Bacterial single-stranded DNA-binding proteins are phosphorylated on tyrosine

Ivan Mijakovic, Dina Petranovic, Boris Macek, Tina Cepo, Matthias Mann, Julian Davies, Peter R. Jensen and Dusica Vujaklija

Microbial Physiology and Genetics group, BioCentrum, Technical University of Denmark, DK-2800 Lyngby, Denmark, 1Center for Experimental Bioinformatics, Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark, 2Department of Molecular Biology, Rudjer Boskovic Institute, 10002 Zagreb, Croatia and 3Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada

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ABSTRACT

Single-stranded DNA-binding proteins (SSBs) are required for repair, recombination and replication in all organisms. Eukaryotic SSBs are regulated by phosphorylation on serine and threonine residues. To our knowledge, phosphorylation of SSBs in bacteria has not been reported. A systematic search for phosphorytire-containing proteins in Streptomyces griseus by immunoaffinity chromatography identified bacterial SSBs as a novel target of bacterial tyrosine kinases. Since genes encoding protein-tyrosine kinases (PTKs) have not been recognized in streptomycetes, and SSBs from Streptomyces coelicolor (ScSSB) and Bacillus subtilis (BsSSB) share 38.7% identity, we used a B.subtilis protein-tyrosine kinase YwqD to phosphorylate two cognate SSBs (BsSSB and YwpH) in vitro. We demonstrate that in vivo phosphorylation of B.subtilis SSB occurs on tyrosine residue 82, and this reaction is affected antagonistically by kinase YwqD and phosphatase YwqE. Phosphorylation of B.subtilis SSB increased binding almost 200-fold to single-stranded DNA in vitro. Tyrosine phosphorylation of B.subtilis, S.coelicolor and Escherichia coli SSBs occurred while they were expressed in E.coli, indicating that tyrosine phosphorylation of SSBs is a conserved process of post-translational modification in taxonomically distant bacteria.

INTRODUCTION

Protein phosphorylation plays an important role in the regulation of cellular processes in all organisms. The demonstration of protein-tyrosine phosphorylation in bacteria (1) challenged the notion that this type of modification is restricted to the eukaryotes. Bacterial protein-tyrosine kinases (PTKs) possess a Walker A motif as their active site (2), while their eukaryotic counterparts contain Hanks motifs (3). Most bacterial PTKs reported are involved in the regulation of exopolysaccharide production (4–6) and were initially believed to be exclusively autophosphorylating enzymes, phosphorylatyng tyrosine(s) at their C-termini (7,8).

Recently, two endogenous substrates of bacterial PTKs have been identified: UDP-glucose dehydrogenases in B.subtilis (9) and E.coli (10), and an RNA polymerase sigma factor in E.coli (11). Growth-related variation in protein tyrosine phosphorylation has been reported in streptomycetes (12) and Myxococcus xanthus (13). However, no direct evidence for tyrosine phosphorylation of a protein involved in bacterial DNA metabolism is available. We describe the isolation and identification of tyrosine phosphorylated single-stranded DNA-binding proteins (SSBs), which are ubiquitous proteins that bind DNA in a sequence independent manner to maintain genome integrity in various stages of DNA metabolism: replication (14,15), recombination (16,17) and repair (18). Besides stabilizing single-stranded DNA (ssDNA), SSBs interact with enzymes such as DNA polymerase (19), RNA polymerase (20) or DNA helicase (21) and modulate their activity. Although accomplishing similar functions, bacterial and eukaryotic SSBs differ considerably in their structure.

Until this study, phosphorylation of SSBs was detected only in eukaryotes (22); such SSBs (23) are hetero-trimers, whereas bacterial SSBs are homo-tetramers. Phosphorylation of eukaryotic SSBs takes place on serine and threonine residues on the central RPA2 subunit, and is cell cycle-dependent (24) or induced by DNA damage (25). The physiological role of SSB phosphorylation is not clear, since in some cases it increases ssDNA binding (26), but in others had no effect (27). Phosphorylation of SSBs did not interfere with replication or...
nucleotide excision repair (28), although a more recent study suggests that it prevents association of SSB with replication centers (29). Induced DNA damage or apoptosis favored SSB phosphorylation, and it has been suggested that hyperphosphorylated SSB participates in DNA repair (30,31).

E. coli SSB has been well characterized and it serves as a model for eubacterial SSBs (32,33). Bacterial SSBs have two distinct domains (34): a conserved N-terminal domain responsible for tetramerization and DNA-binding (35), and a less conserved C-terminal domain important for the interaction of SSBs with various proteins (36). Many bacteria encode two SSBs that differ in size. In B. subtilis, it was shown that the larger SSB is an essential protein and participates in DNA replication, while the short SSB, lacking most of the C-terminal domain, is non-essential but plays a role in natural transformation (37).

We describe the in vivo and in vitro tyrosine phosphorylation of bacterial SSBs from taxonomically distant bacterial species. Phosphorylations in vitro were performed on homologous and heterologous substrates with the B. subtilis PTK YwqD.

MATERIALS AND METHODS
DNA manipulations, E. coli and B. subtilis strains and growth conditions
Bacterial SSB-encoding genes were PCR-amplified from the respective genomic DNAs: ssb (NCBI, GeneID: 937911) and ywqH (NCBI, GeneID: 936910) from B. subtilis, sbb (NCBI, GeneID: 948570) from E. coli and sbb (NCBI, GeneID: 1099343) from S. coelicolor. The point-mutation BsSSB Y82F was obtained using two partially overlapping mutagenic primers. All PCR products were inserted between the BamHI and PstI sites of the vector pQE-30 (Qiagen) and used for gene expression were verified by sequencing. For mutagenic primers. All PCR products were inserted between their respective central regions were inserted into the EcoRI and BamHI sites of pMUTIN-2 (41) and used to transform B. subtilis SPSSBHT. All PCR primers are listed in Table 1. Both E. coli and B. subtilis were grown in Luria–Bertani (LB) medium at 37°C. Ampicillin (100 μg/ml), kanamycin (25 μg/ml), erythromycin (1 μg/ml) and neomycin (5 μg/ml) were added as appropriate. DNA damage in B. subtilis was induced by adding mitomycin C (60 ng/ml), at the onset of exponential growth (OD₆₀₀ ~ 0.2).

S-medium (42) was used to culture Streptomyces. Cells were grown at 30°C until late exponential phase and mycelium (wet weight 20–25 g/500 ml) was collected by centrifugation (7000 g) at 4°C, washed in 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, 10 % glycerol, and resuspended in the same buffer supplemented with protease and phosphotyrosine protein phosphatase (PTP) inhibitors [0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml pepstatin, 2 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM Na₃VO₄]. The biomass was passed twice through a French pressure cell at 68.9 MPa. Cell debris was removed by centrifugation at 20000 g and the proteins in the supernatant fractionated with ammonium sulfate at 0–45%, 45–75% and 75–100% saturation. The precipitates were collected by centrifugation at 20000 g for 40 min, dissolved and dialyzed against the supplemented buffer. Protein concentration was determined by OD₂₈₀, and tyrosine phosphorylated (PY) proteins were detected using anti-PY western blots.

| Table 1. PCR primers used for amplification and mutagenesis with underlined restriction sites and bold typed mutated codons |
|-----------------|-----------------|-----------------|-----------------|
| Amplification primers | B. subtilis sbb forward | CCGGATCCATCTTTAACCGAGTATTGTATTCGG | BanHI |
| B. subtilis sbb reverse | AAAACGCTTGAAGCTGATCCTGAGGC | PstI |
| B. subtilis ywqH forward | AAAAAATCGATTTATGACGCTTCCAATGTTTGAAGG | BanHI |
| B. subtilis ywqH reverse | AAAAACGCTTGAAGCTGATCCTGAGGC | PstI |
| B. subtilis sbb SP forward | CCGGATCCATCCTTTAACCGAGTATTGTATTCGG | EcoRI |
| B. subtilis sbb SP reverse | AAAACGCTTGAAGCTGATCCTGAGGC | PstI |
| B. subtilis sbb HT reverse | AAAAACGCTTGAAGCTGATCCTGAGGC | BanHI |
| E. coli sbb forward | CCGGATCCATCCTTTAACCGAGTATTGTATTCGG | BanHI |
| E. coli sbb reverse | AAAACGCTTGAAGCTGATCCTGAGGC | PstI |
| S. coelicolor sbb forward | CCGGATCCATCCTTTAACCGAGTATTGTATTCGG | BanHI |
| S. coelicolor sbb reverse | AAAACGCTTGAAGCTGATCCTGAGGC | PstI |
| Mutagenic primers | B. subtilis sbb Y82F forward | CAAACACAGAAATTTGAAAACAGCAAGGACAGCTTCTTCGTC | NA |
| B. subtilis sbb Y82F reverse | GCTGTTTTTCAAAGTTCTTCTTTGATCAAAGCCATCTACCGGC | NA |
| Primers for gene inactivation with pMUTIN-2 | B. subtilis ywqD forward | CCGGATCCATCCTTTAACCGAGTATTGTATTCGG | BanHI |
| B. subtilis ywqD reverse | AAAACGCTTGAAGCTGATCCTGAGGC | NotI |
Production, purification and coupling of the anti-PY antibody 4G10 to Affi-gel 10

Monoclonal antibody 4G10 was produced by hybridoma cells (43). The cell supernatant was supplemented with 0.02% sodium azide, adjusted to pH 8 with 1 M Tris–HCl and passed through a column containing 2.5 ml of protein A Sepharose beads, previously washed with borate/EDTA buffer (0.1 M borate, 0.5 M NaCl and 2.5 mM EDTA, pH 8). The bound antibody was eluted with 3.5 M MgCl₂, monitoring protein concentration by OD₂₈₀. The pooled protein fractions were dialyzed and concentrated with Centriprep-10 columns at 4°C. Hybridoma cells produced 7–10 mg of antibody per liter.

Purified 4G10 was covalently coupled to Affi-gel 10 (BioRad) as recommended by the manufacturer, except that the antibody solution was first saturated with 10 mM sodium azide, adjusted to pH 8 with 1 M Tris–HCl and passed through a column containing 2.5 ml of protein A Sepharose beads, previously washed with borate/EDTA buffer (0.1 M borate, 0.5 M NaCl and 2.5 mM EDTA, pH 8). The bound antibody was eluted with 3.5 M MgCl₂, monitoring protein concentration by OD₂₈₀. The pooled protein fractions were dialyzed and concentrated with Centriprep-10 columns at 4°C. Hybridoma cells produced 7–10 mg of antibody per liter.

Isolation of PY-proteins from S.griseus

4G10 affinity chromatography was used for enrichment of PY-proteins from the 0 to 45% ammonium sulfate fraction obtained from cell-free extracts of S.griseus using a modification of the published protocol (44). A typical purification cycle consists of the following steps. The most efficient binding of PY-proteins was obtained when 50 ml of fractionated extract in binding buffer (50 mM Tris–HCl, pH 7.7, 50 mM NaCl, 0.2% Triton X-100, 0.5 mM PMSF, 2 µg/ml pepstatin, 2 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM Na₃VO₄) was mixed with 2 ml of affinity matrix in a 10 ml column and placed on a rocking platform at 4°C for 4 h. The column was washed extensively with 50 mM Tris–HCl, pH 7.7, 50 mM NaCl, 0.2% Triton X-100 and 0.5 mM PMSF. Buffering wash supplemented with PNPP (2.6 mg/ml) was used for the elution of the PY-proteins. The eluates from 10 runs (~30 ml) were concentrated to one third of the initial volume on Centricron-10 columns (Amicon) and washed with 20 mM Tris–HCl, pH 7.7, 0.5 mM PMSF and 1 mM Na₃VO₄. Triton X-100 micelles did not pass through the concentrator membrane and were concentrated to 0.6%. A MonoQ ion exchange column (BioRad) was used for additional protein concentration and removal of Triton X-100 prior to separation of the isolated PY-proteins by preparative SDS–PAGE. Aliquots containing 10 ml of PY-protein fractions were loaded onto a 1 ml MonoQ column equilibrated with the same buffer at a flow rate of 0.3 ml/min. The column was washed with 20 mM Tris–HCl, pH 7.7, 2 mM EDTA, 0.5 mM PMSF, and then with the same buffer supplemented with 0.1 M NaCl. The proteins were eluted batchwise with 0.4 and 0.8 M NaCl, and the eluates analyzed by Western blotting. All fractions containing PY-proteins were pooled, desalted and concentrated 10-fold; an aliquot was run on 12% polyacrylamide–SDS gel and the gel was stained by standard silver staining procedure. The sample was submitted for peptide sequencing.

Synthesis and purification of tagged proteins

Synthesis of all 6xHis-tagged proteins was performed in E.coli NM522, except for YwqD and YwqD-123, whose reduced solubility required the use of a chaperon-overproducing strain (45). Protein purification and desalting were performed as described previously (9). When 6xHis-tagged SSB was purified from B.subtilis SPSSBHT, 10 mM sodium pyrophosphate was added to the cell lysate to inhibit the PTP YwqE (46); 8 M urea was added when the purified SSB was assayed by western blot.

Western blotting

PY-proteins purified by immunoaffinity chromatography were detected using 4G10 monoclonal anti-PY antibodies (Upstate Biotechnology) (12). Western blotting of purified 6xHis-tagged SSBs was performed as described previously (9). Approximately 50 ng of each protein (Bradford assay (Biorad)) were run in parallel on two identical pre-cast 10–20% gradient polyacrylamide–SDS gels (Cambrex) and each was electroblotted on to a PVDF membrane (Biorad). One membrane was treated with 10 µ/ml of alkaline phosphatase in the blocking buffer. Both membranes were incubated for 1 h with the peroxidase-conjugate monoclonal anti-PY (Sigma) diluted 1:40 000. Peroxidase-conjugate anti-phosphoserine/anti-phosphothreonine (Upstate Biotechnology) diluted 1:5000 was used as negative control. The signals were visualized with a peroxidase AEC staining kit (Sigma).

In vitro phosphorylation assays

Protein phosphorylations were performed with 1 µM PTK, YwqD or YwqD-123 (the non-autophosphorylatable form of the kinase), and 1 µM YwqC-NCter, a truncated form of the transmembrane modulator of PTK activity (9). A typical phosphorylation is performed with 50 µM [γ⁻³²P]ATP (20 µCi/µmol), 1 mM MgCl₂, 100 mM Tris–HCl, pH 7.5 and 1–5 µM SSBs. Reactions were incubated at 37°C for 60 min. The ‘phosphate-sliding’ assay, ~50 µg of YwqD were autophosphorylated in a scaled-up reaction described above, without SSB. [³²P]Tyr-YwqD and YwqC-NCter were then desalted (two successive PD-10 columns, Pharmacia) to remove the [γ⁻³²P]ATP, and to exchange initial buffer to 20 mM NH₄HCO₃ buffer. After lyophilization, the proteins were resuspended in reaction buffer without [γ⁻³²P]ATP, mixed with B.subtilis SSBs (BeSSB or YwpH) and incubated overnight at 37°C to permit the transfer of [³²P] from [³²P]Tyr-YwqD to the SSBs. All reactions were stopped by adding SDS–PAGE sample buffer and heating at 100°C for 5 min. Proteins were separated by electrophoresis on 15% SDS–polyacrylamide gels, then washed by boiling in 0.5 M HCl for 10 min to reduce the radioactive background and dried. Signals were visualized with the STORM PhosphoImager and quantified with ImageQuant (Amersham).

In vivo labeling of B.subtilis SSB

B.subtilis strains SPSSBHT, SPSSBHT-ΔywqD and SPSSBHT-ΔywqE were grown in 100 ml of LB medium supplemented with 300 µCi of [³²P]phosphate. Cells were harvested at the late exponential phase (OD₆₀₀ 0.8) and 6xHis-tagged SSB was purified from each strain as described. Purified
proteins were lyophilized in 20 mM NH₄HCO₃, resuspended in 30 μl of 100 mM Tris–HCl, pH 7.5 and separated by electrophoresis on 15% SDS–polyacrylamide gels. Radioactive signals were visualized as described.

Gel filtration

Sephacryl™ S-300 (Pharmacia) gel filtration column (working buffer 100 mM Tris–HCl at pH 6.8) was calibrated with protein standards of following sizes: 14, 45, 57 and 98 kDa. Purified 6xHis-tagged B. subtilis proteins, SSB, SSB-Y82F, SSB-Y82E and YwpH, were loaded separately on the column, eluate was collected in 0.3 ml fractions, and the protein content monitored by the Bradford assay (Biorad).

Gel-shift assays

Random sequence oligonucleotides (50 bases) labeled with [³²P] were purchased from DNA Technology. Different ratios (indicated in the figure legend) of the oligonucleotide and BsSSB were mixed in 100 mM Tris–HCl, pH 7.5, incubated for 10 min at 37°C and loaded on a 12.5% native polyacrylamide gel (without SDS). After migration, the gel was dried, the radioactive signals visualized and quantified as described.

Determination of phosphorylation site by mass spectrometry

SSB purified from B. subtilis SPSSBHT (50 μg) was dissolved in 8 M urea, reduced with 1 μg of DTT, carboxymethylated with 5 μg of iodoacetamide, diluted with 4 vol of 50 mM NH₄HCO₃ and digested O/N with 1 μg of trypsin. The digest was separated into 10 fractions on a Source™ 15RPC ST 4.6/100 column (Amersham Pharmacia Biotech). For phosphopeptide enrichment, each fraction was incubated with 50 μl of PHOS-Select™ IMAC beads (Sigma), according to the manufacturer’s instructions. Eluted peptides were dried, resuspended in 1% trifluoroacetic acid, and LC-MS/MS analysis was performed on an 1100 nano-HPLC system (Agilent Technologies) coupled to a LTQ-FS mass spectrometer (Thermo Electron), as described previously (47), but without Single Ion Monitoring scans in the acquisition cycle. Spectra were acquired in the positive ion mode with acquisition cycle consisting of a full scan in the FT ICR cell, followed by MS/MS scans of the five most intense ions in the linear ion trap. Resulting mass spectra were searched against the NCBInr database using the Mascot search engine (Matrix Science).

RESULTS AND DISCUSSION

Affinity purification of PY SSB from S. griseus and detection of PY SSBS in B. subtilis and E. coli

In order to apply affinity chromatography for purification of the PY-proteins from Streptomyces griseus, we produced a 4G10 monoclonal antibody specific for PY-proteins and coupled the antibody to Affi-gel 10 as described in Materials and Methods. We reported previously (12) that the pattern of PY-proteins varied during the growth phase; accordingly we harvested the cells at the logarithmic growth stage when increased tyrosine phosphorylation was noted. The culture conditions, preparation of the cell-free extracts and ammonium sulphate fractionation are prepared as described in Materials and Methods. The 0–45% ammonium sulphate fraction contained the same number and size of proteins phosphorylated on tyrosine as detected in S. griseus previously (12). The maximal binding capacity of the affinity column was when 60 mg of 0–45% ammonium sulphate fraction was applied to 2 ml affinity matrix, no PY proteins were detected in the flow through fraction. The presence of the PY proteins in the eluates were detected by immunoblotting assay and the samples containing PY-proteins were pooled. According to published purification procedure (44) MonoQ ion-exchange chromatography was used for buffer exchange and protein concentration. The eluates were analysed by western blotting, the fractions with PY-proteins from 10 purification cycles were pooled, desalted and concentrated as described in Materials and Methods. Prior to submitting for sequencing the amount of the proteins was estimated by running an aliquot on the SDS–PAGE and silver staining (data not shown). Several protein bands were detected and estimated to be between 0.5 and 2 μg. The isolated PY-proteins were sequenced and the peptide (Q/K)AAENVAES(I/L)(Q/K)R identified as amino acids 62–73 of a putative S. coelicolor A3(2) SSB protein (NCBI protein databases, accession no. CAB42735).

No gene encoding a PTK has yet been identified in streptomycetes. To determine whether ScSSB could be phosphorylated in vitro with S. coelicolor cell extract the corresponding S. coelicolor gene (ssb) was cloned, and 6xHis-SSB purified from E. coli. In vitro phosphorylation of 6xHis-SSB was carried out with both cytoplasmic and membrane extracts from S. coelicolor and as described previously (48), but no evidence of phosphorylation could be detected. A database search revealed a 38.7% identity in a 163 amino acid overlap between S. coelicolor SSB and B. subtilis SSB. Protein tyrosine kinase (YwqD) has been described in B. subtilis and shown to phosphorylate endogenous substrates (9). We observed that both genes are identically grouped on the chromosome with ribosomal protein genes (37). S. coelicolor and B. subtilis are both Gram-positive spore-forming genera (49,50) and we assumed that SSBs from both bacteria might be similarly phosphorylated on a tyrosine residue.

This prompted us to examine the in vivo phosphorylation of SSB in B. subtilis SPSSBHT that was constructed to constitutively produce endogenous 6xHis-tagged SSB. Purified B. subtilis SSB reacted with the anti-PY antibody (Figure 1A, lane 1). In contrast to eukaryotic SSBs, no signal was obtained with anti-phosphoserine/anti-phosphothreonine antibody (data not shown), B. subtilis SSB was also phosphorylated when overproduced in E. coli (Figure 1A, lane 3), indicating that an E. coli PTK, possibly Wzc (8), might be involved. E. coli ssb was cloned, and 6xHis-SSB overproduced in E. coli. As expected, E. coli SSB strongly reacted with anti-PY antibodies (Figure 1A, lane 5). S. coelicolor SSB was also phosphorylated in the E. coli host (Figure 1A, lane 7). Differences in signal intensity are likely due to the structural variability of the SSB proteins as has been reported for M. tuberculosis and E. coli (51). To confirm the specificity of the signals, the samples were treated with the alkaline phosphatase and all signals were significantly reduced (Figure 1A, lanes 2, 4, 6 and 8). To exclude the possibility that the Western
Tyrosine phosphorylation of different SSBs. (A) In vivo phosphorylation of bacterial SSBs. The odd-numbered lane contains purified 6xHis tagged protein (50 ng) separated on SDS–PAGE, electroblotted on PVDF membrane, and probed with anti-PY antibody, and the even-numbered lane contains the same sample treated with alkaline phosphatase. B. subtilis SSB purified from B. subtilis is in lanes 1 and 2, BsSSB purified from E. coli in lanes 3 and 4, EcSSB purified from E. coli in lanes 5 and 6, ScSSB purified from E. coli in lanes 7 and 8, and the eluate from the Ni-NTA column obtained from a crude extract of wild-type B. subtilis is in lanes 9 and 10. (B) In vitro phosphorylation of S. subtilis SSBs. Autoradiography of in vitro phosphorylation assays separated on SDS–PAGE. The lanes contain the products of reactions with the following proteins: YwpH (lane 1), YwpH and YwqC-NCter (lane 2), YwpH and YwqD (lane 3), YwqD, YwqC-NCter and YwqD (lane 4), BsSSB (lane 5), BsSSB and YwqC-NCter (lane 6), BsSSB and YwqD (lane 7), BsSSB and YwqC-NCter and YwqD (lane 8), BsSSB, YwqC-NCter and YwqD (lane 9), Lanes 5 and 10 were the same as lanes 4 and 9, respectively, only YwqE and MnCl2 were added after 60 min, and the reactions were left for an additional 120 min before loading on SDS–PAGE. (C) In vivo labeling of S. subtilis SSB. 6xHis SSB was purified from SPSSBHT, SPSSBHT-YwqD and SPSSBHT-YwqE grown in [32P]phosphate. Purified BsSSB was separated by SDS–PAGE; the Coomassie-staining of the gel is shown in the right panel; and autoradiography signals in the left: SPSSBHT in lanes 1 and 4, SPSSBHT-YwqD in lanes 2 and 5, and SPSSBHT-YwqE in lanes 3 and 6.

signal came from a co-migrating protein, co-purifying with the BsSSB, we prepared an extract from the wild-type B. subtilis strain (no 6xHis-tagged SSB) that underwent the same purification procedure as that from SPSSBHT. Since this sample gave no signal (Figure 1A, lane 9), we concluded that the assay was specific for PY-SSB.

These findings provide the evidence that bacterial SSBs, until this study unidentified substrates of bacterial PTKs, are tyrosine phosphorylated and that this post-translational modification is conserved in Gram-positive and Gram-negative bacteria.

B. subtilis PTK YwqD phosphorylates cognate SSBs in vitro

We also tested the ability of YwqD to modify bacterial SSBs in vitro. YwqD interacts with the intracellular domain of a transmembrane modulator YwqC-NCter during phosphorylation (9). The results of in vitro phosphorylation are presented in Figure 1B. Only when YwqD and YwqC-NCter were present in the phosphorylation mixture did YwpH and BsSSB phosphorylation occur (Figure 1B, lanes 4 and 9). YwqD-123, the non-autophosphorylable form of YwqD (9), retained full capacity for SSB phosphorylation (data not shown). Although we have shown that E coli, B. subtilis and S. coelicolor SSBs can be phosphorylated in E. coli (Figure 1A), the E. coli and S. coelicolor SSBs were not phosphorylated by B. subtilis YwqD in vitro (data not shown). This may reflect the low efficiency of the in vitro phosphorylation system with the artificial modulator (9).

The phosphorylation of B. subtilis SSBs by YwqD was compared with that of the UDP-glucose dehydrogenase YwqF (9). While YwpH was the most rapidly phosphorylated substrate in initial velocity measurements (9.8 nM/min), BsSSB phosphorylation (2.6 nM/min) was comparable with the phosphorylation of YwqF (3 nM/min) (9). As in the case of YwqF, phosphorylation of SSBs by YwqD was strictly ATP-dependent; [32P]YwqD did not transfer phosphate to either BsSSB or YwpH in absence of ATP (data not shown). Optimal BsSSB phosphorylation occurred at a lower MgCl2 concentration (1 mM) compared with YwqF (5 mM).

To confirm the identity of the modified residues in B. subtilis SSBs, the phosphorylation mixtures containing [32P]YwpH and [32P]BsSSB were treated with a specific PTP, YwqE (46) to remove the radioactive label from both phosphoproteins (Figure 1B, lanes 5 and 10), confirming the presence of labeled PY in the SSBs.

B. subtilis PTK YwqD and PTP YwqE control the phosphorylation state of BsSSB in vivo

Since in vitro phosphorylation assays show that YwqD and YwqE act on B. subtilis SSB, we examined if the same enzymes control the phosphorylation state of the SSB in vivo. B. subtilis strains SPSSBHT, SPSSBHT-YwqD and SPSSBHT-YwqE were grown in the presence of [53P]inorganic phosphate, and the purified BsSSB assayed by autoradiography (Figure 1C). A weak radioactive signal comigrated with BsSSB purified from SPSSBHT (Figure 1C, lane 1), indicating in vivo phosphorylation. This signal was no longer detectable in the kinase deficient strain (Figure 1C, lane 2), and the signal was strongly enhanced in the phosphatase deficient strain (Figure 1C, lane 3). These results confirm that the kinase YwqD is indeed responsible for phosphorylating B. subtilis SSB, and the phosphatase YwqE carries out its dephosphorylation in vivo.

Phosphorylation of B. subtilis SSB increases ssDNA binding in vitro

Effective binding to ssDNA requires tetramers of bacterial SSBs (32). The oligomerization state of purified BsSSB and YwpH was checked by gel filtration, indicating that both proteins are tetramers in solution (data not shown). The efficiency of ssDNA binding was assayed by gel-shift analysis using a 50 base ssDNA fragment as substrate (Figure 2A, lanes 1–4). The ssDNA fragment binds one or two SSB tetramers, confirming observation that the bacterial SSB tetramer could wrap 25–30 bases of ssDNA (52). To test whether B. subtilis SSB phosphorylation affects ssDNA binding, 2 pmol of BsSSB were pre-phosphorylated with YwqD and YwqC-NCter. As
SSB phosphorylation in *B. subtilis* decreases during DNA damage response

Investigating all potential implications of SSB phosphorylation is beyond the scope of the present study. To initiate this research, we examined the relation of this post-translational modification to the DNA lesion repair since a consensus exists regarding the link between SSB phosphorylation and DNA lesion repair in *Eukarya* (23). Concomitant to DNA damage, the ssDNA-dependent protein kinase is activated to phosphorylate the SSB, but it is not known how hyper-phosphorylated SSB enhances lesion repair.

When the lesions are induced, the fraction of ssDNA is likely to increase in the cells. As presented, the ssDNA exerted an inhibitory effect on *in vitro* phosphorylation of YwpH (Figure 3A) and SSB (similar results, data not shown). An equivalent amount of double-stranded DNA had a less pronounced inhibitory effect; ssDNA also inhibited YwqD autophosphorylation, irrespective of the presence of SSBs (data not shown), suggesting that ssDNA exerts its effect directly on the kinase. Furthermore, we examined SSB phosphorylation *in vivo* while DNA damage was induced by adding mitomycin C at the onset of exponentially growing *B. subtilis* SPSSBHT cultures. The phosphorylation state of 6xHis-SSB was checked at different time intervals using anti-PY antibodies (Figure 3B). BsSSB was phosphorylated to a significantly lower extent in the mitomycin-treated cells, suggesting that dephosphorylated BsSSB after induction of DNA damage might play a role in DNA repair. According to this *Eukarya* and *Bacteria* might respond differently to DNA damaging conditions.

Therefore, we compared the growth of *B. subtilis* SPSSBHT-ΔywqD cells with *B. subtilis* SPSSBHT in the presence of mitomycin C. Cultures of *B. subtilis* SPSSBHT-ΔywqD reached a higher OD<sub>600</sub> than *B. subtilis* SPSSBHT before growth finally stopped (Figure 3C). Since DNA damaging agents often cause cell filamentation, thus rendering OD measurements non-reliable for estimating cell proliferation (54), we also compared the colony-forming ability of the two strains after induction of DNA damage (Figure 3D). The CFU of both strains fell dramatically after the addition of mitomycin, but *B. subtilis* SPSSBHT-ΔywqD retained a 2-fold higher colony forming ability throughout the experiment. This could indicate that kinase activity might affect DNA repair. However, our data could also be ascribed to a pleiotropic effect of ywqD deletion. Nevertheless, it can not be neglected, as depicted in Figure 3B, that BsSSB phosphorylation is inhibited in the mitomycin-treated cells with wild type kinase activity. The reduction in the level of BsSSB phosphorylation that decreases its affinity for ssDNA is in agreement with published data. SSB pre-bound to ssDNA inhibits the nucleation stage of RecA and its affinity for ssDNA is in agreement with published data. SSB

SSB needs to be removed from and re-attached to ssDNA during bacterial growth in concert with various stages of DNA metabolism, such as DNA replication or lesion repair (32,53). The results of this study suggest that SSB phosphorylation could participate in this process by changing its affinity to bind to ssDNA.
B. subtilis SSB is phosphorylated on tyrosine 82 in vivo

In order to identify the phosphorylated residue, purified SSB from B. subtilis SPSSBHT was analyzed by mass spectrometry (Figure 4A). LC-MS analysis detected a doubly charged peptide at m/z 608.748, the mass of which matched (within 12 p.p.m.) the theoretical mass of the B. subtilis SSB peptide NYENQQGQR containing one phosphate group. An MS/MS spectrum of the peptide showed pronounced y-ion series, confirming its sequence and pointing to Y82 as the phosphorylation site. Identification was furthermore confirmed by comparison with the fragmentation spectrum of the non-phosphorylated peptide (data not shown). As shown in Figure 4B, when the residue Y82 in B. subtilis SSB was replaced by phenylalanine, in vitro phosphorylation of the mutant protein was severely impaired, with residual phosphorylation still detectable. The BsSSB Y82F migrated as a mutant protein was severely impaired, with residual phosphorylation still detectable. The BsSSB Y82F migrated as

CONCLUSION

We have shown that SSBs are the target of bacterial PTKs and that this specific post-translational modification occurs in Streptomyces spp., B. subtilis and E. coli. This suggests that tyrosine phosphorylation of SSBs may be a general process in Gram-positive and Gram-negative bacteria. Focusing on the B. subtilis system, we have identified the kinase (YwqD) and the phosphatase (YwqE) that affect the phosphorylation state of the SSB on its residue Y82. Phosphorylation of BsSSB increased almost 200-fold its binding affinity for ssDNA. During DNA damage response, tyrosine phosphorylation of

Figure 3. Effect of DNA damage on BsSSB phosphorylation. (A) Control kinase reaction is shown in lane 1. The same reaction was performed in presence of increasing amounts of ssDNA (lanes 2–5) and dsDNA (lanes 6–9). (B) BsSSB phosphorylation followed by anti-PY western blotting with equal quantities (about 50 ng) of purified BsSSB. Growth of B. subtilis strains was compared in DNA damaging conditions. Mitomycin treatment of increasing amounts of ssDNA (lanes 2–5) and dsDNA (lanes 6–9). (C) Sequence alignment of the region surrounding the phosphorylated residue Y82 in B. subtilis SSB. Aligned bacterial SSBs are from B. subtilis (Bs), E. coli (Ec), L. lactis (Lac), S. aureus (Saur), E. faecalis (Efue), S. coelicolor (Eco), Yersinia pseudotuberculosis (Ypse), E. coli SSB has no tyrosine at this position, but was also found to be tyrosine-phosphorylated. How can this be explained? E. coli possesses four tyrosine residues in the SSB protein. Among these, two tyrosine residues (78 and 98) are located close to tyrosine residue 82 in BsSSB. This suggests that its overall structure was not disrupted by this mutation. This residue is highly conserved in SSBs from Gram-positive and Gram-negative bacteria. Focusing on the B. subtilis system, we have identified the kinase (YwqD) and the phosphatase (YwqE) that affect the phosphorylation state of the SSB on its residue Y82. Phosphorylation of BsSSB increased almost 200-fold its binding affinity for ssDNA. During DNA damage response, tyrosine phosphorylation of

Figure 4A. MS/MS spectrum of the precursor ion at m/z 608.748, corresponding to the B. subtilis SSB peptide NYENQQGQR with one phosphorylated residue. C-terminal y-ion series confirms the peptide sequence and indicates Y82 as the phosphorylation site. The low intensity of the spectrum reflects low occupancy of the phosphorylation site. (B) In vitro phosphorylation of mutant protein BsSSB Y82F compared with wild-type BsSSB. Reactions were performed with YwqD-123. (C) Sequence alignment of the region surrounding the phosphorylated residue Y82 in B. subtilis SSB. Aligned bacterial SSBs are from B. subtilis (Bs), E. coli (Ec), L. lactis (Lac), S. aureus (Saur), E. faecalis (Efue), S. coelicolor (Eco), Yersinia pseudotuberculosis (Ypse), Salmonella enterica (Sent) and Neisseria meningitidis (Nm). B. subtilis SSB and YwpH are denoted by ‘S’ and ‘Y’, respectively.
BsSSB was reduced and this could suggest one biological aspect of this process in the regulation of DNA metabolism.

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