Immunoelectron Microscopic Localization of Small, Acid-Soluble Spore Proteins in Sporulating Cells of Bacillus subtilis

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Small, acid-soluble spore proteins SASP-α, SASP-β, and SASP-γ as well as a SASP-β-lacZ gene fusion product were found only within the forespore compartment of sporulating Bacillus subtilis cells by using immunoelectron microscopy. The α/β-type SASP were associated almost exclusively with the forespore nucleoid, while SASP-γ was somewhat excluded from the nucleoid. These different locations of α/β-type and γ-type small, acid-soluble spore proteins within the forespore are consistent with the different roles for these two types of proteins in spore resistance to UV light.

Approximately 10% of the protein of dormant spores of Bacillus subtilis is composed of a group of small, acid-soluble spore proteins (SASP) (18). The SASP are synthesized in parallel during sporulation within the developing forespore and are rapidly degraded during spore germination, thus providing amino acids for protein synthesis during this period of development. Three proteins, termed SASP-α, SASP-β, and SASP-γ, make up approximately 75% of the SASP pool. SASP-α and SASP-β are almost identical in primary sequence, and there are a number of minor SASP with primary sequences similar to those of SASP-α and SASP-β. In contrast, SASP-γ has a more different primary sequence, and there is only a single γ-type SASP (18).

Other than its role in providing amino acids for protein synthesis during spore germination, SASP-γ appears to have no function in spores (6). However, the α/β-type SASP also play a key role in the resistance of spores to UV light, as they are intimately involved in the modification of the UV photochemistry of spore DNA which is essential for spore UV resistance (11, 12, 14). While the mechanism whereby α/β-type SASP affect spore DNA photochemistry is not known, it seems likely that it involves direct SASP-DNA interactions, and it is known that SASP are localized within the spore core, the site of spore DNA (18). SASP can also bind to DNA in vitro (18). However, this binding is weak, and attempts to isolate spore DNA or spore nucleoids with significant associated SASP have failed (17). One study using intact Bacillus megaterium spores and UV-induced protein-DNA cross-linking did provide evidence that significant α/β-type SASP was associated with spore DNA in vivo (15). However, from this study it was not possible to determine what percentage of these SASP were DNA associated.

Because of the limitations of these previous techniques, we decided to attempt to localize various SASP within B. subtilis spores and forespores by using immunoelectron microscopy. However, initial attempts to localize SASP in dormant spores by using this technique were unsuccessful, since the cross-linking agents used in fixation (parafomaldehyde with or without glutaraldehyde) did not penetrate dormant spores sufficiently to prevent SASP movement during subsequent steps. The lack of penetration of dormant spores by cross-linking agents is not surprising in light of what is known of dormant-spore permeability (5) as well as the resistance of spores to killing by glutaraldehyde (13), but it did preclude the use of dormant spores in this study. However, the impermeability of the spore to hydrophilic agents such as fixatives, and thus glutaraldehyde resistance, is only achieved during sporulation well after the developing spore has acquired its complement of SASP and full UV resistance, both of which are acquired in parallel late in stage III of sporulation (1, 4, 13, 14, 18). Consequently, we turned to studies of SASP localization in cells in stages III and IV of sporulation.

In initial experiments, we used a B. subtilis strain carrying a lacZ gene fusion to the sspE gene, which codes for SASP-γ; previous work has shown that the β-galactosidase synthesized by this gene fusion is made in parallel with SASP (10). Cells were grown in 2 × SG medium (11) at 37°C, and samples (1 ml) were harvested by centrifugation when 80 to 90% of the maximum amount of β-galactosidase had been accumulated (10). The cell pellet was suspended in 1 ml of 40 mM NaPO4 (pH 6.8), and a 50-μl aliquot was added to 300 μl of fresh modified Karnovsky’s fixative (2% paraformaldehyde, 0.1% glutaraldehyde in 100 mM cacodylate buffer [pH 7.2] [9]) in a 400-μl microcentrifuge tube. The tube was centrifuged and then stored at 4°C for −5 h, and the supernatant fluid was removed, replaced with 300 μl of 0.5 M NH4Cl, and incubated overnight. On the following morning, the fixed cell pellet was removed and dehydrated in successive 1-h incubations in 30, 50, and 70% ethanol at 4°C, followed by 1 h in 70% ethanol at −20°C, and then successive 1-h incubations in 90, 95, and 100% ethanol (twice) at −20°C. Samples were then mixed with Lowicryl K4 M embedding resin to give a 2/1 ratio of Lowicryl to ethanol. After agitation for 4 days at −20°C, the samples were transferred to pure Lowicryl which was changed daily for 6 days. After the final change, to which appropriate initiators were added, the resin was polymerized with UV light for 1 day at −20°C and for 6 days at room temperature. Samples were then sectioned and placed on Butvar-coated nickel grids (8). Grids were treated with blocking buffer (1% glycine, 1% gelatin) for 10 min at room temperature, incubated for 20 min in a 1/25 dilution of affinity-purified rabbit anti-Escherichia coli β-galactosidase (S.S. Biochemicals) in dilution buffer (0.5 M NaCl, 50 mM Tris hydrochloride [pH 7.5], 0.1% bovine serum albumin, 0.05% Tween 20, 5% fetal calf serum), washed with five changes of 50 mM NaPO4 (pH 7.2), incubated for 10 min with a 1/40 dilution of gold-

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FIG. 1. Electron micrographs of fixed sporulating cells treated with antibodies to various forespore components. Cells were grown, harvested, fixed, embedded, treated with primary antibodies and gold-conjugated second antibody, and stained as described in the text. (A and B) Grids were treated with anti-\textit{E. coli} \(\beta\)-galactosidase antibody. (C and D) Grids were treated with anti-SASP-\(\alpha/\beta\) antibody. (E and F) Grids were treated with anti-SASP-\(\gamma\) antibody. Bar, 100 nm. C and N, Spore cortex and forespore nucleoid, respectively.
conjugated (5-nm) goat anti-rabbit immunoglobulin (Boehringer Mannheim) in dilution buffer, washed five times as described above, stained with 0.2% uranyl acetate for 30 s, washed three times in filtered glass-distilled water, air dried, incubated for 5 s with a 1/100 dilution of Reynolds Lead (7), washed three times with filtered glass-distilled water, and dried. Grids were observed and photographed on a Zeiss model EM 10-A electron microscope. All cells of which electron micrographs were taken for subsequent analysis appeared to be in stage IV of sporulation, since forespore engulfment was complete and significant deposition of spore cortex could be seen around the engulfed forespore (Fig. 1A to F) (4). Previous work has indicated that acquisition of UV resistance by the developing forespore precedes spore cortex synthesis, although SASP synthesis is required (14, 18). Consequently, the forespores we were examining should have acquired full UV resistance.

Strikingly, essentially all β-galactosidase antigen appeared to be localized within the developing forespore (Fig. 1A and B), in agreement with results of enzyme assays with isolated forespores (10). Grids of sporulating cells were also treated as described above with 1/75 or 1/25 dilutions of affinity-purified rabbit anti-SASP-α/β or anti-SASP-γ immunoglobulin G as the primary antibody. These antisera were prepared as described previously by using either glutaraldehyde-cross-linked SASP-α plus SASP-β or cross-linked SASP-γ as immunogen, and the immunoglobulin G fractions were isolated and affinity purified in columns of SASP-α plus SASP-β or SASP-γ coupled to CNBr-Sepharose (2, 3). Grids treated with these antibodies were stained for 3 min with uranyl acetate and for 2 to 3 min with Reynolds Lead. Both anti-SASP antibodies bound only within the forespore compartment of the sporulating cell, but the α/β antigen was associated only with the ribosome-free fibrillar nucleoid of the forespore (1, 4) (Fig. 1C and D), while the SASP-γ antigen was more uniformly distributed (Fig. 1E and F).

Analysis of micrographs of 10 sporulating cells stained with either anti-SASP-α/β or anti-SASP-γ antisera indicated that the density of the gold particles from anti-SASP-α/β-treated grids was 10-fold higher over the nucleoid area than over nonnucleoid areas of the forespore. In contrast with grids treated with anti-SASP-γ, the density of gold particles in nonnucleoid areas of the forespore was 25% higher than over the nucleoid itself, suggesting that SASP-γ is somewhat excluded from the forespore nucleoid. Analysis of comparable grids from sporulating cells of a B. subtilis strain which lacks the genes for SASP-α, SASP-β, and SASP-γ (6) also showed that with both antibodies the density of gold particles in the forespore was reduced by 10-fold (anti-SASP-α/β) to 40-fold (anti-SASP-γ) (data not shown).

The finding that both α/β-type and γ-type SASP are located only in the developing forespore is consistent with previous work in which SASP levels were determined directly in the mother cell and forespore compartments (17). However, the striking association of α/β-type SASP with the forespore nucleoid and the partial exclusion of γ-type SASP from the nucleoid have not been demonstrated previously. The partial exclusion of γ-type SASP from the nucleoid is consistent with the lack of a role for this protein in the UV photochemistry of spore DNA which leads to spore UV resistance (6). In contrast, the association of the α/β-type SASP with the forespore nucleoid is consistent with the key role for these proteins in bringing about the novel UV photochemistry of spore DNA by affecting spore DNA conformation or hydration or both (P. Setlow, Commun. Mol. Biol. Biophys., in press). It also suggests that α/β-type SASP alter spore DNA photochemistry by a direct interaction with the DNA, rather than by an alteration in some intraforespore physiological parameter. This further predicts that in vitro it should be possible to alter DNA photochemistry from cell type to spore type by using purified α/β-type SASP; experiments to test this prediction are currently in progress.

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