staining as a band corresponding to the deduced molecular mass ($\sim$20 kDa). This band was absent in cell-free extracts of cultures grown in the absence of isopropyl-1-thio-β-D-galactopyranoside. With this heterologous expression system, SSB proteins accumulated to levels representing 5–10% after induction of transcription with isopropyl-1-thio-β-D-galactopyranoside. This enabled us to monitor the progress of purification by analyzing samples at various steps by SDS-PAGE. A rapid method was utilized for purification of SSB proteins involving lysis of cells, fractionation with polymin P and $(NH_4)_2SO_4$, and chromatography over DEAE-cellulose as described under "Materials and Methods." SSB proteins, obtained in yields of up to 1.5 mg/g cell paste, were judged to be homogeneous by SDS-PAGE analysis (Fig. 2). Mass spectrophotometric analysis of SSB proteins yielded a molecular mass of 17,257 and 17,302 Da corresponding to MtSSB and MsSSB, respectively, indicating the absence of *E. coli* SSB (18,885 Da) or DNA in these preparations (see below). Purified SSB proteins were devoid of ssDNA-dependent or independent exo- or endonuclease activities (data not shown).

Electrophoretic Mobility Shift Assays—Mobility shift assay is a rapid and convenient method to explore the specificity of interaction of proteins with nucleic acids, and as a means of determining the extent of the formation of nucleic acid-protein complexes. To assess the ability of purified mycobacterial SSBs to bind ssDNA, increasing concentrations of SSBs from *E. coli*
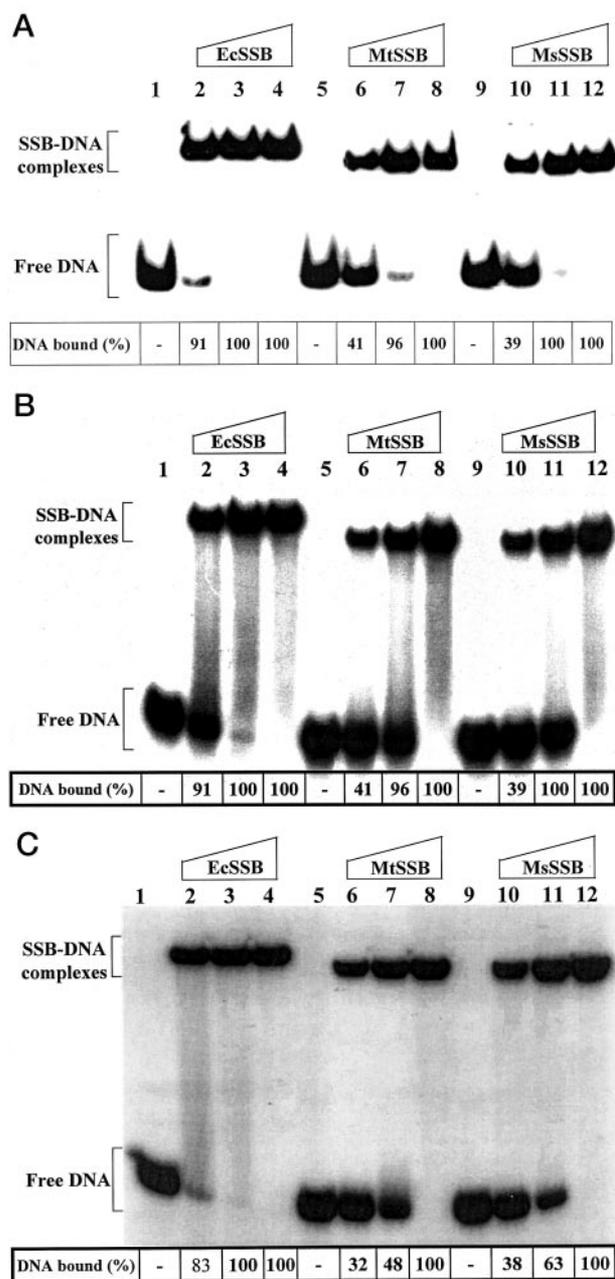

FIG. 3. **Comparison of single-stranded DNA binding activity of SSB proteins of *M. smegmatis*, *M. tuberculosis*, and *E. coli*.** Reactions were performed as described under "Materials and Methods." After the addition of gel loading solution (3 μl), samples were separated by electrophoresis on a 8% native polyacrylamide gel. The bands corresponding to the unbound and SSB-DNA complexes were visualized by autoradiography. The extent of binding of SSB to ssDNA was quantified using UVItech gel documentation system, which is indicated *below* each *lane*. *Lanes 1*, *5*, and *9* contained 40-mer DNA lacking any added protein. Identical reaction mixtures were incubated with the indicated SSB at a stoichiometric ratio (monomer of SSB:ssDNA) of 1:16 (*lanes 2*, *6*, and *10*), 1:8 (*lanes 3*, *7*, and *11*), or 1:4 (*lanes 4*, *8*, and *12*). Reactions performed in the presence of 100 mM NaCl (*panel A*), 200 mM NaCl (*panel B*), and 12 mM $MgCl_2$ (*panel C*).

as well as mycobacteria were separately incubated with a fixed amount of ^{32}P-labeled 40-mer DNA of mixed sequence. Analysis of reaction mixtures by native polyacrylamide gel electrophoresis showed that all three SSBs bound 40-mer DNA, as indicated by the reduced mobility of the band corresponding to free DNA as increasing amounts of SSBs were added (Fig. 3). This observation is consistent with earlier studies showing that ssDNA fragment containing 30–36 nucleotide residues is suf-

ficient for the formation of a stable complex between *E. coli* SSB and DNA (37). Furthermore, the crystal structure of SSB[c] tetramer bound to 35-mer DNA has been reported recently (38). However, the amount of SSB required for incorporating all of 40-mer DNA into nucleoprotein complex differed for each SSB and was sensitive to the ionic strength in the incubation buffer. In the presence of 100 mM NaCl, a monomer of *E. coli* SSB/16 nucleotide residues was required to fully saturate DNA. Under identical conditions, a 2-fold higher amount of mycobacterial SSBs was required to achieve a comparable effect (compare *lane 2* with *lanes 7* and *11*, Fig. 3*A*). We have verified the validity of this conclusion by performing experiments in buffers containing 2 or 50 mM NaCl. Results similar to that carried out in the presence of 100 mM were obtained (data not shown). To further evaluate the relative efficiencies of binding of mycobacterial SSBs to ssDNA, a second set of experiments were performed in the presence of 12 mM $MgCl_2$ or 0.2 M NaCl or with the natural solute, potassium glutamate. In the presence of 0.2 M NaCl (Fig. 3*B*) or 12 mM $MgCl_2$ (Fig. 3*C*), the extent of formation of protein-DNA complexes was comparable with *E. coli* SSB, whereas a 4-fold higher amount of mycobacterial SSBs was required to produce an equivalent effect. Similar results were obtained in buffers containing 200 mM potassium glutamate (data not shown). The reduction in binding of mycobacterial SSBs has been observed in multiple experiments. Together, these observations suggest that the formation of nucleoprotein complexes between mycobacterial SSBs and ssDNA is distinct from that of *E. coli* SSB. In this regard, binding of mycobacterial SSBs to DNA compared with *E. coli* SSB is weakened by increasing salt concentration, suggesting that nonelectrostatic forces contribute significantly to DNA binding with the latter than the former.

Differential Proteolytic Signatures of SSB Proteins—To further explore possible differences among SSB proteins, each of them was treated with chymotrypsin in the absence or presence of ssDNA. The partial proteolysis approach may offer useful insights into conformational changes emanating from interaction of a protein with an effector molecule. The cleavage products were isolated from SDS-PAGE gels. Mass spectroscopy and amino-terminal sequencing methods were used to map the cleavage sites in the isolated fragments. Consistent with previous studies (39), cleavage of *E. coli* SSB with chymotrypsin in the absence of ssDNA produced a larger product and a smaller product by cleavage after Trp[135] and Phe[147], respectively (Fig. 4*A*). In the presence of ssDNA, cleavage also occurred after Trp[135] and Phe[147]; however, continued incubation generated a single product, which was resistant to further digestion. Similar treatment of mycobacterial SSB proteins in the absence of ssDNA resulted in cleavage after Phe[126] in the case of *M. tuberculosis*, but at Phe[156] near the carboxyl terminus of *M. smegmatis* SSB (Fig. 4*A*). However, the rate of the cleavage reaction was much slower with mycobacterial SSBs compared with *E. coli* SSB. In addition, sites of cleavage changed with mycobacterial SSBs when bound to ssDNA; two larger and one smaller fragment were generated in the case of *M. smegmatis*, whereas one intense fragment was produced with *M. tuberculosis* SSB. Strikingly, unlike *E. coli* SSB, ssDNA-induced sensitivity was enhanced in the case of mycobacterial SSBs. Fig. 4*B* summarizes the cleavage pattern of all three SSBs in the absence or presence of ssDNA. These data demonstrate that, on addition of ssDNA, the conformation of the carboxyl terminus of each SSB changes, resulting in an alteration of the sensitivity of carboxyl terminus to cleavage by chymotrypsin.

Characterization of Amino-terminal Domain of M. smegmatis SSB—To explore the consequence of removal of the carbox-

yl-terminal domain of mycobacterial SSBs on their function, MsSSB was subjected to partial digestion with chymotrypsin. The reaction was stopped by the addition of PMSF to a concentration of 0.2 mM. The resulting mixture was then chromatographed on a Superdex 75 column, which had been equilibrated with a buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 10% glycerol, 0.1 mM EDTA, and 1 mM 2-mercaptoethanol. Aliquots from each fraction were analyzed on SDS-PAGE and visualized by silver staining (Fig. 5). Fractions in peak 1 contained a mixture of native as well as the proteolytic product, whereas fractions in peak 2 contained the MsSSB[c] resulting from cleavage after Phe[130] with an estimated size of 13.9 kDa. We note that a homotetramer of SSB is likely to remain associated after the removal of carboxyl-terminal domain (38). The fractions from peak 2 were pooled, and the yield of MsSSB[c] was 32%.

To investigate the DNA-binding ability of MsSSB[c], in comparison with its native form, we made use of the BIAcore, which utilizes SPR, and gel mobility shift assays. ^{32}P-Labeled 40-mer DNA was incubated with increasing concentrations of either native MsSSB or MsSSB[c], and the resulting nucleoprotein complexes was separated by native polyacrylamide gels. The data indicate that both proteins formed single protein-ssDNA complex with comparable efficiencies at all concentrations tested (Fig. 6). However, MsSSB[c]-DNA complex displayed faster mobility consistent with relatively smaller size of SSB[c].

We examined the affinity and kinetic parameters of interaction for native SSBs as well as MsSSB[c] with ssDNA using SPR, which permits macromolecular interactions to be measured in real time (40). A 3′-biotinylated 60-mer DNA was immobilized on a streptavidin-coated surface. The indicated SSB in a buffer (30 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.1 mM EDTA, and 1 mM 2-mercaptoethanol) containing 12 mM $MgCl_2$ was injected onto a biosensor chip containing a low level of immobilization (400 RU) of 60-mer DNA for 600 s, followed by 300 s of buffer injection period for dissociation. The sensograms showed that all three native SSBs bound quantitatively to ssDNA with fast association and slow dissociation rates (Fig. 7, *A–C*). By contrast, MsSSB[c] bound to DNA somewhat less efficiently and required higher concentrations (Fig. 7*D*). Further, both k_{on} and k_{off} were significantly different from that of native SSB. Satisfactory global fits were obtained using the simple (1:1 or Langmuir) binding model $(A + B \leftrightarrow AB)$. The values of K_D calculated from equilibrium and kinetic data for native SSB and MsSSB[c] to ssDNA is summarized in Table I. Within the limits of experimental error, association and dissociation rate constants for all three SSBs was indistinguishable. However, the K_D value for MsSSB[c] was ~100-fold lower than its native form, suggesting that binding is relatively weak.

Direct Interaction between Mycobacterial SSBs and Their Cognate RecA—One poorly understood aspect of homologous genetic recombination in eubacteria is the extent to which the components of the multi-enzymatic process interact with each other. Although there have been attempts to decipher direct interaction between *E. coli* RecA and its cognate SSB, experimental evidence is still lacking. To gain insights into direct interaction between RecA and SSB, we made use of the BIAcore. RecA was immobilized on a sensor chip and designated as the ligand, while a solution containing SSB designated as analyte was passed over the sensor chip in a microfluidics chamber. To abrogate nonspecific binding, we performed kinetic analysis in assay buffer containing 0.2 M NaCl and 10% glycerol. Under these conditions, increasing concentrations (20–125 μM) of the indicated SSB was injected on to a biosensor chip containing 2000 RU of specified RecA for 10 min, and then followed by a 5-min buffer injection period for dissociation. The
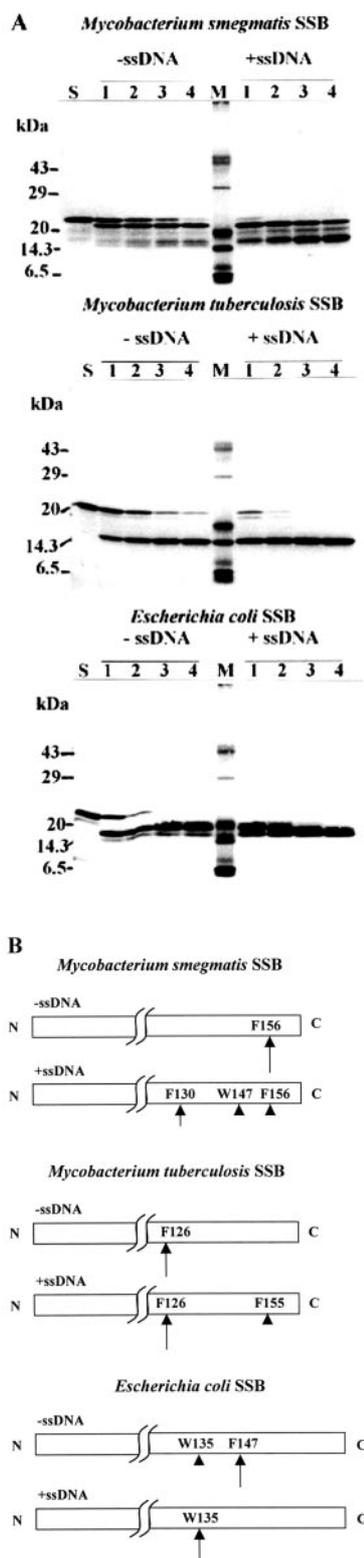
Interaction of SSB with Its Cognate RecA

A

B

FIG. 4. **Comparison of proteolytic signatures of SSB proteins from *M. smegmatis*, *M. tuberculosis*, and *E. coli* in the presence and absence of single-stranded DNA.** Reactions were performed as described under "Materials and Methods." Samples were analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. *A*, SSB proteins were incubated with chymotrypsin in the presence (*plus symbol*) or absence (*minus symbol*) of ssDNA for 10 min (*lane 1*), 20 min (*lane 2*), 40 min (*lane 3*), or 60 min (*lane 4*). *Lane S*, SSB incubated in the absence of ssDNA and chymotrypsin. *Lane M*, molecular size markers. *Numbers* on the *left* correspond to the size (kDa) of molecular mass markers. *B*, schematic representation of the cleavage pattern from *panel A*. *Arrow* and *arrowhead* represent major and minor sites of cleavage, respectively.
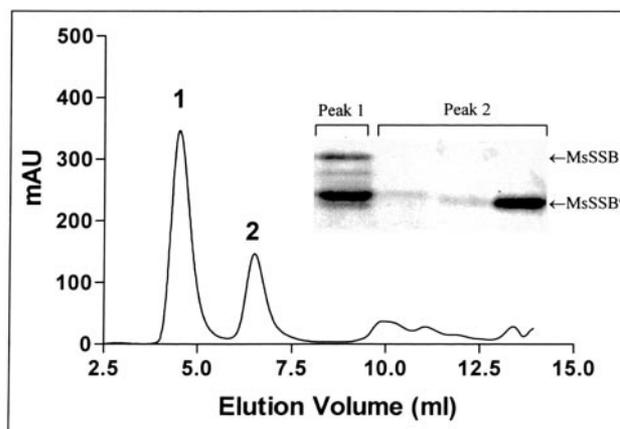

FIG. 5. **Isolation of MsSSB^c.** Reaction was performed in the absence of ssDNA as described under "Materials and Methods." After stopping the reaction with the addition of PMSF to a final concentration of 0.2 mM, sample was subjected to gel filtration on a Superdex 75 column. Chromatographic profile of chymotryptic digest of MsSSB is shown. *1* and *2* indicate the position of peaks in the elution profile. *mAU*, milliampere units. *Inset*, aliquots of different fractions were subjected to 12.5% SDS-PAGE and visualized by silver staining. Fractions from peak 2, which contained MsSSB^c, were pooled and used for assaying its ssDNA binding activity.

sensograms showed that EcSSB failed to interact with its cognate RecA in the absence (Fig. 8*A*) or presence of cofactors (Table II). The apparent binding seen with EcRecA surface at time 0 of sample injection is because of the presence of glycerol in the solution containing analyte (experiments performed in the absence of glycerol resulted in nonspecific binding to the chip). Under physical parameter of interaction between EcRecA and its cognate SSB, we note the absence of slope during the association phase (0–600 s) and indistinguishable signals in the sensogram at increasing concentrations. By contrast, interaction of MsRecA or MtRecA with their cognate SSB proteins resulted in significant increase (~6-fold) in resonance signals approaching equilibrium over several minutes and declining during wash (Fig. 8, *B* and *C*). The display of complete binding curves suggested 1:1 binding behavior between RecA and SSB. Although MtRecA formed a stable complex with its cognate SSB in the absence of Mg^{2+} and ATP, a 1000-fold increase in affinity was displayed in the presence of cofactors (Table II). By contrast, MsSSB displayed low affinity of interaction with its cognate RecA as well as with *M. tuberculosis* RecA in the presence of cofactors. The mechanism of cofactor-induced association between SSB and RecA is under investigation.

To explore the specificity of interaction between SSB and RecA, we used two different approaches. With paralogous combination, binding activity was observed between MsSSB and MtRecA or *vice versa*, whereas, in heterologous combination, EcSSB failed to interact with mycobacterial RecA proteins. Likewise, mycobacterial RecAs did not recognize EcSSB in the presence or absence of cofactors. Furthermore, the specificity of interaction of MsSSB with its cognate RecA was investigated by monitoring the binding of MsSSB^c with its cognate RecA. Injection of varying concentrations of SSB^c produced weak resonance signals equivalent to that of background values, suggesting that the carboxyl-terminal domain of MsSSB possess amino acid residues required for specific interaction with its cognate RecA (Fig. 8*D*). The results of affinity and kinetic analyses of the equilibrium binding response for all proteins (with background response subtracted) are summarized in Table II. Taken together, these data argue for protein-protein interaction between SSB proteins of mycobacteria with their cognate RecA.

FIG. 6. **Comparison of single-stranded DNA binding activity of MsSSB[c] with native MsSSB.** Increasing concentrations of native MsSSB or MsSSB[c] were incubated with ^{32}P-labeled 40-mer DNA in the presence of NaCl (100 mM) or MgCl$_2$ (12 mM). Samples were analyzed as described in the legend to Fig. 3. *Lanes 1, 5, 9,* and *13* contained 40-mer DNA lacking any added protein. Identical reaction mixtures were incubated with MsSSB or MsSSB[c] at a stoichiometric (monomer of SSB:ssDNA) ratio of 1:16 (*lanes 2, 6, 10,* and *14*), 1:8 (*lanes 3, 7, 11,* and *15*), or 1:4 (*lanes 4, 8, 12,* and *16*).
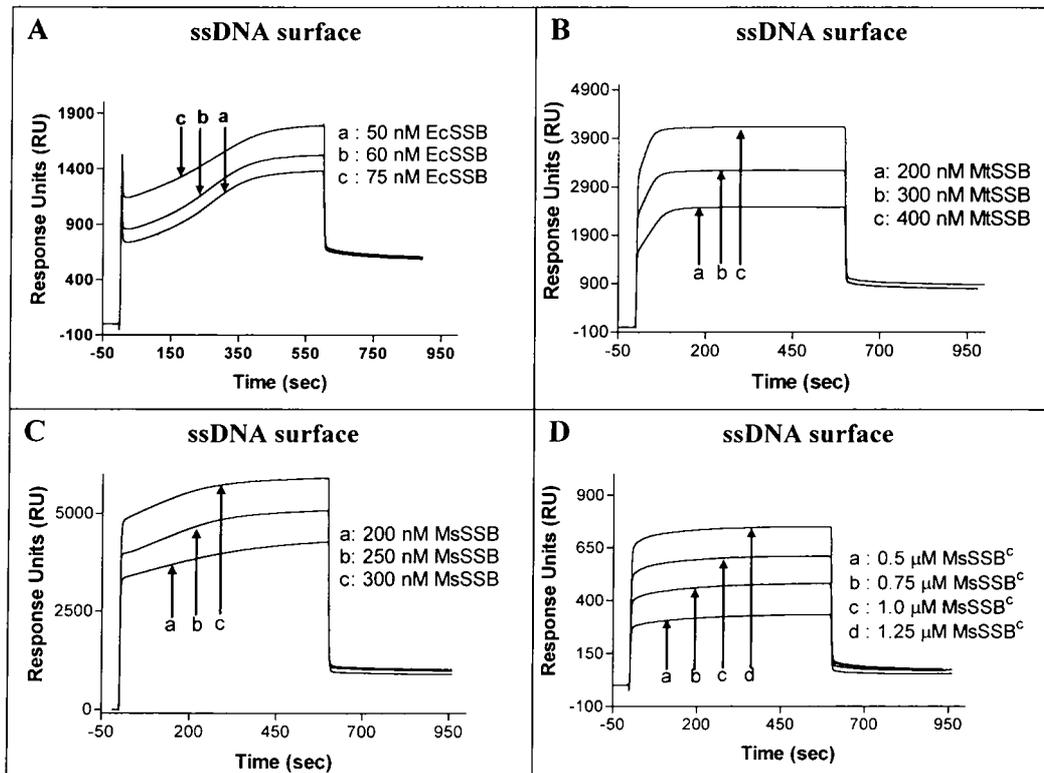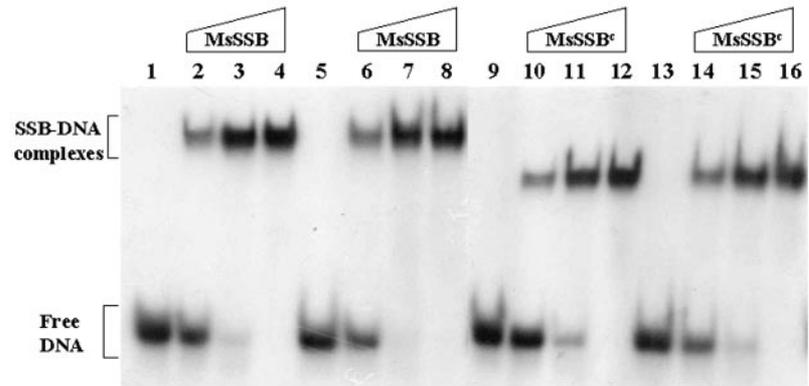
FIG. 7. **Kinetic analysis of interaction of SSB with single-stranded DNA.** Representative sensograms depicting changes in the surface plasmon resonance signal (*y axis*) as a function of time (*x axis*) are shown. Native SSB or MsSSB[c] were injected onto a 60-mer DNA surface. Both sample injection (association phase) and buffer injection (dissociation phase) were carried out as described under "Materials and Methods." *Panel A*, EcSSB; *panel B*, MtSSB; *panel C*, MsSSB; *panel D*, MsSSB[c]. The curves (*a–d*) represent increasing concentrations of the specified native SSB or MsSSB[c].

TABLE I
Kinetic and steady-state parameters for the interaction between native SSB and MsSSB[c] with single-stranded DNA

Binding reactions were performed as described under "Materials and Methods." The kinetic data of binding of native SSB proteins or MsSSB[c] (analyte) to the 60-mer DNA (ligand) were fitted using the simple (1:1 or Langmuir) binding model (A + B ↔ AB). The data shown are means of at least three independent measurements. The errors between different experiments were insignificant.

Ligand	Analyte	k_{on}	k_{off}	K_A	K_D
		$M^{-1}s^{-1}$	s^{-1}	M^{-1}	M
60-mer DNA	EcSSB	1.4×10^5	3.4×10^{-4}	4.1×10^8	2.5×10^{-9}
	MtSSB	6.3×10^4	2.3×10^{-4}	2.7×10^8	3.6×10^{-9}
	MsSSB	2.6×10^4	2.2×10^{-4}	1.2×10^8	8.4×10^{-9}
	MsSSB[c]	4.9×10^3	8.7×10^{-4}	5.6×10^6	1.8×10^{-7}

M. smegmatis RecA Is Physically Associated with SSB in Vivo—As a second independent method to ascertain physical association between SSB and RecA *in vivo*, immunoprecipitation was performed with preimmune serum or antibodies directed against RecA or SSB using cell lysates from isogenic strains of *M. smegmatis* mc^{2}155 wild-type and its mutant

$\Delta recA$ strain (HS42). In one set of experiments, RecA and SSB in the cell lysate was coimmunoprecipitated with preimmune serum or anti-RecA antibody and immunoblotted with anti-SSB antibody. In the second set of experiments, SSB and RecA in the cell lysate was coimmunoprecipitated with preimmune serum or anti-SSB antibody, and immunoblotted with anti-
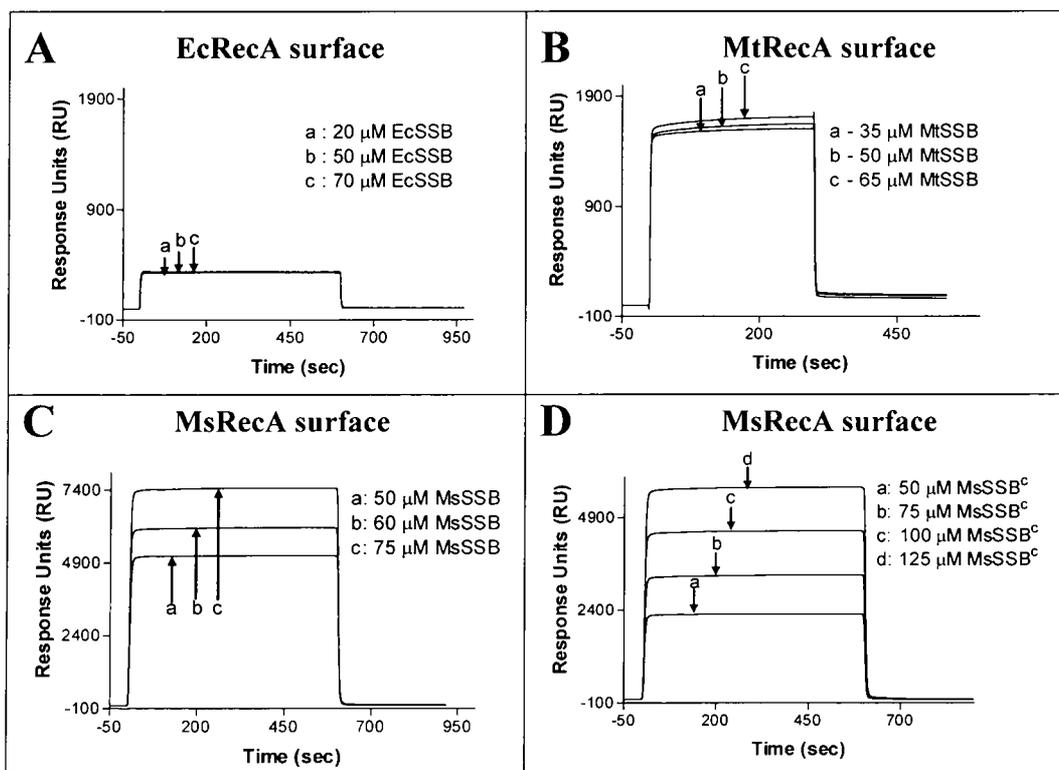
FIG. 8. **Kinetic analysis of interaction of SSB with RecA protein.** Representative sensograms depicting changes in the surface plasmon resonance signal (*y axis*) as a function of time (*x axis*) are shown. Increasing concentrations of SSB or MsSSB[c] were injected onto a surface with the indicated RecA protein. The curves (*a–d*) represent increasing concentrations of the specified native SSB or MsSSB[c] injected on a EcRecA surface (*panel A*), MtRecA surface (*panel B*), and MsRecA surface (*panels C and D*). Both sample injection (association phase) and buffer injection (dissociation phase) were carried out as described under "Materials and Methods."

TABLE II

Kinetic and steady-state parameters for the interaction of native SSB and MsSSB[c] with RecA proteins

Binding reactions were performed as described under "Materials and Methods." The kinetic data for binding of native SSB or MsSSB[c] (analyte) to the specified RecA protein (ligand) were fitted using the simple (1:1 or Langmuir) binding model ($A + B \leftrightarrow AB$). The data shown are means of at least three independent measurements. The errors between different experiments were insignificant. "Plus" and "minus" symbols correspond to the presence or absence of the specified cofactors, respectively. ND, no detectable binding in the presence (+) or absence (−) of ATP or $MgCl_2$.

Ligand	Analyte	$MgCl_2$ (12 mM)	ATP (1 mM)	k_{on}	k_{off}	K_A	K_D
				$M^{-1} s^{-1}$	s^{-1}	M^{-1}	M
EcRecA	EcSSB	−	−	ND	ND	ND	ND
	EcSSB	+	+	ND	ND	ND	ND
	MtSSB	−	−	ND	ND	ND	ND
	MtSSB	+	+	ND	ND	ND	ND
	MsSSB	−	−	ND	ND	ND	ND
	MsSSB	+	+	ND	ND	ND	ND
	MsSSB[c]	−	−	ND	ND	ND	ND
	MsSSB[c]	+	+	ND	ND	ND	ND
MtRecA	EcSSB	−	−	ND	ND	ND	ND
	EcSSB	+	+	ND	ND	ND	ND
	MtSSB	−	−	1.2×10^2	5.2×10^{-4}	2.3×10^5	4.3×10^{-6}
	MtSSB	+	+	6×10^4	2.9×10^{-4}	2.1×10^8	4.8×10^{-9}
	MsSSB	−	−	ND	ND	ND	ND
	MsSSB	+	+	0.4×10^2	1.9×10^{-3}	1.9×10^4	5.1×10^{-5}
	MsSSB[c]	−	−	ND	ND	ND	ND
	MsSSB[c]	+	+	ND	ND	ND	ND
MsRecA	EcSSB	−	−	ND	ND	ND	ND
	EcSSB	+	+	ND	ND	ND	ND
	MtSSB	−	−	ND	ND	ND	ND
	MtSSB	+	+	ND	ND	ND	ND
	MsSSB	−	−	ND	ND	ND	ND
	MsSSB	+	+	1.82×10^2	1.7×10^{-3}	1.08×10^5	9.3×10^{-6}
	MsSSB[c]	−	−	ND	ND	ND	ND
	MsSSB[c]	+	+	ND	ND	ND	ND

RecA antibody. A similar approach has been used for the detection of interaction between Rad51-Brca2 in ES cells (35). The data presented in Fig. 9 suggest that RecA and SSB are physically associated with each other. Significantly, RecA or

SSB could not be detected in lysates from *M. smegmatis ΔrecA* cells. In control experiments, we found that interaction between RecA and SSB was resistant to treatment with DNase I, thereby excluding the possibility of indirect interaction
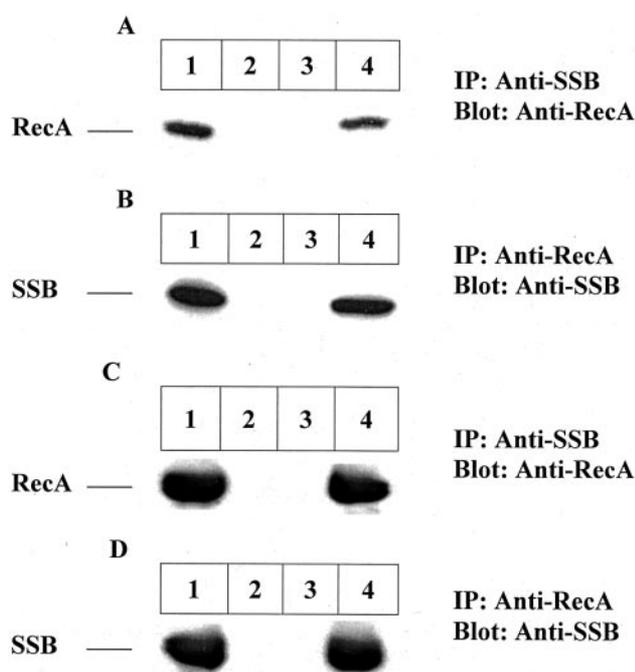
FIG. 9. **RecA-SSB association *in vivo* in *M. smegmatis*.** Figures shows immunoprecipitation of SSB-RecA in cell lysates using anti-RecA antibody or anti SSB antibody, followed by Western blot analysis with anti-SSB or anti-RecA antibody as a probe. Approximately 20–30 μg of total protein was used for coimmunoprecipitation as described under "Materials and Methods." *Panel A*, immunoprecipitation with α-SSB antibody and immunoblotted with α-RecA antibody. *Panel B*, immunoprecipitation with α-RecA antibody and immunoblotted with α-SSB antibody. *Lane 1*, cell lysate (5 μg of protein) from *M. smegmatis* mc[2]155 wild-type; *lane 2*, immunoprecipitate obtained from cell lysate of *M. smegmatis* mc[2]155 wild-type with preimmune serum; *lane 3*, immunoprecipitate obtained from cell lysate of *M. smegmatis* $\Delta recA$ (HS42) with α-RecA or α-SSB antibody; *lane 4*, immunoprecipitate from cell lysate of *M. smegmatis* mc[2]155 wild-type with α-RecA or α-SSB antibody. *Panels C* and *D*, as in *panels A* and *B*, respectively, except that cell lysates were treated with DNase I prior to immunoprecipitation as described under "Materials and Methods."

through contaminating DNA (Fig. 9, *C* and *D*). Thus, these results provide persuasive evidence regarding stable association between SSB and RecA and raise the possibility that these proteins might function together during DNA repair and/or recombination.

DISCUSSION

Single-stranded DNA is a transient intermediate in most aspects of DNA metabolism including DNA replication, repair, and recombination (1, 2). Data from genetic studies as well as biochemical and structural analyses is consistent with the notion that these processes entail the interconversion of DNA between duplex and single-stranded forms. The eponymous *E. coli* SSB has been the subject of intense study over the past 20 years and, consequently, has become the benchmark by which all other single-stranded DNA-binding proteins are measured (1, 2). In recent years, a large family of SSBs have been characterized both biochemically and at structural level. These proteins display differences in their oligomerization status and DNA-binding properties. Thus, the binding modes include monomeric (T4 gp32 (Refs. 41 and 42), *Sulfolobus solfataricus* SSB (Ref. 43)), dimeric (M13 g5p (Ref. 44)), homotetrameric (EcSSB (Ref. 45), *M. tuberculosis* SSB (Ref. 46)), heterotrimeric (RPA (Refs. 47 and 48)) and homohexameric (phage GA-1 SSB (Ref. 49)) forms.

In this study, we have purified and characterized the DNA-binding properties of SSB proteins of mycobacteria in comparison with the prototype EcSSB. *ssb* genes of *M. smegmatis* and

M. tuberculosis were amplified and cloned into *E. coli*. Comparative sequence analysis of SSB proteins from mycobacteria and *E. coli* revealed extensive sequence similarities between SSB proteins of mycobacteria, but not to the latter. However, sequence comparison of *M. smegmatis* SSB with *M. tuberculosis* SSB over its entire length also revealed significant differences. Although the sequence conservation is restricted to a large amino-terminal domain and to extreme carboxyl-terminal tail, the spacer connecting the former with the latter differs markedly in primary sequence. The spacer region of MsSSB is unusually rich in glycine residues. The biological significance of sequence divergence of the spacer region is obscure. Consequently, the spacer would be a good candidate for mutational and structural analyses to understand the phylogenetic significance of sequence divergence between SSB proteins of mycobacteria.

The DNA binding studies disclosed similarities and differences between *E. coli* and mycobacterial SSB proteins. The apparent equilibrium association and dissociation constants are similar among all three SSBs under comparable experimental conditions. The binding of SSB proteins of mycobacteria to ssDNA is substantially weaker in buffers containing increasing ionic conditions. Recently, the crystal structure of SSB[c] tetramer bound to 35-mer DNA has been determined (38). Consistent with biochemical and fluorescence measurements, in the crystal structure of SSB[c]-DNA complex, Trp[40], Trp[54] and Phe[60] make extensive contacts with DNA. In mycobacterial SSB proteins, the aromatic amino acid residues are conserved at the same or adjacent positions. The strong conservation of key residues important for DNA binding imply that, although maintaining the main functions of SSB, the subtle differences may permit mycobacteria to exert species-specific control over regulatory steps.

Three specific and interdependent findings reported here provide insights into specific interaction between RecA and SSB. First, the cleavage patterns of the carboxyl terminus of all SSBs change upon binding to DNA. Therefore, to investigate the role of carboxyl-terminal domain, we generated MsSSB[c]. The MsSSB[c] was able to bind DNA with comparable efficiency, but failed to interact with its cognate RecA. Of course, we do not know either the nature and/or the number of amino acid residues involved in SSB-RecA interactions. It is possible that the carboxyl-terminal tail serves as interface for interaction with RecA and possibly other recombination/repair proteins. Second, kinetic data from surface plasmon resonance studies suggest specific and high affinity interaction between these two proteins. Third, coimmunoprecipitation assays confirm that MsSSB exists as a complex with RecA *in vivo*. This is, to our knowledge, the first pair of eubacterial SSB proteins described that display high affinity interaction with their cognate RecA.

What is the biological significance of physical interaction between SSB and RecA *in vivo*? The simplest interpretation is that the two proteins function together in some aspect of DNA repair and/or recombination. It is possible, for example, that cognate SSB may increase the kinetic lifetime of the nucleoprotein filament of RecA-ssDNA. In addition, or alternatively, it might be involved in recruitment of RecA to form a stable nucleoprotein filament. Considering the high affinity interaction of SSB proteins of mycobacteria with their cognate RecAs, a question arises as to how SSB partakes in diverse processes related to DNA metabolism. We speculate two possibilities. There is sufficient excess of SSB existing in the cells to meet the demands of DNA metabolism. Alternatively, or in addition, it is possible that SSB functions dynamically between different complexes via combinatorial flexibility. Experiments aimed at gaining insights into these issues are in progress.

Interaction of SSB with Its Cognate RecA

Several lines of evidence corroborate the functional interaction between SSB and several proteins involved in DNA metabolism. Human RPA interacts with proteins involved in DNA replication, repair, and recombination. For example, RPA interacts with uracil-DNA glycolyase (50), nucleotide excision repair protein XPA (40, 51, 52), the Bloom's syndrome helicase (53), Rad51 (54), and Rad52 (55, 56) proteins. In eubacteria, genetic studies have elucidated the role of carboxyl-terminal domain of EcSSB in some detail. Deletion of the carboxyl-terminal domain or substitution of amino acid residues in this region resulted in severe repair-deficient phenotypes, suggesting a role for protein-protein interactions (1, 2). Consistent with this observation, studies have shown association of *E. coli* exonuclease I with its cognate SSB (57, 58). Although a similar interaction between *E. coli* RecA and its cognate SSB is not apparent (this study), it is possible that the interaction of EcSSB with RecA is relatively weak or indirect via protein(s) that participate in DNA repair and/or recombination.

In eubacteria, the mechanistic aspects of homologous pairing and strand exchange have been elucidated mainly with the *E. coli* paradigm. It will be important, however, for analogous studies to be performed in other organisms to establish the generality of the phenomenon. Although homologues exist, there are some important differences in *rec* genes between the genomes of *M. tuberculosis* and *E. coli* (59). Knowledge of the identity of Rec proteins is essential for understanding of their functions and mechanistic aspects. The identification and functional characterization of SSB proteins from mycobacteria in conjunction with their cognate RecA are beginning to provide new insights and focuses on the relationships between the process of recombination and specific interaction among the factors.

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