A Monoclonal Antibody That Recognizes the Functional Domain of
Escherichia coli Single-stranded DNA Binding Protein That Includes
the ssb-113 Mutation*

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We have isolated a monoclonal antibody against
Escherichia coli single-stranded DNA binding protein
(SSB) that recognizes the functional domain specified by
the ssb-113 temperature-sensitive mutation, a domain which is distinct from the DNA-binding site. Although the ssb-113 and ssb-1 mutations result in many similar phenotypic defects, they differ significantly in others, indicating that they affect different functional domains of the protein. Whereas the SSB-1 mutant protein is clearly defective in tetramer formation and is also unable to bind single-stranded DNA at nonpermissive temperatures, no similar in vitro defects have yet been found in the SSB-113 mutant protein. In fact, the only reported in vitro effect of the ssb-113 mutation on the protein is a slight increase in its helix destabilizing ability. Competition radioimmunoassays using a monoclonal antibody demonstrated that SSB-113 mutant protein, containing a single amino acid substitution at position 176 (the penultimate residue), did not compete with SSB while SSB-1 protein (with a single change at position 55) did compete with SSB. This analysis was refined by studies with a proteolysis fragment and with peptides derived from both SSB and SSB-113. The results indicate that the antibody recognizes a determinant near the COOH-terminal end of the protein and that the SSB-113 mutation lies within or very close to this determinant.

These analyses together with studies of the purified wild-type protein and its partial proteolysis products (Williams et al., 1983) have allowed us to define several functional domains of SSB.

At present, there are two mutations in ssb, ssb-1 and ssb-113, that have been well studied. Either one confers temperature-sensitive lethality, which emphasizes the essential cellular role of SSB. Both mutant proteins are reported to be defective in in vitro DNA replication systems at 42 °C (Meyer et al., 1979, 1980). The ssb-1 and ssb-113 alleles confer similar phenotypic defects under certain conditions, including defects in recombination (Glassberg et al., 1979; Golub and Low, 1983), in λ prophage induction (Vales et al., 1980), and in SOS repair (Lieberman and Witkin, 1983; Whittier and Chase, 1983). In addition, both mutants exhibit increased UV sensitivity (Glassberg et al., 1979; Vales et al., 1980) and failure to amplify the synthesis of RecA protein as a response to DNA damage (Bahuch et al., 1980). Nevertheless, there are significant differences between the effects of the two alleles. Both ssb mutations lead to a temperature-sensitive defect in DNA replication and hence in growth, but at the permissive temperature for growth, ssb-1 mutant strains appear relatively normal in all respects. In contrast, ssb-113 mutant strains are defective in certain aspects of DNA recombination and repair even at the permissive temperature for growth. Thus, the ssb-1 mutation appears to alter a function of the SSB protein generally required for DNA replication, recombination, and repair, whereas the effects of the ssb-113 mutation appear more complex. Strains containing the ssb-113 mutation appear to be defective in all functions tested, except DNA replication, even at the temperature permissive for growth. There are also differences in the rates of degradation of DNA that are observed when strains carrying these two different mutations are irradiated with ultraviolet light (Whittier and Chase, 1983). In vitro studies demonstrate that in the presence of SSB, several RecA-mediated reactions occur more efficiently, and various deficiencies in these reactions are associated with the SSB-1 and SSB-113 mutant proteins (McEntee et al., 1980; Resnick and Sussman, 1982). 1

Recently, we have isolated a monoclonal antibody against SSB, which provides a useful tool for extending the analysis of SSB functional domains. Defining the determinants or epitopes that the monoclonal antibody recognizes will aid in understanding the structural alterations caused by a particular mutation (Van Regenmortel, 1984). This report describes competition radioimmunoassays which examine the ability of

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† The abbreviations used are: SSB, E. coli single-stranded DNA binding protein; FSSB, single-stranded DNA binding protein encoded by F plasmid; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline.

1 E. Egner, E. Ashderian, S. S. Tsang, J. Chase, and C. Radding, unpublished results.
two SSB mutant proteins (SSB-1 and SSB-113), a partial proteolysis product of SSB (SSBc), peptides isolated from SSB and SSB-113, and the single-stranded DNA binding protein encoded by F plasmid (FSSB), which has extensive homology to E. coli SSB, to compete with SSB in binding to antibodies against SSB. These studies enable us to define one antigenic determinant on SSB near the COOH-terminal end of the protein and to demonstrate the inability of a monoclonal antibody directed against SSB to react with the SSB-113 mutant protein, which contains a single amino acid substitution at position 176.

MATERIALS AND METHODS

Isolation of Single-stranded DNA Binding Proteins and Peptides from SSB—Single-stranded DNA binding proteins were purified by published procedures: Chase et al., 1980, 1984 for E. coli wild-type SSB and SSB-113 mutant protein; Williams et al., 1984 for SSB-1 mutant protein; Williams et al., 1983 for partial proteolysis of SSB (SSBc); Chase et al., 1983a for the SSB-like single-stranded DNA binding protein from F-plasmid. Tryptic digestion of SSB and SSB-113 and the HPLC isolation of the COOH-terminal tryptic peptide, T-14,15, from each of these proteins was performed as described by Williams et al. (1984). As noted previously (Williams et al., 1982), reverse-phase HPLC of T-14,15 at pH 2.5 resulted in two closely spaced peaks with identical amino acid compositions. Both peaks gave identical competition curves in our radioimmunoassays. The major, earlier eluting peak of T-14,15 was dried in vacuo, dissolved in 0.5 ml of 6 M guanidine hydrochloride, and further purified by reverse-phase HPLC over a Waters µBondapak C18 column equilibrated with 10 mM potassium phosphate at pH 6.1. Peptides were then eluted using the same acetonitrile gradients used at pH 2.5 (Williams et al., 1984).

Cyanogen bromide cleavage of SSB was accomplished as previously described (Williams et al., 1981) after trichloroacetic acid precipitation of 60 nmol of SSB. After cyanogen bromide cleavage, the resulting peptide mixtures were dried in vacuo, redissolved in 0.5 ml of 6 M guanidine hydrochloride, and then injected onto a Vydac C-18 column (300-A pore size) equilibrated with 0.05% trifluoroacetic acid. Peptides were eluted with acetonitrile gradients as shown in Fig. 1. The peptide containing residues 112–169 eluted in two peaks (presumably due to the presence of homoserine and homoserine lactone at its COOH terminus) which gave identical competition curves in our radioimmunoassays. As expected for a COOH-terminal peptide, the peptide containing residues 170–177 gave a single symmetrical peak on reverse-phase HPLC (Fig. 1).

Preparation of Monoclonal and Polyclonal Antibodies against SSB Protein—A male BALB/c mouse was initially sensitized by injecting 100 µg of purified wild-type SSB protein mixed with Freund's complete adjuvant subcutaneously into the tail. Four booster injections followed, each consisting of 50 µg of SSB protein in phosphate-buffered saline introduced into a tail vein. Two days after the last boost, spleen cells from this mouse were fused with P3 NS-1 myeloma cells (Kohler and Milstein, 1976; Buchanan et al., 1981) and plated onto a feeder layer of macrophage cells obtained from a mouse peritoneal exudate. Hybridoma cells were selected by growth in hypoxanthine/aminopterin/thymidine medium for 18 days, and cell populations were screened for production of anti-SSB antibody using an enzyme-linked immunosorbent assay (Buchanan et al., 1981) of the culture medium. Two positive populations were cloned in soft agar. The resulting clones were tested in another enzyme-linked immunosorbent assay, and five stable clones from the two original populations producing high titers of anti-SSB activity were selected for growth in the peritoneal cavities of mice to produce ascites fluid. The titer of anti-SSB activity in ascites fluid from these mice exceeded 1:50,000 for all five cell lines. Ascites fluid from mice injected with one clone, 3H2 c1.5, was selected for use in the studies described here. Ouchterlony double-diffusion analysis using type-specific antisera indicated that the monoclonal antibody was of the IgG class.

SSB polyclonal antibody (IgG fraction from rabbits) was prepared as previously described (Whitter and Chase, 1980). The initial protein concentrations of the antibody preparations used in these studies were 36 mg/ml for the monoclonal antibody and 18 mg/ml for the polyclonal antibody. They were diluted as indicated below.

Competition Radioimmunoassay—In initial studies using SSB polyclonal antibodies, SSB was labeled by the chloramine-T procedure (Syvanen et al., 1973). Later studies using monoclonal antibodies employed SSB labeled by the iodobead (Pierce Chemical Co.) procedure (Markwell, 1982) which allowed us to increase the sensitivity of the assay. The amount of 125I-SSB required to saturate a given amount of antibody was determined, and then quantities of antigen and antibody were chosen which assured that the assays were performed during the assays. The mixture of cyanogen bromide peptides was injected onto a Vydac C-18 column equilibrated with 0.05% trifluoroacetic acid (solvent A). Peptides were eluted at a flow rate of 0.7 ml min⁻¹ with linear gradients of 0.05% trifluoroacetic acid in acetonitrile (solvent B) into solvent A as follows: 0–86 min (0–30% B), 86–109 min (30–60% B), and 109–123 min (60–100%).

![Fig. 1. HPLC separation of cyanogen bromide peptides derived from SSB.](image-url)
in antigen excess. Assays employing polyclonal antibodies contained 1.4 μg of the antibody preparation and 0.06 μg of 125I-SSB (3.4–5.4 × 10^5 cpm/μg). Assays employing monoclonal antibodies contained 0.72 μg of the antibody preparation and 0.0125 μg of 125I-SSB (3.2–4.9 × 10^5 cpm/μg). Polyvinyl 96-well microtiter plates (Dynatech) were coated for 2.5 h at room temperature with antibody appropriately diluted in 0.02 M sodium phosphate buffer (pH 6.5) and then rinsed with cold 0.02 M sodium phosphate (pH 6.5), 0.15 M NaCl (PBS). The wells were then filled with 1% bovine serum albumin in cold PBS, and the plates were incubated overnight at 4 °C. Control wells were treated exactly the same except that they did not contain antibody. Wells were then washed with cold PBS, a mixture of 0.05 ml of 125I-SSB in 1% bovine serum albumin and PBS and 0.05 ml of competing protein in 0.1% bovine serum albumin in PBS was added, and the plates were incubated overnight at 4 °C. The solution from each well was removed, and the wells were rinsed twice with cold PBS. Individual wells were cut out, and radioactivity was determined in a Beckman γ counter. Except where noted, each data point is an average of multiple determinations.

RESULTS

Competition Radioimmunoassay with Polyclonal Antibodies against SSB—A series of competition radioimmunoassays using polyclonal antibodies against E. coli SSB are shown in Fig. 2. No significant difference between the wild-type and SSB-1 mutant protein was apparent, and only a very slight difference was observed between wild-type SSB and SSB-113 at high levels of competing antigen. Incomplete competition was observed between SSB and SSBc, a proteolysis product of SSB lacking 42 amino acid residues from the COOH terminus (Williams et al., 1983). This result indicates that one or more antigenic determinants recognized by a significant fraction of the antibody population were either missing or structurally altered in SSBc. FSSB has extensive homology with SSB, particularly within the NH2-terminal end of the protein where 87 of the first 115 amino acid residues of FSSB exactly correspond to E. coli SSB. Despite this homology, significant competition with SSB was observed only at concentrations of FSSB greater than 0.1 μM. Evidently, the epitopes on these two proteins are not recognized to the same extent by the predominant antibody species. This result suggests that many of the epitopes on these proteins are not identical even though their primary structures are identical or nearly identical in many regions.

![Fig. 2. Competition radioimmunoassays using SSB-polyclonal antibody. Assays were performed as described under "Materials and Methods." SSBc refers to a partial proteolysis product of wild-type SSB containing amino acid residues 1–136 produced by treatment with chymotrypsin. FSSB refers to the single-stranded DNA binding protein encoded by F plasmid (the E. coli sex factor) which has extensive homology to E. coli SSB. Assays and symbols are as in Fig. 2.]

Competition Radioimmunoassays with a Monoclonal Antibody against SSB—Similar experiments to those described above were performed with a monoclonal antibody to SSB (Fig. 3). Surprisingly, both the SSB-113 (proline → serine replacement at residue 176) protein and SSBc were completely incapable of competition with SSB. One interpretation of these results is that the antigenic determinant which is recognized by this monoclonal antibody is at the COOH terminus of the protein and the single amino acid replacement in SSB-113 is as effective in disrupting the antigen-antibody interaction as is complete removal of the epitope from the protein. Peptide analysis presented below lends further support to this idea.

In contrast to SSBc, the SSB-1 mutant protein does compete with SSB. Although larger amounts of the SSB-1 mutant protein are required, complete competition is observed with 0.5 μM protein. The ssb-1 mutation causes the substitution of tyrosine for histidine at amino acid residue 55. Our previous studies demonstrate that this single alteration decreases the stability of the SSB-1 tetramer to such an extent that in the range of protein concentration where competition is poor, SSB-1 is monomeric whereas wild-type SSB is tetrameric (Williams et al., 1984). At protein concentrations where competition of SSB-1 and SSB are similar (>0.2 μM), significant quantities of SSB-1 tetramer do exist, however, and therefore, it is conceivable that the monoclonal antibody only recognizes the tetrameric structure of SSB-1. Alternatively, the ssb-1 mutation may result in the alteration of the structure of an individual SSB-1 monomer enough so that its interaction with the antibody is impaired.

Significant competition with F plasmid single-stranded DNA binding protein required higher protein concentrations than for SSB-1, and complete competition could not be obtained. It is interesting to consider this result in light of the sequence similarities and differences between SSB and FSSB, particularly at the COOH terminus (see "Discussion").

Peptide Analysis of the Interaction of SSB with a Monoclonal Antibody against SSB—The results presented above demonstrate the complete disruption of the interaction of this monoclonal antibody and SSB as a result of the removal of a large portion of the COOH-terminal portion of the protein (SSBc) and the somewhat surprising observation that a single amino acid substitution within this portion of the SSB-113 protein is just as effective in disrupting this interaction. In order to better define this protein-antibody interaction, we isolated several peptides from SSB and SSB-113 and performed competition radioimmunoassays with them. The re-
Monoclonal Antibody That Recognizes E. coli SSB

DISCUSSION

The antigenic determinant of *E. coli* SSB that we have identified occurs within the last eight amino acids of the protein (residues 170–177) in the sequence Asp-Phe-Asp-Asp-Ile-Pro-Phe. This is demonstrated in Fig. 4 by competition radioimmunoassays with peptides isolated from wild-type SSB. That other determinants exist in the protein is clearly indicated by the fact that we observed significant competition between SSB and SSB-113 for the polyclonal antibody (Fig. 2). It is interesting but not surprising that several antigenic determinants exist on SSB.

The only change caused by the ssb-113 mutation is the substitution of proline for serine 176 (the penultimate amino acid residue) which apparently alters a region necessary for stable interaction of the protein and this monoclonal antibody. The results presented in Fig. 4 argue strongly that this mutation occurs at or very near to this antigenic determinant. It is, of course, difficult to distinguish between effects of altered sequence recognition and altered conformation. In the case of the proline to serine substitution in SSB-113, it is clear that the conformational possibilities in the region of the mutation would increase, so that the mutant protein would be structurally more flexible in this region (Haber et al., 1967). It is therefore very likely that a significant structural alteration occurs within this region of the SSB-113 mutant protein affecting the structure of the antigenic determinant. It is also clear that other regions of the protein are necessary for efficient binding of this monoclonal antibody since even a large COOH-terminal peptide (residues 116–177) is 1000-fold less efficient in competition than the intact protein. Structures occurring in the NH-terminal portion of the protein and/or conferred to the COOH-terminal portion are clearly necessary for recognition. That cross-linking to significant competition with SSB; tends to favor the latter notion.

Although the ssb-1 and ssb-113 mutations confer similar phenotypic effects, there are significant differences which we are trying to understand and relate to the functional domains of SSB (see Introduction). Conclusions derived both from *in vitro* and *in vivo* studies provide a plausible explanation for the action of SSB-1 mutant protein (Williams et al., 1984). That is, the tetrameric structure of SSB-1 is unstable compared to wild-type SSB and gradually dissociates to monomer as the protein concentration is decreased from 10 to less than 0.5 μM. The SSB-1 tetramer appears to be stable at elevated temperature (45 °C) but the monomer is not. We estimate the normal cellular concentration of SSB-1 (single chromosomal gene) to be 0.5–1 μM. Thus, there is a plausible physical explanation for the ssb-1 temperature-sensitive phenotype and for our previous finding (Chase et al., 1983b) that increased expression of ssb-1 reverses the effects of a single gene (chromosomal) copy amount of SSB-113. In contrast, the *in vitro* defects that we have found associated with SSB-113 appear quite subtle, and we have not yet been able to determine their significance in vivo. We have shown that SSB-113 is defective in DNA binding and, surprisingly, has slightly increased helix destabilizing ability (Chase et al., 1984). This increase in helix destabilizing activity is qualitatively similar to that which accompanies the actual removal of the COOH terminus by partial proteolysis. These results suggest that structural alterations in the COOH-terminal region interfere with the presumed function of this domain which is to suppress the helix destabilizing ability of SSB. These results and those presented here also support the notion that the COOH-terminal portion of the protein is exposed and should be readily accessible for interaction with other proteins. Although such interactions for SSB have not yet been established, results of studies of phage T4 gene 32 protein suggest that such interactions might occur (Formosa et al., 1983). This possibility is now less difficult to conceptualize, since the results presented here show the complete disruption of a specific SSB-protein interaction as a result of this single amino acid substitution. We have also speculated about the importance of the structure that SSB confers upon single-stranded DNA as a result of its binding to it (Chase, 1984). Again, it is not difficult to imagine the deleterious effects that altered SSB-113 structure, particularly within a region that may regulate or modulate DNA binding, could have on a delicate protein-nucleic acid structure.

We also observed very significant competition between SSB...
and FSSB for our monoclonal antibody (Fig. 3). The seven COOH-terminal amino acids of FSSB are Phe-Ser-Asp-Asp-Le-Pro-Phe. Six of these 7 amino acid residues are identical to the corresponding region of SSB, and the last 5 completely correspond, including the penultimate amino acid residue, proline. Although peptide analysis of the type reported for SSB (Fig. 4) was not performed for FSSB, it is reasonable to speculate that the conservation of this COOH-terminal region in FSSB is important in permitting this competition between these proteins. Further studies of FSSB are required to determine the structural and functional similarities between SSB and FSSB and particularly the role that FSSB plays in F plasmid replication and conjugation.

Although much is known about the general requirements for SSB in DNA replication, recombination, and repair, there is little data which provide direct insight into the biochemical mechanism(s) by which SSB mediates its effects. The results we have presented here and the use of these polyclonal and monoclonal antibodies as reagents may be valuable in understanding the protein-protein and protein-nucleic acid interactions which are the basis of the biochemical mechanism of action of SSB and related proteins.

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