CpgA, EF-Tu and the stressosome protein YezB are substrates of the Ser/Thr kinase/phosphatase couple, PrkC/PrpC, in *Bacillus subtilis*

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The conserved *prpC*, *prkC*, *cpgA* locus in *Bacillus subtilis* encodes respectively a Ser/Thr phosphatase, the cognate sensor kinase (containing an external PASTA domain suggested to bind peptidoglycan precursors) and CpgA, a small ribosome-associated GTPase that we have shown previously is implicated in shape determination and peptidoglycan deposition. In this study, in a search for targets of PrkC and PrpC, we showed that, *in vitro*, CpgA itself is phosphorylated on serine and threonine, and another GTPase, the translation factor EF-Tu, is also phosphorylated by the kinase on the conserved T384 residue. Both substrates are dephosphorylated by PrpC *in vitro*. In addition, we identified YezB, a 10.3 kDa polypeptide, and a component of the stressosome, as a substrate for both enzymes *in vitro* and apparently *in vivo*. We propose that the PrpC/PrkC/CpgA system constitutes an important element of a regulatory network involved in the coordination of cell wall expansion and growth in *B. subtilis*.

INTRODUCTION

We previously defined the first Ser/Thr 2D phosphoproteome of *Bacillus subtilis*, demonstrating that this is dynamic, changing with growth phase and stress conditions (Lvine *et al.*, 2006). The 29 phosphorylated proteins identified in stationary- or exponential-phase cells are involved in many cellular processes, including carbon and energy metabolism, stress and development, protein synthesis and transport. Use of a gel-free approach and the direct identification of phospho- residues in an enriched phosphopeptide fraction (Macek *et al.*, 2007) confirmed that many cellular processes in exponentially growing *B. subtilis* involve Ser/Thr phosphorylation. In combination, these two studies plus another recent report (Eymann *et al.*, 2007) now identify more than 100 distinct Ser/Thr/Tyr phosphoproteins in *B. subtilis*. It is now a major challenge

to assign these to their corresponding kinases and determine their physiological roles.

We have previously characterized the kinase and autophosphorylation activities of PrkC, a eukaryotic-like Ser/Thr protein kinase (STPK), and demonstrated that both PrkC and its cognate phosphatase, PrpC, are implicated in biofilm formation and sporulation in *B. subtilis* (Madec *et al.*, 2002, 2003). PrkC is a membrane-linked kinase with an external domain containing three repeated PASTA motifs (Madec *et al.*, 2002) that are able to bind penicillin (Jones & Dyson, 2006; Yeats *et al.*, 2002). This suggests a possible role for these domains in monitoring the status of the cell wall. However, the nature of the signal activating the PrkC kinase is unknown. Nevertheless, there is growing evidence that PrkC-like enzymes, including PknB from *Mycobacterium tuberculosis*, are indeed implicated in cell wall production or maintenance (Canova *et al.*, 2008; Fernandez *et al.*, 2006; Jin & Pancholi, 2006; Kang *et al.*, 2005, 2008; Novakova *et al.*, 2005).

Crystal structures of the kinase domain of the PrkC homologues PknB (Young *et al.*, 2003) and PknD (Good *et al.*, 2004) demonstrated a two-domain fold with the ATP-binding site situated between the folds, as in eukaryote STPKs. In such eukaryote receptor STPKs, ligand binding promotes dimerization of the external domain and stimulates autophosphorylation of the kinase

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Abbreviations: MBP, myelin basic protein; STPK, Ser/Thr protein kinase.

A supplementary table of primers is available with the online version of this paper.

domain. We previously provided evidence for dimerization of PrkC *in vivo* (Madec *et al.*, 2002), and Greenstein *et al.* (2007) have now shown that dimerization of the kinase domain, and phosphorylation of its catalytic loop, both contribute to the regulation of activity of PknD.

In *B. subtilis* the two adjacent genes *prpC* and *prkC* are followed by *cpgA*, encoding an essential GTPase, and this gene order is conserved in many Gram-positive bacteria. All three genes are transcribed from a promoter upstream of *prpC* (Madec *et al.*, 2002; Iwanicki *et al.*, 2005). Interestingly, depletion of CpgA, a small circularly permuted GTPase (Levdikov *et al.*, 2004), leads to marked morphological changes (Cladière *et al.*, 2006) and the accumulation of peptidoglycan precursors in cells (Absalon *et al.*, 2008). We have proposed previously that CpgA may function as a translation factor responsible for the synthesis of a subset of proteins concerned in some way in peptidoglycan synthesis (Cladière *et al.*, 2006; Levdikov *et al.*, 2004). However, although the three genes might act in the same functional pathway – regulation of cell wall production – *cpgA* appears to be essential for normal growth in some media (Absalon *et al.*, 2008; Cladière *et al.*, 2006), while *prkC* and *prpC* individually can be deleted in *B. subtilis* without detectable effect on growth (Gaidenko *et al.*, 2002; Madec *et al.*, 2002; Obuchowski *et al.*, 2000). Notably, however, homologues of PrkC, PknB in *M. tuberculosis* and StkP in *Streptococcus pneumoniae*, both implicated in cell shape development, are also essential for normal growth (Fernandez *et al.*, 2006; Saskova *et al.*, 2007).

In eukaryotes, STPKs regulate many cellular processes, involving a wide variety of known substrates whose functional roles have been analysed in some detail. In contrast, much less is known about bacterial STPKs. However, recently, a number of specific target substrates for different STPKs have been identified *in vitro* and in some cases *in vivo*. These include proteins with FHA (forkhead-associated) domains and a transcription factor in *M. tuberculosis* (Grundner *et al.*, 2005; Molle *et al.*, 2003a, b, 2004; Sharma *et al.*, 2006), GuaB (purine biosynthesis) in *Streptococcus agalactiae* (Rajagopal *et al.*, 2005), a transcription factor in *Streptomyces coelicolor* (Lee *et al.*, 2002), a histone-like protein in *Streptococcus pyogenes* (Fernandez *et al.*, 2006), and a metabolic factor and transcription factors involved in development in *Myxococcus xanthus* (Nariya & Inouye, 2005, 2006). Notably, in *Mycobacterium tuberculosis*, proteins involved in division or cell wall formation, Wag31, Rv1422, MmpL7, EmbR, FtsZ, PbpA and Rv2175c (Kang *et al.*, 2005; Molle *et al.*, 2003b; Pérez *et al.*, 2006; Thakur & Chakraborti, 2006; Dasgupta *et al.*, 2006; Canova *et al.*, 2008, respectively), and an enzyme, phosphoglucosamine mutase, required for cell wall biosynthesis in *Streptococcus pneumoniae* (Novakova *et al.*, 2005; Saskova *et al.*, 2007), have also been identified as substrates for STPKs. In *B. subtilis*, studies of substrates have so far been limited to different classes of kinases, unrelated to the Hanks sensor

kinases. Thus, single-stranded DNA-binding proteins (SSBs) were identified as targets of the tyrosine kinase YwqD (Mijakovic *et al.*, 2006). Phosphorylation of the UDP-glucose dehydrogenases YwqF and TuaD by YwqD has also been demonstrated (Mijakovic *et al.*, 2003, 2004). In addition, phosphorylation on serine and threonine, also by a distinct class of kinases and phosphatases, STPKs, controls the action of target proteins involved in regulation of the σ^B dependent general stress response (Hecker *et al.*, 2007).

In this study, we have identified CpgA together with another GTPase, EF-Tu, as targets of PrkC and PrpC. In addition, we have shown that a novel stressosome protein YezB is also a target of both enzymes *in vitro* and probably *in vivo*.

METHODS

Bacterial strains and growth conditions

***Bacillus subtilis* strains.** Strains W168 *trpC2* (Anagnostopoulos & Spizizen, 1961), OMG 344 *trpC2*, *prkCK40R kan* (Madec *et al.*, 2002), OMG 401 *trpC2*, *prpC::kan* (Obuchowski *et al.*, 2000) were used. These strains were routinely grown at 37 °C with vigorous agitation either in synthetic B-medium [final concentrations: 15 mM (NH₄)₂SO₄, 8 mM MgSO₄·7H₂O, 27 mM KCl, 7 mM sodium citrate dihydrate, 50 mM Tris/HCl pH 7.5; and added before use, 2 mM CaCl₂·2H₂O, 1 µM FeSO₄·7H₂O, 10 µM MnSO₄·4H₂O, 0.6 mM KH₂PO₄, 4.5 mM glutamic acid, 862 µM lysine, 784 µM tryptophan and 0.5% glucose (Antelmann *et al.*, 1997)], LB or Spizizen medium (Anagnostopoulos & Spizizen, 1961).

***Escherichia coli* strains.** These were DH5 α (Hanahan, 1983) and BL21 α (DE3) (Studier & Moffatt, 1986). Strain DH5 α , carrying one of the following plasmids, was used to overproduce the respective proteins: pOMG318 (pBAD-6His-*fusA*); pOMG767 (pET302-6His-*tufA*); pOMG769 (pET302-6His-*tufA* T384V), pOMG360 (pBAD-6His-*cpgA*; Cladière *et al.*, 2006); pOMG500 (pBAD-6His-*cpgA* S178A; Cladière *et al.*, 2006). *E. coli* strains were cultured with shaking in Luria–Bertani (LB) medium (Miller, 1972). Antibiotics were added to the following final concentrations: for *E. coli* ampicillin 100 µg ml⁻¹, and for *B. subtilis* chloramphenicol 5 µg ml⁻¹, kanamycin 5 µg ml⁻¹.

Construction of overexpressing clones and purification of His-tagged proteins. For construction of the clones for overproduction of YezB, YflT, YbcM, CspD and RsbV, High Fidelity PCR Enzyme Mix (Fermentas) was used to amplify the corresponding gene from total *B. subtilis* DNA, using appropriate PCR primers (see Supplementary Table S1, available with the online version of this paper, for primer sequences). The PCR fragment was restricted and ligated into the cloning vector pBAD-GFPuv (Cramer *et al.*, 1996) opened with *NheI* and *SmaI* for *yezB*, and with *NheI* and *EcoRI* for the other PCR products. Clones were streak-purified to single colonies three times on LB plates plus 100 µg ampicillin ml⁻¹ and the construct confirmed by sequencing. In order to overproduce and to purify the proteins, the strains with the corresponding plasmids were grown in LB medium supplemented with 100 µg ampicillin ml⁻¹ at 37 °C with shaking (200 r.p.m.) to an OD₅₇₀ of 1.2. Then 0.05% arabinose was added to the medium and the cultures were grown for a further 3 h. The cells were harvested by centrifugation at 4500 r.p.m. for 15 min (Sorvall RC-5C, SS-34 rotor) and the resulting pellets were washed twice with 50 mM Tris/HCl, pH 8, 300 mM NaCl. The cells

were then disrupted by sonication (3×45 s at 4°C) and centrifuged at 14 000 r.p.m. (Eppendorf S417C). The supernatants were adjusted to a final concentration of 20 mM imidazole and the proteins were purified with the Ni-NTA Spin kit (Qiagen) as recommended by the manufacturer.

Site-directed mutagenesis. Substitution of alanine for serine in CpgA at codons 196 and 226 or substitution of alanine for threonine at codons 192, 205, 206 and 222 was achieved by PCR of *cpgA* encoding the His-tagged version of the gene, using the High Fidelity PCR Enzyme Mix (Fermentas), followed by 1 h digestion with *DpnI* and subsequent transformation into *E. coli* DH5 α . Mutagenic primers were used to introduce the desired mutation into the known sequence of pOMG360 plasmid DNA (see Supplementary Table S1). The gene of all constructs was then sequenced in order to confirm the presence of the mutation and no other changes.

Deletion of *yezB*. The deletion of *yezB* was made by insertion of a chloramphenicol-resistance cassette into *yezB*. Two fragments corresponding to the upstream and the downstream regions of *yezB* were amplified by PCR and inserted on each side of a chloramphenicol-resistance cassette carried by a derivative of the plasmid pMTL20E, using *Bam*HI and *Sac*I restriction sites, respectively (see Supplementary Table S1). The resulting plasmid, named pMTL20E-cat-*yezB*, was used to transform *B. subtilis* 168 by double crossing-over.

Purification of PrkCc and CpgA. The catalytic subunit of PrkC (PrkCc) was overexpressed and purified as a His-tagged protein as described previously (Madec *et al.*, 2002). His-tagged CpgA and the CpgA mutated versions were overexpressed and purified as described previously (Cladière *et al.*, 2006), except for the use of 50 mM Tris/HCl pH 8, 300 mM NaCl and 50 mM Tris/HCl pH 8, 100 mM NaCl instead of buffer A and B, respectively.

Cellular fractionation of *B. subtilis* 168 total protein extract. Isolation of membrane and cytosolic fractions was carried out essentially as described previously (Eymann *et al.*, 2004). Briefly, cells were grown in 200 ml LB medium at 37°C with shaking to an OD₅₇₀ of 0.6. The cells were harvested by centrifugation (14 000 r.p.m., Sorvall RC-5C, SS-34 rotor) and washed twice with TE buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA). The cells were disrupted by sonication and then subjected to ultracentrifugation (100 000 g, 60 min, 4°C). The resulting supernatant was designated the cytosolic fraction. The remaining pellet was homogenized in 8 ml high-salt buffer (20 mM Tris/HCl, pH 7.5, 10 mM EDTA, 1 M NaCl, 1 mM Pefabloc) and incubated for 30 min at 4°C on a rotary shaker and again subjected to ultracentrifugation. The pellet was homogenized in 100 mM Na₂CO₃/HCl, pH 11, 10 mM EDTA, 100 mM NaCl. After a final washing step with 8 ml TE buffer and subsequent ultracentrifugation (100 000 g, 60 min, 4°C), the resulting pellet was homogenized in 200 μ l TE buffer. All supernatants were added to the cytosolic fraction. Protein was extracted from the membrane by solubilizing the pellet with 15 mM n-dodecyl β -D-maltoside. Protein concentrations were determined by the Bradford method.

Kinase assays. To test the possible phosphorylation of the purified proteins by PrkCc, reactions were performed in kinase buffer (10 mM HEPES, 5 mM MgCl₂, pH 8) containing 1 μ g purified PrkCc plus 1–10 μ g of the purified protein substrate and 1 μ Ci [³²P]ATP in 20 μ l reaction volume. Mg²⁺ was chosen as the activating cation since in titration experiments this gave higher activities than Mn²⁺. After 10 or 30 min incubation at 30°C , the reactions were terminated by addition of 0.2 vol. $5 \times$ SDS-PAGE buffer [2% (w/v) SDS, 2.5% (v/v) β -mercaptoethanol, 25 mM Tris/HCl, pH 7.5] and 10% (v/v) glycerol. The mixture was boiled for 5 min and separated by SDS-PAGE (12 or 15%). After electrophoresis, gels were soaked in boiling

16% (w/v) trichloroacetic acid for 20 min at 90°C in order to avoid non-specific [³²P]ATP binding, stained with Coomassie brilliant blue (G-250), dried and exposed for autoradiography using a Molecular Dynamics Storm phosphorimager; labelled bands were quantified using ImageQuant 5.2 (Molecular Dynamics). The same protocol was used to assay P10 phosphorylation in extracts of *B. subtilis* 168, using 50–100 μ g of total protein extract. Poly-L-lysine was purchased from Sigma-Aldrich.

Phosphatase assays. Kinase reactions were first performed as described above, then after 30 min, 1 μ g PrpC, 5 mM ATP (in order to mask the kinase activity) and 5 mM Mn²⁺ were added to the mixture and incubation continued at 37°C . The reaction was stopped at the desired time by addition of 0.2 vol. $5 \times$ SDS-PAGE buffer. Reaction products were separated by SDS-PAGE and analysed by autoradiography as above.

Thin-layer chromatography for phosphoamino acid analysis. Phosphoamino acid analysis was essentially performed as described previously (Madec *et al.*, 2002) with P10 and CpgA labelled as described in the kinase assay section.

³²P-radiolabelling of cultures and 2D PAGE separation of proteins. Radiolabelling of cultures with [³²P]orthophosphate, preparation of the protein extracts and 2D PAGE separation of proteins were performed as described previously (Lévine *et al.*, 2006).

Purification of P10. Twenty-litre batches of strain 168, grown in LB medium, were harvested by centrifugation at 4500 r.p.m. for 5 min and the pellet was resuspended in 50 mM Tris/HCl pH 8, 300 mM NaCl. Cells were broken in a Dry-ice press and centrifuged at 14 000 r.p.m. (Sorvall RC-5C, SS-34 rotor) for 30 min to remove debris. The cell lysate was heated to 100°C for 20 min and centrifuged at 14 000 r.p.m. for 30 min to remove denatured protein. The supernatant was then adjusted to 40% saturation with ammonium sulfate in the cold for 1 h and centrifuged at 14 000 r.p.m. for 30 min. The P10 protein was subsequently precipitated by adding ammonium sulfate to the supernatant until it reached 60% saturation. The precipitate was collected by centrifugation at 14 000 r.p.m. for 30 min, redissolved in 50 mM Tris/HCl (pH 8), dialysed to remove ammonium sulfate and NaCl and then subjected to chromatography on a MonoQ anion-exchange column (GE Healthcare). The peak containing P10 was eluted by 350 mM NaCl. The eluted fraction was then subjected to gel filtration on a Hi-load 16/60 Superdex 200 column (GE Healthcare) and the fraction containing P10 was retained. Finally, in some preparations the partially purified material from the last gel filtration step was concentrated and separated by 2D electrophoresis using a 4–5 pH gradient. The P10 spot was then identified by autoradiography. The presence of P10 was detected at each purification step by the addition of purified PrkCc and ³²P-labelled ATP in kinase buffer. The radioactivity in the P10 band was determined by phospho-imaging, following SDS-PAGE.

Mass spectrometry. MS/MS analysis was carried out on protein samples and peptides were identified as described by Lévine *et al.* (2006).

RESULTS

Effect of poly-L-lysine on autophosphorylation and kinase activity of PrkCc

We showed previously that the region of PrkC constituting the cytoplasmic kinase domain (PrkCc), when purified as a

His-tagged protein, displayed autophosphorylation activity and phosphorylated the synthetic substrate myelin basic protein (MBP) on threonine *in vitro*. In addition, we demonstrated that kinase activity was completely abolished in the PrkCc mutants K40R and K40A with a residue substituted in the presumed catalytic site (Madec *et al.*, 2002). These studies established minimal conditions suitable for analysis of PrkCc kinase activity. However, in eukaryote STPKs, full kinase activity can depend on several allosteric regulatory mechanisms, including ligand-induced dimer formation and/or binding to the kinase domain of specific regulatory proteins (Huse & Kuriyan, 2002). Thus, when studying the isolated kinase domain *in vitro*, the observed (constitutive) kinase activity may not be optimal. Indeed, several earlier studies have shown that the activity of isolated eukaryotic tyrosine or serine/threonine kinase domains can be stimulated by basic proteins or poly-L-lysine (Hubler *et al.*, 1992). Hubler *et al.* (1992) provided evidence that this stimulation effect, in the case of the EGF receptor kinase, reflects an increased $V_{\max}$ and decreased K_m , suggesting an effect on substrate binding/utilization by the kinase. We therefore first investigated the effect of poly-L-lysine on the autophosphorylation activity of PrkCc in the presence of [32 P]ATP. As shown in Fig. 1, the basic amino acid clearly stimulated autophosphorylation activity with a maximum concentration of around $4 \mu\text{g ml}^{-1}$. Similarly, MBP ($250 \mu\text{g ml}^{-1}$) also stimulated kinase activity. In most subsequent experiments, therefore, poly-L-lysine or MBP was included in the reaction mixture in order to maximize the kinase activity of PrkCc.

CpgA phosphorylated on Thr and Ser is a target for both PrkC and PrpC *in vitro*

In view of the relatively conserved occurrence of *cpgA* downstream of *prpC* and *prkC* in Gram-positive bacteria, we considered the possibility that the GTPase, a putative translation factor (Cladière *et al.*, 2006), might be a target for the kinase and phosphatase. In addition, we demonstrated that the activity of PrkC was stimulated by both

poly-L-lysine and MBP, as shown in Fig. 2(a, b). In contrast, several other proteins, for example GroEL, CspB, ClpP, CspD, and YfiT and RsbV, were not phosphorylated by the kinase, when tested under similar conditions (data not shown and see Fig. 6, respectively). In control experiments in the absence of the kinase, no labelling of CpgA or any other substrates was detected. The 32 P-phosphorylated CpgA was shown to be labelled on Ser and Thr (Fig. 2c). Finally, we were able to show that phosphorylated CpgA was rapidly dephosphorylated by PrpC (Fig. 2d). In contrast, a mutant of PrpC, PrpC D234A, substituted in the active site (Obuchowski *et al.*, 2000), failed to dephosphorylate CpgA or the autophosphorylated PrkCc (data not shown).

Attempts to identify the phosphorylated residues on CpgA

We previously identified eight serine/threonine phosphorylated residues in CpgA, mostly located in the catalytic loop (Madec *et al.*, 2003). This permitted an attempt to identify a possible CpgA residue that might be phosphorylated by PrkCc, based on conserved serine/threonine residues present within the catalytic site (Levdikov *et al.*, 2004). Four threonines and three serines were identified and the corresponding codons were subjected to directed mutagenesis, resulting in replacement by alanine. The presence of only the desired mutation in all the mutant constructs was then confirmed by sequencing. With the exception of T205A (data not shown), the His-tagged mutant proteins were all purified with good yield and ran like the wild-type protein in SDS-PAGE, although S226A showed a marked mobility change (see Fig. 3). The mutant proteins were then tested as substrates for PrkCc. As also shown in Fig. 3, three of the mutants (T222A, S178A, S196A) and possibly T206A, still appeared to be phosphorylated similarly to the wild-type CpgA. In contrast, the mutants S226A, and in particular T192A, showed markedly reduced phosphorylation. However, it was clear that with the mutant protein T192A (and to a lesser extent S226A), autophosphorylation of PrkCc in the same reaction mix was also greatly diminished, perhaps suggesting a reduced ability of the kinase to bind to these substrates. Conceivably, on the other hand, if these mutants, in particular S226A, have *increased* affinity for the kinase, this might interfere with the binding of poly-L-lysine and consequently reduce autophosphorylation of PrkCc. Thus, although these results are consistent with T192 and S226 in CpgA being involved in a specific interaction with PrkCc, it is not clear if they are necessarily specific phosphorylation targets for PrkCc.

Fig. 1. Stimulation of PrkCc autophosphorylation in the presence of poly-L-lysine. Purified PrkCc ($1 \mu\text{g}$) was incubated in the presence of [γ - 32 P]ATP ($1 \mu\text{Ci}$; 37 kBq) and different concentrations of poly-L-lysine for 30 min at 37°C . Reaction mixtures were then separated by SDS-PAGE and analysed by autoradiography (see Methods for details).

PrkCc specifically phosphorylates EF-Tu on T384

The highly conserved translation factor EF-Tu is known to be phosphorylated in *E. coli* on threonine 382 (Lippmann *et al.*, 1993) and EF-Tu is also reported to be a substrate for the homologue of PrpC in *Listeria*, Stp (Archambaud *et al.*, 2005). Moreover, phosphorylated EF-Tu was detected *in*

Fig. 2. Phosphorylation and dephosphorylation of CpgA. (a, b) CpgA (10 µg) was incubated with PrkCc (1 µg) for 30 min at 37 °C plus 1 µCi (37 kBq) [γ - 32 P]ATP and either 0.1 µg poly-L-lysine (a) or 5 µg MBP (b) in a 20 µl reaction mixture. Samples were separated by SDS-PAGE and analysed by autoradiography. (c) The phosphoamino acids of CpgA phosphorylated by PrkCc were detected by thin-layer chromatography as described in Methods. (d) Dephosphorylation of CpgA (10 µg) was carried out by PrpC (1 µg), following phosphorylation by PrkCc. Samples were separated by SDS-PAGE and analysed by autoradiography. PrkCc normally runs as a double band as in (b) and (d), although in (a) this appears as a smear. For all other details, see Methods.

vivo in previous phosphoproteome studies, although the residue was not identified (Eymann *et al.*, 2007; Lévine *et al.*, 2006). Thus, in view of the phosphorylation of the G-protein CpgA by PrkC, it was important to test EF-Tu as a possible target. The His-tagged EF-Tu from *B. subtilis* was therefore overexpressed, purified and tested for phosphorylation by PrkCc. As shown in Fig. 4(a), EF-Tu was not phosphorylated in the absence of PrkCc, but it was phosphorylated in the presence of the kinase plus either MBP (left panels) or poly-L-lysine (right panels). Moreover, the phosphorylated form of EF-Tu was efficiently dephosphorylated by PrpC (Fig. 4c, d). Importantly, as shown in Fig. 4(b), when we constructed the EF-Tu mutant T384V with the conserved phosphorylated residue (T382 in *E. coli*)

substituted for valine, and tested this protein as a substrate for PrkCc, no phosphorylation could be detected. This strongly suggests that the T384 residue is also a target of PrkC *in vivo*.

The phosphorylated form of another GTPase translation factor in *B. subtilis*, EF-G, using antiphosphoprotein antibodies, was reported by Gaidenko *et al.* (2002) to be phosphorylated and dephosphorylated by PrkC and PrpC. In contrast, however, despite several attempts under different conditions *in vitro*, including the presence of poly-L-lysine, no phosphorylation of EF-G by PrkC could be demonstrated in this study. The reason for the different result obtained by Gaidenko *et al.* (2002) is not clear.

Fig. 3. Phosphorylation of mutants of CpgA, following site-directed mutagenesis of conserved serine and threonine residues. Purified samples of different mutant forms of CpgA (6 μ g) prepared as described in Methods, were phosphorylated by PrkCc (1 μ g) in a kinase assay containing 0.1 μ g poly-L-lysine and analysed by SDS-PAGE as in Fig. 2.

A 10 kDa cytoplasmic protein is phosphorylated in crude extracts by exogenous PrkCc

In a different approach to identify possible targets of PrkC, purified PrkCc was added to a crude extract of *B. subtilis* 168 and incubated in the presence of [32 P]ATP. Some weakly phosphorylated species were detected, but one major labelled product in the extract migrated with an apparent molecular mass of 10 kDa when the extract was analysed by SDS-PAGE. This protein, designated P10, was present in the cytoplasmic fraction (Fig. 5a). When 32 P-labelled P10 was hydrolysed and analysed by thin-layer chromatography, the labelled residue was clearly shown to be threonine (Fig. 5b). After phosphorylation by PrkCc, P10 was also shown to be a substrate for purified PrpC added to the crude extract, while in contrast, the mutant PrpC (D234A) failed to dephosphorylate P10 (Fig. 5c, d).

In contrast to P10, the purified substrates of PrkC demonstrated above, CpgA and EF-Tu, were not detectable in these experiments with the crude extracts, even with added poly-L-lysine (data not shown). This may be due to the presence of inhibitors in the extract including phosphatases, but this was not investigated further.

Identification of P10

Exhaustive attempts were made to purify P10, using an assay based on the capacity of P10 to be phosphorylated by purified PrkCc. Purification involved heat treatment, ammonium sulfate fractionation, anion-exchange chromatography, gel chromatography and in some cases a final step with a 2D gel analysis of the partially purified preparation by isoelectric focussing and SDS separation. From different partially purified preparations, the 10 kDa band or the corresponding 2D gel spot, in some preparations, were analysed by mass spectrometry. CspB, LicA or CspD were identified by mass spectrometry as major components, enriched in different partially purified

preparations. These proteins indeed have predicted molecular masses and isoelectric points similar to P10 and were therefore possible candidates for P10. These proteins were therefore all purified by Ni-affinity chromatography from the corresponding overexpressing clones (see Methods) and tested *in vitro* as substrates for PrkCc. However, all proved to be negative as possible targets, as no phosphorylation was detected (data not shown).

Using a completely different approach, four additional proteins, YbcM, YezB, YfT and RsbV, were then identified directly from the *B. subtilis* database, with sizes and isoelectric points (10–13 kDa, pI 4.6–4.8) close to those of the phosphorylated P10. His-tagged versions of these proteins were also purified from the corresponding overexpressing clones (prepared as in Methods) and tested as substrates *in vitro* with PrkCc. As shown in Fig. 6(a), one candidate protein, YezB, was indeed shown to be a substrate for PrkCc. Moreover, when a strain deleted for *yezB* was constructed (see Methods), phosphorylated P10 was no longer detected in the crude extract after addition of purified PrkCc (Fig. 6b). These results confirmed that P10 was identical to YezB, a component of the general stressosome, but of so far unknown function (Akbar *et al.*, 2001; Hecker *et al.*, 2007). Interestingly, the protein RsbV, involved in regulation of the general stress response and also tested in this screen, is known to be phosphorylated on S56 by the RsbW kinase (Yang *et al.*, 1996), but was not found to be a substrate for PrkCc (Fig. 6a).

yezB encodes a 10.3 kDa polypeptide in *B. subtilis*

The sequence of the *B. subtilis* genome (Kunst *et al.*, 1997) indicates that the gene *yezB*, located immediately downstream of *yetI*, encodes a protein of 10.3 kDa similar to P10. Curiously, however, from recent genome sequencing in other bacilli such as *Bacillus amyloliquefaciens* and *Bacillus* strains NRRL B-14911 and GG-1, *yetI* and *yezB*

Fig. 4. PrkCc phosphorylation and dephosphorylation by PrpC of wild-type EF-Tu and mutants of this protein. (a) Purified EF-Tu (3 μ g) was phosphorylated by PrkCc (1 μ g) in the presence of MBP (5 μ g) or poly-L-lysine (0.1 μ g) and the reaction mix was separated by SDS-PAGE and analysed by autoradiography as described in Fig. 2. (b) Wild-type EF-Tu or the mutant T384V were phosphorylated by PrkCc as above. (c, d) EF-Tu phosphorylated as in (a) was dephosphorylated by PrpC (1 μ g) as in Methods. Samples were taken at intervals for analysis of residual 32 P-labelling of the EF-Tu, then separated by SDS-PAGE and analysed by autoradiography; 32 P-labelled bands were quantified with a Molecular Dynamics Storm phosphorimager.

appear to constitute a single gene, generating a polypeptide with the C-terminal domain showing 50% identity with YezB. It was therefore important to determine whether *yezB* was in fact an independent gene in *B. subtilis*. Accordingly, the genes *yetI* and *yezB* and flanking regions were sequenced on both strands. The results (Fig. 5e) confirmed the presence of an intact independent *yezB*, with

a start codon (GTG) separated by 5 bp from the stop codon for *yetI*. Thus, *yezB* does appear to constitute a single independent gene. We identified a Shine–Dalgarno motif for translation of *yezB* messenger but no clear evidence of sequences corresponding to an independent promoter. Thus, it appears likely that *yezB* is transcribed from an upstream promoter as part of an operon.

Fig. 5. P10 phosphorylation by PrkCc, dephosphorylation by PrpC and sequence of *yezB*. (a) P10 phosphorylation by PrkCc (1 μ g) in a total protein extract of *B. subtilis* 168 and in cytosolic and membrane fractions (all 100 μ g protein). Assays were performed as described in Methods. (b) The phospho-amino acid residue of P10 was determined by thin-layer chromatography. (c, d) Dephosphorylation of P10 by PrpC (1 μ g) (c) and by the PrpC D234A inactive mutant (d), as a function of time. Assay and analysis conditions as in Fig. 2. (e) Sequence of the 3' end of *yetI* and the entire *yezB* gene with start and stop codons boxed; the apparent Shine-Dalgarno motif is underlined.

P10 (YezB) is a substrate for both PrkCc and PrpC *in vivo*

Phosphorylated P10 can readily be identified in 2D gels of total extracts of strain 168 following incubation *in vitro* with PrkCc and [32 P]ATP. In addition, we were able to detect the same phosphorylated protein in a 2D gel of a total cell extract prepared from a culture grown into stationary phase in the presence of [32 P]orthophosphate (see Methods). This result is shown in Fig. 7(a, b). As shown in Fig. 7(c, d), when total extracts were analysed from a strain lacking *prpC* or carrying a mutation, K40R, inactivating PrkCc, P10 was apparently hyperphosphorylated or not detectably phosphorylated, respectively. These results provided evidence that P10/YezB is also a substrate of PrkC and PrpC *in vivo*. Notably, the results in Fig. 7 also show that the level of phosphorylated RsbV *in vivo* remains essentially unchanged in these different mutant strains, as expected for a non-PrkC or PrpC target.

DISCUSSION

Our previous studies have shown that the co-transcribed genes *prpC*, *prkC* and *cpgA* are involved in shape determination and deposition of cell wall (Cladière *et al.*, 2006; Absalon *et al.*, 2008). Further evidence for a role of this locus in cell wall biogenesis comes from our recent demonstration that a strain deleted for *prkC* was approximately twofold more sensitive to the cephalosporin cefotaxime, while the *prpC* mutant was four times more sensitive: MIC 2.5 μ g ml $^{-1}$ versus 10 μ g ml $^{-1}$ for the parental strain 168 (all unpublished data). Interestingly, deletion of the PrkC homologue in *Enterococcus faecalis* results in reduced growth rate and a substantial increase in sensitivity to cephalosporins but not to ampicillin (Kristich *et al.*, 2007). There is indeed growing evidence that kinases similar to PrkC in Gram-positive bacteria are implicated in some way in the control of morphogenesis (see Introduction). These findings are consistent with our

Fig. 6. *In vitro* phosphorylation of putative candidates for P10 and phosphorylation of a $\Delta yezB$ cell extract with purified PrkCc. (a) Phosphorylation assays with the four different purified His-tagged proteins (5 μ g) were carried out by incubation for 10 min at 37 °C in the presence or absence of PrkCc (1 μ g) and analysed as in Fig. 2. (b) *In vitro* phosphorylation of a cell extract from the wild-type or the $\Delta yezB$ strain, incubated for 10 min at 37 °C in the presence or absence of added purified PrkCc.

hypothesis that the *prpC*, *prkC*, *cpgA* locus encodes a coordinated signalling system implicated in the normal control of peptidoglycan expansion or deposition (Absalon *et al.*, 2008; Jones & Dyson, 2006). This hypothesis is now greatly strengthened with the demonstration here that, *in vitro*, CpgA is a substrate for both the PrkC kinase and the cognate phosphatase, PrpC. CpgA is phosphorylated on both serine and threonine, and two residues located in the catalytic site, S226 and T192, were identified as possible targets for PrkCc. There remains the apparent paradox that, while CpgA is required for normal growth, PrkC under the same conditions is not. This may indicate that the PrkC function is redundant or, alternatively, that the phosphorylated form of CpgA is not itself engaged in a process essential for normal growth, at least under our test conditions.

The ligand that normally activates PrkC, presumably by binding to the PASTA domain, has not been identified, but

is likely to be a transiently exposed component of the cell wall peptidoglycan, a free peptidoglycan precursor or a degradation product. We could envisage that PrkC responds to general cell wall stress or, more specifically, that PrkC is activated when expansion of the cell wall sacculus fails to keep pace with growth. The evidence in this study that a key factor determining biomass increase, another GTPase, the elongation factor EF-Tu, is a substrate of PrkCc and PrpC *in vitro* lends support to the hypothesis that these enzymes are normally involved in monitoring the coupling of wall expansion to growth. Phosphorylated EF-Tu has been identified previously *in vivo* in exponentially growing *B. subtilis* (Eymann *et al.*, 2007; Lévine *et al.*, 2006). Moreover, a phosphorylated EF-Tu from *Listeria monocytogenes* was shown to be a substrate of a serine-threonine phosphatase *in vitro* (Archambaud *et al.*, 2005). It is reasonable therefore to suggest that in *B. subtilis*, EF-Tu may be a PrkC substrate *in vivo*, since our evidence shows that the residue phosphorylated by PrkCc in EF-Tu, T384, is the same as that previously described for EF-Tu in *E. coli*. Alexander *et al.* (1995) reported that phosphorylation of EF-Tu in *E. coli* was stimulated *in vitro* by EF-Ts and that the preferred substrate was the GDP-bound form. On the basis of *in vitro* studies these authors suggested that phosphorylation of EF-Tu *in vivo* during protein synthesis could accelerate its release from aa-tRNA and therefore from the translation site. However, no further studies have been reported and nothing is known about the role of this phosphorylation step in *B. subtilis*. Finally, we also note with interest that the elongation factor EF-G was previously reported to be phosphorylated by the *B. subtilis* PrkC, using anti-threonine antibodies (Gaidenko *et al.*, 2002). However, despite extensive efforts, we were unable to confirm this using our 32 P-labelling assay conditions with the purified protein *in vitro*.

YezB is a 10.3 kDa polypeptide in *B. subtilis* and we confirmed that it is encoded by an independent gene, while in the genomes of some other bacilli such as *B. amyloliquefaciens*, *yezB* is apparently fused to the upstream gene *yetI*. Interestingly, YezB, a component of the *B. subtilis* stressosome involved in the general stress response, was also demonstrated in this study to be a substrate of PrkC *in vitro*. In addition, we present evidence that YezB is also phosphorylated *in vivo* since the absence of *prkC* or *prpC* appears to abolish or enhance, respectively, the levels of phosphorylated P10/YezB in a phosphoproteome analysis. The stressosome (Pane-Farre *et al.*, 2005) normally transduces effects of stress (for example, ethanol or energy limitation) into activation of σ^B (for a review, see Hecker *et al.*, 2007). The components of the stressosome YkoB, YojH and YqhA have been shown previously to be substrates for the kinase RsbT. Unlike other stressosome components, expression of YezB, whose function is unknown, is not induced by either ethanol or energy stress and was not shown previously to be phosphorylated (Akbar *et al.*, 2001). It therefore appears reasonable to propose that phosphorylation of YezB, responding to changes in the cell wall, could provide a distinctive signal to activate the general stressosome.

Fig. 7. 2D gel analysis of the *in vivo* phosphorylation of YezB in cell extracts of mutants inactivated for PrkC or deleted for PrpC. *In vivo* phosphorylation of YezB was determined by ^{32}P -labelling of stationary-phase cultures of strain 168, $\Delta prpC$ or the K40R *prkC* mutant. ^{32}P -labelled protein extracts (1 mg protein) from stationary-phase cultures were prepared as indicated in Methods, separated by 2D electrophoresis (pH range 4.5–5.5) and stained with Coomassie blue (not shown). Radioactive phosphoproteins were detected by autoradiography. (a) Wild-type (strain 168 extract); (b) enlargement of inset in (a); (c) extract from $\Delta prpC$ strain; (d) extract from *prkC*-inactivated point mutant. Molecular mass markers are indicated at the right and pI values at the top of the panel in (a). The arrows indicate the position of YezB and its differential phosphorylation in the three strains.

We have previously proposed (Absalon *et al.*, 2008; Cladière *et al.*, 2006) that CpgA acts as a translation factor, responsible for the synthesis of a subset of proteins implicated in coordination of cell wall biogenesis with growth. It is now tempting to speculate that such proteins may include EF-Tu and YezB, modulated by the PrkC-dependent phosphorylated form of CpgA PrkC, in response to inappropriate changes in the status and/or rate of expansion of the peptidoglycan sacculus.

NOTE ADDED IN PROOF

We note that Shah *et al.* (2008) have recently confirmed that the PASTA domain of PrkC does indeed specifically bind *B. subtilis* muropeptides and that this is essential to provide the signal at least for spore germination.

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