PkwA, a WD-repeat protein, is expressed in spore-derived mycelium of *Thermomonospora curvata* and phosphorylation of its WD domain could act as a molecular switch

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WD-repeat proteins are found in all eukaryotes and are implicated in a variety of regulatory functions as a result of protein–protein interactions. PkwA from *Thermomonospora curvata* CCM3352 is a first potential example of a WD-repeat protein in a prokaryotic actinomycete. A mAb (3G2) was generated against the carboxy terminus of PkwA and was used to analyse the expression of PkwA in *T. curvata*. PkwA was detected in exponential growth phase following inoculation with spores, but could not be found at any stage of growth following inoculation with vegetative mycelium. PkwA and its WD domain were expressed in *Escherichia coli* as His-tag derivatives and purified on a Talon metal affinity matrix. The WD domain was phosphorylated by Pkg2, a membrane-spanning protein Ser/Thr kinase from '*Streptomyces granaticolor*'. A membrane fraction from an exponential, spore-derived culture of *T. curvata* was found to phosphorylate the WD domain specifically in the presence of Mn²⁺. These data confirm that PkwA is expressed in spore-derived exponential growth phase of *T. curvata* and could play a role as a molecular switch in a signalling pathway.

Keywords: PkwA, WD-repeat protein, protein Ser/Thr kinase, phosphorylation, *Thermomonospora curvata*

INTRODUCTION

The so-called WD-repeat proteins contain four to eight copies of a highly conserved motif, which is usually flanked by the characteristic dipeptides GH (Gly-His) and WD (Trp-Asp) (Neer *et al.*, 1994; Neer & Smith, 1996; Smith *et al.*, 1999). They form an ancient regulatory protein family, which was originally thought to be confined to eukaryotes (for a review, see Neer *et al.*, 1994). The first reported WD-repeat protein was the β subunit of a heterotrimeric GTP-binding protein (G protein) which transduces signals across the plasma membrane (Fong *et al.*, 1986) and folds in a propeller-like structure (Neer & Smith, 1996; Smith *et al.*, 1999). All WD-repeat proteins in eukaryotes seem to play a regulatory role and are involved in diverse functions mediated through protein–protein interactions (Neer *et al.*, 1994). Some interact with kinases to activate or inactivate and target them to their physiological sub-

strates (Ron *et al.*, 1994, 1995; Kolman & Egelhoff, 1997; Kwak *et al.*, 1997; Datta *et al.*, 1998; Bhalerao *et al.*, 1999). There have been only a few reports where a single polypeptide carrying a WD-repeat domain also possesses a functional domain for an enzyme activity. These cases include MHCK (myosin heavy chain kinase) A and MHCK B reported in *Dictyostelium* (Futey *et al.*, 1995; Claney *et al.*, 1997), SPA1 (suppressor of phyA-105) in *Arabidopsis* (Hoecker *et al.*, 1999). MHCK A, MHCK B and SPA1 have a protein kinase domain in the amino-terminal region whilst the WD-repeat domain is located in the carboxy-terminal region.

The *pkwA* gene of *Thermomonospora curvata* CCM3352 (Janda *et al.*, 1996) was the first reported example of a gene encoding a WD-repeat protein in prokaryotes. The DNA sequence indicated that it encodes a protein with seven tandem repeats of 31 amino acids, having the characteristic dipeptides GH (Gly-His) and WD (Trp-Asp). There is also a putative Ser/Thr-type kinase domain at its amino terminus, which represents the first case of a eukaryotic-type

Abbreviations: DIG, digoxigenin; MBP, maltose-binding protein; WD, Trp-Asp; WDA, WD domain of PkwA.

Ser/Thr kinase (for a review, see Zhang, 1996) with a WD repeat in actinomycetes. Interestingly, Ser/Thr-type kinases found in *Streptomyces* spp., for instance AfsK from *Streptomyces coelicolor* A3(2) (Matsumoto *et al.*, 1994), and Pkg3, Pkg4 (Vomastek *et al.*, 1998) and Pkg2 (Nadvornik *et al.*, 1999) from '*Streptomyces graticolor*' show a conserved region of seven tandem repeats of 11–12 amino acids with similarity to the tryptophan-docking motif at their carboxy terminus, which could also fold into a propeller-like structure (Vomastek *et al.*, 1998; Nadvornik *et al.*, 1999).

In the last three years, several genes encoding WD-repeat proteins have been discovered in the genus *Streptomyces*, such as *wdpA* from *S. coelicolor* A3(2) (AJ131817) and *wlA* from *Streptomyces lincolnensis* RIA 1246 (GenBank accession no. AF116463) (both cloned, sequenced and expressed in our laboratory; unpublished data). In addition, two genes (AL078635, AL021411) have been detected during the *S. coelicolor* A3(2) sequencing project. A recently found gene (AL136500) encodes a hypothetical protein very similar to PkwA. Putative WD-repeat-encoding genes in some other prokaryotes such as *Synechocystis* PCC6803 (Q55563), *Thermotoga maritima* (AE001822), *Myxococcus xanthus* (AF162663), *Deinococcus radiodurans* (AE000513) and *Chlorobium tepidum* have also been reported. However, all of these data are just nucleotide sequences; there is no direct evidence for the expression of WD-repeat proteins in any prokaryotes and hence their role in these organisms remains unknown. Nevertheless, the fact that a number of WD-repeat-protein-encoding genes is present in prokaryotes suggests that this ancient regulatory protein family can no longer be regarded as confined to eukaryotes. Since it is known that these proteins are involved in diverse functions, their very presence in prokaryotes may indicate novel regulatory systems.

In this paper we demonstrate the expression of PkwA, a WD-repeat protein, in the thermophilic actinomycete *T. curvata* CCM3352. We show that the expression of PkwA is limited to exponential phase of spore-derived growth and that the WD domain of PkwA can undergo phosphorylation in the presence of membrane fractions of *T. curvata*.

METHODS

Overexpression and purification of the carboxy-terminal part of PkwA. DNA manipulations were carried out according to the standard procedures described by Sambrook *et al.* (1989). *Escherichia coli* NM522 (Promega) was used as a recipient strain in all cases. Plasmid pMPT2 [pTZ19R (USB) carrying 3.2 kb *SalI* fragment of *T. curvata* chromosomal DNA] (Janda *et al.*, 1996) (Fig. 1) with the *pkwA* and *pkwR* genes was shortened using the restriction enzyme *SmaI*. The resulting plasmid was named pMPT2-S (Fig. 1). This carries only a fragment of the *pkwA* gene encoding the WD repeats plus 65 amino acid residues from the spacer region. This fragment was recloned in the pMAL-c2 expression system (New England Biolabs) using restriction enzymes *EcoRI* and *HindIII*. The fusion between the gene encoding maltose-binding protein (MBP) and the WD-repeat-encoding part of

Fig. 1. Plasmid constructs used in overexpression experiments. Newly introduced restriction sites are shown in bold and underlined.

pkwA was confirmed by sequencing. Overexpression and purification of the fusion protein on an amylose affinity matrix were performed according to a standard protocol (New England Biolabs). The fusion protein was digested with Factor Xa (New England Biolabs) and the two proteins [MBP (42 kDa) and the carboxy-terminal part of PkwA (45.5 kDa)] were separated on an amylose affinity matrix (New England Biolabs) and Sephacryl S-200 HR (Amersham Pharmacia Biotech) into two fractions according to the suppliers' protocols. The fraction with the carboxy-terminal part of PkwA was used for immunization.

mAb preparation. Two female BALB/c mice were immunized intraperitoneally at 14 d intervals with three doses (40 µg each) of the carboxy-terminal part of the PkwA protein (45.5 kDa) electroblotted on a nitrocellulose disc. The disc was homogenized and injected into the mice. Fourteen days after the third dose and four days before fusion, mice were given an intravenous booster dose of 120 µg protein. Fusion with a Sp2/mL-6 plasmacytoma cell line (Harris *et al.*, 1992), hybridoma cloning and production of ascetic fluid were performed according to the standard protocol (Galfre *et al.*, 1977). mAbs were screened using ELISA and Western blotting.

Preparing constructs for PkwA and WDA expression in *E. coli*.

A plasmid that expresses full-length PkwA (residues 1–742) in *E. coli* with a hexa-His-tag at the carboxy terminus was constructed. An *EcoRI* site at the start of *pkwA* was generated in pMPT2 by PCR and the resulting construct was named pMPT2-E (Fig. 1). The suitable restriction sites *XhoI* and *HindIII* were also generated and the termination codon was removed from the 3' end of *pkwA* in pMPT2-E by PCR. The 2.2 kb *EcoRI*–*XhoI* fragment carrying *pkwA* was then subcloned in pET24a (+) (Novagen) and named pMPT2-EX (Fig. 1). In this construct there are an extra 14 codons from the vector at the N terminus of PkwA.

A plasmid that expresses a His-tag derivative of the WD domain of PkwA (WDA) was also constructed. The *EcoRI* site was generated at the 3300 position in *pkwA* (GenBank accession no. AF115313) by PCR using pMPT2-E (Fig. 1) as template. The amplified fragment (~1.0 kb) was digested with *EcoRI*/*HindIII*, subcloned in pET28b (+) (Novagen) and named pMPT2-E2 (Fig. 1). This construct contains an extra 36 codons from the vector at the N terminus of the WD domain, which includes a hexa-His-tag and a T7-tag.

Expression and purification of PkwA and WDA. Both pMPT2-EX and pMPT2-E2 constructs were individually transformed into *E. coli* strain BL21 (DE3) (Novagen). Transformants were grown at 37 °C in LB medium containing 50 µg kanamycin ml⁻¹ until the optical density at 600 nm reached 0.6. Cells were induced with 1 mM (final concentration) IPTG for 3 h and then placed on ice for 5 min; they were then harvested by centrifugation at 13000 g (BiofugeA; Heraeus Sepatech). Cells were disrupted by sonication and native protein purification of both PkwA and WDA His-tag derivatives was performed on a metal affinity matrix according to the standard protocols supplied with the Talon metal affinity resin (Clontech).

Maintenance and growth curve of *T. curvata* CCM3352. *T. curvata* strain CCM3352 was obtained from the Czechoslovak Culture Collection (Brno, Czech Republic) and maintained on SPTC agar medium as described by Janda *et al.* (1997). Cultivation and growth measurements of *T. curvata* in mineral salt-vitamin minimal medium (MM-medium) was performed as described by Petricek *et al.* (1989).

Detection of PkwA in *T. curvata* CCM3352. To examine PkwA expression in *T. curvata*, spores stored in 20% (v/v) glycerol at –70 °C were inoculated (10⁸ spores in 100 ml medium) into MM-medium (Petricek *et al.*, 1989) and incubated on an orbital shaker (Gallenkamp) (200 r.p.m.) at 52 °C in a 500 ml Erlenmeyer flask. Samples were collected at 6 h intervals. Pellicles were harvested by centrifugation (35000 g, 15 min) (SS34, RT5C Sorvall; Dupont), washed once with ice-cold X-buffer (20 mM Tris/HCl pH 7.5 containing 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF) and stored frozen at –20 °C. The samples were resuspended in ice-cold X-buffer (~50 mg in 0.5 ml) and disrupted by ultrasonic treatment (Labsonic 2000; USA). Each cycle consisted of sonication for 1 min followed by cooling for 2 min. These two steps were repeated until the pellicles were completely disrupted. The whole sonication process was carried out on ice. The lysate was centrifuged and supernatant was separated. The crude extract was boiled for 3 min with SDS-PAGE loading buffer and the proteins were separated on 0.1% SDS/10% acrylamide PAGE gels (Laemmli, 1970). The gel was electroblotted onto Hybond C nitrocellulose membrane (Amersham) using Tris/glycine/methanol buffer (pH 8.0) and 150 mA constant current for 2.5 h. PkwA was probed using anti-PkwA mAb 3G2 (1:100) and the signal was developed by horse-radish-peroxidase-labelled anti-mouse antibody (1:1000) (Sevac) as

described in the standard ECL (enhanced chemiluminescence) protocol manual (Amersham).

Total RNA extraction from *T. curvata*. Total RNA from 18, 24, 30, 36 and 48 h spore-derived cultures of *T. curvata* was prepared as described by Petricek *et al.* (1992). The integrity of RNA was checked on agarose electrophoresis containing 2.2 M formaldehyde.

Preparation of digoxigenin (DIG)-labelled RNA probe by *in vitro* transcription. The 1.4 kb *EcoRI*–*BamHI* fragment (pMPT2-E, Fig. 1) from *pkwA* was subcloned in pGEM-4Z (Promega) and the resulting plasmid was named pGEMEB. pGEMEB was linearized by *SmaI*, separated by electrophoresis, isolated from the agarose gel and used for *in vitro* transcription. The reaction was performed according to the standard procedure supplied with the DIG RNA Labelling Kit (SP6/T7) (Roche Molecular Biochemicals) using 1 µg linearized pGEMEB. The 145 bp *BamHI*–*SmaI* fragment of *pkwA* was transcribed in the opposite direction using the T7 promoter and T7 RNA polymerase.

Northern hybridization. Samples of total RNA (~25 µg) were denatured, separated by 2.2 M formaldehyde-agarose gel electrophoresis and vacuum-blotted onto Amersham Hybond N+ membranes following the procedure of Bormann *et al.* (1992). RNA–RNA hybridization and disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo-[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD)-based chemiluminescent detection were performed with the DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim), following the standard procedure recommended by the suppliers.

cDNA synthesis and PCR amplification. cDNA was synthesized in 20 µl of a reaction mixture using a sequence-specific primer WD-Rev2 (5' AGG GTT GTG TGT TCT TC 3') annealing at nucleotides 4150–4166 of *pkwA* (GenBank accession no. AF115313) and MMLV reverse transcriptase (Amersham Pharmacia Biotech) according to the standard procedure described in the *PCR Application Manual* (Boehringer Mannheim). A 5 µl aliquot from the first-strand cDNA synthesis mixture was subjected to 30 cycles of amplification by PCR. In addition to amplification buffer and template cDNA, the amplification mixture contained (in a final volume of 100 µl) 2.5 U *Taq* polymerase (Promega), 1.5 mM MgCl₂, 250 µM of each deoxynucleotide triphosphate and 50 pmol forward primer WD-For1 (5' AAC ACG CCG TCC TCA AA 3') annealing at nucleotides 3779–3795 of *pkwA*. Reverse primer WD-Rev2 was added to a final concentration in the reaction mixture of 50 pmol.

Assessing the effect of the WD domain on Pkg2. *E. coli* strain BL21 (DE3) harbouring pEX2, which encodes Pkg2, was grown and induced by a method similar to that described for PkwA and WDA. The induced *E. coli* cells from 1 ml culture were harvested, washed three times in ice-cold phosphorylation buffer (50 mM Tris/HCl pH 7.5 containing 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA and 10 mM β-mercaptoethanol), resuspended in 150 µl of the same buffer and disrupted by sonication. The lysate was centrifuged (18000 g) at 4 °C for 10 min. The supernatant was collected and used as the source of Pkg2. Partially purified His-tag derivatives of both PkwA and WDA were transferred in ice-cold phosphorylation buffer using microcon-30 (Amicon). PkwA, WDA and Pkg2 were subjected to autophosphorylation as controls. The reaction was carried out at 37 °C for 10 min after mixing 5 µCi (1.85 × 10⁵ Bq) [γ -³²P]ATP (Amersham Pharmacia Biotech). To assess the effect of the WD domain, PkwA (~1.0 µg) and WDA (~3.0 and ~5.0 µg) were separately mixed with Pkg2 and subjected to phosphorylation.

All the above reactions were terminated by adding SDS-PAGE loading buffer. After electrophoresis, gels were soaked in boiling 16% trichloroacetic acid (Mannai & Cozzone, 1982), dried under vacuum and exposed to an image processor (Fuji film). The signal was developed with Fuji-BAS-5000 (Fuji film).

WDA phosphorylation in *T. curvata*. Twenty-four-hour spore-derived pellicles of *T. curvata* (~50 mg) were harvested and washed three times in ice-cold phosphorylation buffer (500 µl each time), resuspended in 500 µl of the same buffer and disrupted by sonication as described above. The lysate was ultracentrifuged (100000 g) at 4 °C for 60 min. The supernatant (cytoplasmic fraction) was collected separately. The sediments containing membrane fractions were washed three times in ice-cold phosphorylation buffer (500 µl each time) and resuspended in 100 µl of the same buffer. Both cytoplasmic fraction and membrane fractions were subjected to auto-phosphorylation as negative controls. To assess WDA phosphorylation, ~2.0 µg purified WDA was mixed separately with 5 µl cytoplasmic fraction and 5 µl membrane fractions of *T. curvata*, and phosphorylation was carried out by mixing with 5 µCi (1.85×10^5 Bq) [γ - 32 P]ATP (Amersham Pharmacia Biotech). Various conditions were used for both control and test samples, such as different cations, different temperatures and different times of incubation. Termination of reaction, electrophoresis, gel treatment, drying and signal development were performed as described above.

RESULTS

Preparation of mAbs

To follow the expression of PkwA in *T. curvata*, we generated a specific mAb against the WD domain of PkwA. It was desirable for the mAb to react specifically with the WD-repeat domain of the protein rather than with the kinase domain. Hence, the 3' end of the gene carrying the WD-repeat region and part of the spacer region (Fig. 1) was subcloned into the expression vector pMAL-c2 to produce a fusion protein with MBP. This protein was overexpressed in *E. coli* NM522 and purified on an amylose affinity matrix (Fig. 2a, lanes 1, 2, 3). After digesting purified MBP-WD with Factor Xa, the WD-repeat part of the fusion protein could be separated from MBP (Fig. 2a, lane 4) and purified (Fig. 2a, lane 5). This protein was used to generate mAbs. A number of mAbs from several clones strongly reacted with the fused MBP-WD-repeat protein (Fig. 2b, lanes 1). But, with the exception of 3G2 and 2H7 (Fig. 2b, lanes 3), they also cross-reacted with purified MBP alone (Fig. 2b, lanes 2). Since only mAb 3G2 reacted with the purified His-tag derivative of PkwA (Fig. 2c, lane 1), it was used for further experiments.

PkwA expressed as a His-tag derivative

The purpose of expressing PkwA in *E. coli* was to produce larger amounts of protein for its characterization, and to use it as a substrate for phosphorylation. Its His-tag derivative was intended to facilitate purification. Hence, *E. coli* strain BL21 (DE3) harbouring pMPT2-EX (Fig. 1) was subjected to induction under the control of the T7 promoter. No IPTG-responsive PkwA band could be seen in the SDS-

Fig. 2. (a) Coomassie-blue-stained gel showing expression and purification of the WD domain of PkwA. Lanes 1 and 2, uninduced (lane 1) and IPTG-induced (lane 2) *E. coli* NM522 crude cell extracts harbouring pMAL-c2 with the WD-encoding fragment. Overexpressed ~90 kDa MBP-WD fusion protein is visible in lane 2. Lane 3, amylose affinity-purified MBP-WD. After digestion with Factor Xa, MBP and WD domain were separated by amylose affinity and gel filtration. Lane 4, purified MBP at ~42 kDa; lane 5, purified WD domain at ~45.5 kDa. (b) Western blots showing the specificity of mAbs. Lanes: 1, MBP-WD fusion protein; 2, purified MBP; 3, purified WD domain. 3G2, 2H7, 3E1, 2Cd and 4A7 represent several mAb clones, each reacting with MBP-WD. Only 3G2 and 2H7 react with purified WD domain, whereas 3E1, 2Cd and 4A7 cross-react with MBP. (c) Western blot showing attempts to detect recombinant PkwA expressed in *E. coli* with 3G2 (lane 1) and 2H7 (lane 2). A protein of ~100 kDa and some low-molecular-mass bands were detected by 3G2, but not by 2H7.

PAGE gel stained with Coomassie blue (Fig. 3a, lane 2). However, when the Western blot of the gel was probed with the mAb 3G2, a strong positive signal at an apparent molecular mass of ~100 kDa was detected in

Fig. 3. Overexpression of PkwA His-tag derivative. (a) PkwA overexpression after induction is not visible on SDS-PAGE gels. Lanes 1 and 2, uninduced (lane 1) and IPTG-induced (lane 2) *E. coli* BL-21 (DE3) crude cell extract harbouring pMPT2-EX (no IPTG-responsive band in SDS-PAGE gel). Corresponding Western blot (lane 3, uninduced; lane 4, induced) and mAb 3G2 detection show expression of PkwA at ~100 kDa. (b) Western blot showing mAb 3G2 detection of PkwA in *T. curvata* and its overexpression in *E. coli* BL-21 (DE3). Lanes: 1 and 2, IPTG-induced (lane 1) and uninduced (lane 2) *E. coli* BL-21 (DE3) crude cell extract harbouring pMPT2-EX (overexpressed PkwA at ~100 kDa); 3–7, *T. curvata* crude extract from spore-derived cultures after 18, 24, 30, 36 and 48 h of growth, respectively; 8, empty; 9–11, *T. curvata* crude extract after 18, 24 and 30 h of growth, respectively, from cultures inoculated with vegetative mycelium. The same amount of total protein was applied in each lane. ~100 kDa PkwA is observed in 18, 24, 30 and 36 h samples but almost disappears after 48 h of spore-derived growth. No PkwA was observed at any stage of growth initiated by vegetative mycelium. *T. curvata* PkwA (lane 3) and recombinant PkwA (lane 1) have similar mobility.

the lysate from the induced culture (Fig. 3a, lane 4) but not in the uninduced culture sample (Fig. 3a, lane 3). There were also weaker bands of lower molecular mass. The observed molecular mass of recombinant PkwA is somewhat higher than that deduced (~80 kDa) from the amino acid sequence (742 residues) of the *pkwA* gene (Janda *et al.*, 1996).

PkwA is expressed in *T. curvata* CCM3352

Crude protein extracts of *T. curvata* samples harvested at different times of growth after spore germination were separated by SDS-PAGE and used for Western blotting. A strong band with an apparent molecular mass of about 100 kDa (Fig. 3b, lanes 3–6) was repeatedly detected by mAb 3G2 after 18, 24, 30 and 36 h, while there was active growth. The signal became weaker at 48 h (lane 7), which was about 8 h after the dry weight of the culture had stopped increasing. The slow migration of PkwA from *T. curvata* on SDS-PAGE gels was however very much in agreement with the expressed recombinant PkwA in *E. coli* (Fig. 3b, lanes 3 and 1). Interestingly, no PkwA expression was observed at any stage of growth (18, 24 or 30 h) when vegetative mycelium that had grown for 30 h was used as the inoculum (Fig. 3b, lanes 9–11).

Fig. 4. Northern hybridization and RT-PCR show the presence of *pkwA* transcript. (a) Specificity of the DIG-labelled RNA probe. Lanes: 1, linearized pGEMEB; 2, linearized pGEM-4Z as a negative control. DIG-labelled RNA probe specific to *pkwA* hybridizes with linearized pGEMEB but not with pGEM-4Z. (b) Northern hybridization. Lanes 1–5, total RNA samples extracted after 18, 24, 30, 36 and 48 h of growth, respectively. The DIG-labelled RNA probe hybridized with the *pkwA* transcript of ~2.4 kb in total RNA extracted after 18, 24 and 30 h of growth. (c) PCR amplification from cDNA of a ~210 bp product. Lanes: M, size marker (12000–100 bp); 1, 3, 5, 7 and 9, controls without MMLV reverse transcriptase (total RNA samples extracted after 18, 24, 30, 36 and 48 h of growth, respectively, subjected to cDNA synthesis followed by PCR); 2, 4, 6, 8 and 10, total RNA samples extracted after 18, 24, 30, 36 and 48 h of growth, respectively, subjected to cDNA synthesis with MMLV reverse transcriptase. A ~210 bp PCR-amplified product is visible in all cDNAs synthesized from total RNA samples, except those extracted after 48 h of growth and controls.

Northern analysis and RT-PCR further confirm PkwA expression

To further analyse *pkwA* expression, a *pkwA*-specific DIG-labelled RNA probe, generated by *in vitro* transcription, was used in Northern analysis. Prior to hybridization, the specificity of the probe for *pkwA* was determined against linearized pGEMEB by DNA–RNA hybridization (Fig. 4a, lane 1). After 18 and 24 h growth, RNA from *T. curvata* showed a strong signal at approximately 2.4 kb, decreasing after 30 h growth (Fig.

Fig. 5. Overexpression of WDA as a His-tag derivative and its phosphorylation analysis. (a) Overexpression of WDA. Lanes 1 and 2, uninduced (lane 1) and IPTG-induced (lane 2) *E. coli* BL-21 (DE3) crude cell extract harbouring pMPT2-E2, which encodes WDA as a His-T7-tag derivative. An IPTG-responsive band is visible at ~39 kDa in induced crude cell extract analysed on SDS-PAGE gels. Lanes 3 and 4, corresponding Western blot and mAb 3G2 detection (lane 3, uninduced; lane 4, induced) confirm overexpression. (b) Pkg2 phosphorylates WDA. Lanes 1, 2, 3, 7 and 8, controls for phosphorylation analysis. Lanes 1 and 2, induced *E. coli* BL21 (DE3) crude cell extracts harbouring pET28b (+) mixed with purified PkwA (~1.0 µg) and WDA (~3.0 µg), respectively. Neither were phosphorylated in *E. coli* crude extract. Lane 3, induced *E. coli* BL21 (DE3) crude cell extract harbouring pEX2 (expresses Pkg2) show active autophosphorylated Pkg2. Lanes 7 and 8, ~1.0 µg purified PkwA and ~3.0 µg purified WDA, respectively, do not show autophosphorylation. Lanes 4–6, induced *E. coli* BL21 (DE3) crude cell extract harbouring pEX2 with purified ~1.0 µg PkwA (lane 4), and with purified ~3.0 µg (lane 5) and ~5.0 µg (lane 6) WDA. The same amount of Pkg2 was used in lanes 3–6. Strongly phosphorylated WDA with molecular mass ~39 kDa is visible in lanes 5 and 6. A significant decrease in Pkg2 autophosphorylation is visible in lanes 3–6. (c) Corresponding Western blots of lanes 3, 4 and 5 shown in (b) probed with polyclonal antibody anti-Pkg2 (lane 1) and anti-WD mAb 3G2 (lanes 2 and 3). Lanes 1, 2 and 3 verify the presence of Pkg2, PkwA and WDA, respectively. (d) Membrane fractions from *T. curvata* phosphorylate WDA. Lanes 1 and 3, negative controls for phosphorylation show membrane fractions and cytoplasmic fraction from *T. curvata*, respectively. Neither shows a phosphorylated band of the molecular mass of WDA. Lanes 2 and 4, purified WDA (~2.0 µg) mixed with the membrane fractions and cytoplasmic fraction from *T. curvata*, respectively. A phosphorylated band of ~39 kDa in lane 2 represents WDA. A faint band of similar molecular mass is also visible in lane 4.

4b, lanes 1–3). These data confirm that *pkwA* was expressed 18, 24 and 30 h after inoculation, but that there was little or no observed expression after 36 and 48 h growth (Fig. 4b, lanes 4 and 5).

To further confirm this, a sequence-specific primer, WD-Rev2, was designed and cDNA for RT-PCR was prepared. As shown in Fig. 4(c), PCR amplification of the cDNA prepared from the *T. curvata* total RNA samples of the 18 h (lane 2), 24 h (lane 4) and 30 h (lane 6) gave a PCR product of the expected size of 210 bp, amplified by WD-For1 and WD-Rev2 primers. In contrast to Northern analysis, a weak band could also be seen in the 36 h sample (lane 8). No band appeared in the 48 h sample (lane 10).

Pkg2 phosphorylates WDA

To examine the effect of the WD domain of PkwA on protein Ser/Thr kinase, the Pkg2 from '*S. granaticolor*' (Nadvornik *et al.*, 1999) was used as a positive control. WDA, the WD domain of PkwA, was overexpressed as a His-tag derivative in *E. coli* BL21 (DE3), verified on Western blot probed by mAb 3G2 (Fig. 5a, lanes 2 and 4) and partially purified using a Talon metal affinity matrix. In a control phosphorylation reaction, PkwA and WDA did not undergo autophosphorylation (Fig. 5b, lanes 7 and 8) whereas the active Pkg2 was positively phosphorylated (Fig. 5b, lane 3). When Pkg2 was subjected to phosphorylation in the presence of partially purified PkwA or WDA, a decrease in the auto-phosphorylation ability of Pkg2 was reproducibly observed (Fig. 5b, lanes 4–6). At the same time, Pkg2 phosphorylated WDA (Fig. 5b, lanes 5 and 6). To rule out the possibility that this phosphorylation of WDA was due to an *E. coli* endogenous kinase, WDA was incubated under the same conditions with *E. coli* BL21 (DE3) crude extract. However, the WDA was not phosphorylated (lane 2), thus proving that it is Pkg2 that specifically phosphorylates WDA. Fig. 5(c), showing a Western blot of lanes 3, 4 and 5 from Fig. 5(b) run in parallel in the SDS-PAGE gels, demonstrates the presence of corresponding proteins in the reaction mixtures.

Membrane fractions of *T. curvata* phosphorylate WDA

Encouraged by the phosphorylation of WDA by Pkg2, we looked for a similar putative kinase in *T. curvata*. Partially purified WDA was mixed separately with the cytoplasmic fraction and the membrane fractions obtained from the spore-derived culture of *T. curvata*. Under a variety of conditions, phosphorylation assays were carried out. In the presence of Mn^{2+} (10 mM) at 37 °C for 10 min with 5 μCi (1.85×10^5 Bq) [γ - ^{32}P]ATP, a positive band of ~39 kDa (the molecular size of WDA) was observed in the membrane fractions of *T. curvata* incubated with WDA (Fig. 5d, lane 2). A faint band of similar size was also observed in the cytoplasmic fraction incubated with WDA (Fig. 5d, lane 4). Neither the cytoplasmic fraction (lane 3) nor the membrane

fraction controls (lane 1) showed any phosphorylated band at the position of WDA.

Phosphorylation reactions were also performed in the presence of Ca^{2+} (10 mM) or Mg^{2+} (10 mM), alone or in combination; however, the results were negative (not shown).

DISCUSSION

In an earlier study, we reported the presence of the *pkwA* gene encoding a WD-repeat protein in *T. curvata* (Janda *et al.*, 1996). It was the first of its kind found in a prokaryote. Thereafter, it was important to demonstrate that PkwA is indeed expressed. For this purpose, a mAb, 3G2, was generated against the WD-repeat domain of PkwA.

During the examination of the expression of PkwA on Western blots, it was found that 3G2 reacted with the exponential-growth-phase extracts of *T. curvata* (Fig. 3b, lanes 3–7) derived from spores but interestingly, no PkwA could be detected at any stage of growth initiated from vegetative mycelium (Fig. 3b, lanes 9–11). This indicates possible involvement of PkwA in some crucial spore-derived developmental stage of the growth of *T. curvata*. The pattern of PkwA expression in spore-derived growth was further confirmed by Northern hybridization. Northern analysis showed the presence of *pkwA* transcript (Fig. 4b) during early exponential growth, which was in good agreement with the results obtained by Western blot analysis. It also suggested that the transcription of *pkwA* could be over by the end of the early exponential phase. However, the results obtained by the more sensitive RT-PCR method (Fig. 4c) enabled us to detect the presence of transcript during up to 36 h of growth (to late exponential phase).

The apparent molecular mass of PkwA in SDS-PAGE gels was approximately 100 kDa instead of the 80 kDa predicted from the amino acid sequence. However, when PkwA was expressed as a His-tag derivative in *E. coli*, it also yielded a band of about 100 kDa on Western blots (Fig. 3a, b). Such unexpectedly slow migration of protein on SDS-PAGE gels has also been reported in other cases such as Pkg2 (Nadvornik *et al.*, 1999). Expression of recombinant PkwA could not be seen on a stained gel, but immunodetection using mAb 3G2 on Western blots confirmed expression (Fig. 3a) similar to other reported cases (Matsumoto *et al.*, 1994; Urabe & Ogawara, 1995).

The deduced amino acid sequence of *pkwA* (Janda *et al.*, 1996) suggests the presence of a putative Ser/Thr-type kinase domain at the amino terminus of PkwA. However, we failed to detect autophosphorylation under various conditions. Pkg3 reported from '*S. granaticolor*' represents another such case (Vomastek *et al.*, 1998). According to the primary structure, PkwA and Pkg3 belong to the family of so-called RD protein kinases, which requires phosphorylation of an activation loop between domains VII and VIII for their activation (Johnson *et al.*, 1996). It is very likely that the kinase

domain of PkwA could be phosphorylated and activated by some other kinase that could be functioning together with PkwA in *T. curvata*.

The WD-repeat proteins are also known for their interaction with kinases (Ron *et al.*, 1994, 1995; Kolman & Egelhoff, 1997; Kwak *et al.*, 1997; Datta *et al.*, 1998) and some can even exert an inhibitory effect (Bhalerao *et al.*, 1999). A sequence alignment of the WD domain of PkwA showed significant sequence similarity with other such WD-repeat proteins. We therefore wanted to examine the possible effect of WDA on active Pkg2, a protein Ser/Thr kinase from '*S. granaticolor*' (Nadvornik *et al.*, 1999). The aim of an attempt to autophosphorylate Pkg2 with partially purified PkwA or WDA was to prove that whatever the effect may be, it is due to the WD domain. The results showed that Pkg2 autophosphorylation was lower in the presence of PkwA or its WD domain, WDA (Fig. 5b, lanes 4, 5 and 6). However, at the same time Pkg2 phosphorylated WDA (Fig. 5b, lanes 5 and 6). It is very likely that the WDA could exert a dual effect, influencing Pkg2 autophosphorylation and at the same time acting as a substrate for Pkg2. This scenario would be similar to Skp2, p21^{Cip1/WAF1} or p27^{Kip1} that inhibit CDK (cyclin-dependent kinase) activity and at the same time act as substrates and undergo phosphorylation (Yam *et al.*, 1999; Zhang *et al.*, 1994; Sheaff *et al.*, 1997). However, the WDA phosphorylation by Pkg2 encouraged us to look for the putative kinase in *T. curvata*. The cytoplasmic fraction and the membrane fractions obtained from 24-h-old mycelia of *T. curvata* were used as a possible source. When testing under various conditions in the presence of partially purified WDA, the membrane fractions of *T. curvata* showed a phosphorylated band of the molecular size of WDA (Fig. 5d, lane 2). However, the control reaction with the membrane fractions alone was negative (Fig. 5d, lane 1), implying that the phosphorylated band in the test was indeed WDA. This result was obtained in the presence of Mn²⁺; the reactions carried out in the presence of other cations such as Ca²⁺ or Mg²⁺, alone or in combination, were negative. This implies that the putative kinase phosphorylating WDA prefers Mn²⁺, as in the case of Pkn2 protein kinase of *Myxococcus xanthus* or MbK of *Mycobacterium tuberculosis* (Udo *et al.*, 1997; Peirs *et al.*, 1997). We also observed a faint band in the test reaction carried out with the cytoplasmic fraction and WDA (Fig. 5d, lane 4). This could be due to contamination of the membrane fractions caused by disruption of the mycelia by sonication. According to these results, if the putative kinase phosphorylating WDA in *T. curvata* is membrane-bound then it would be strikingly similar to Pkg2, as the latter is also membrane-spanning (Nadvornik *et al.*, 1999). It is tempting to speculate that PkwA could be a member of a signalling pathway occurring in early exponential stages of growth derived from spores of *T. curvata*. The phosphorylation of WDA and the presence of a putative protein Ser/Thr kinase domain in PkwA further strengthen this hypothesis. It is also very likely that the WD domain of PkwA

could serve as a molecular switch in a phospho-relay signal transduction mechanism in *T. curvata*.

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