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 substrates (Ron et al., 1994, 1995; Kolman & Egelhoff, 1997; Kwak et al., 1997; Datta et al., 1998; Bhalarao 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.
In this paper we demonstrate the expression of PkwA, a regulatory systems. Their very presence in prokaryotes may indicate novel functions; these proteins are involved in diverse functions, regarded as confined to eukaryotes. Since it is known that this ancient regulatory protein family can no longer be encoded genes present in prokaryotes suggests that nevertheless, the fact that a number of WD-repeat-protein-encoding genes have been detected during the 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.

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 pklwA and pkwR genes was shortened using the restriction enzyme SphI. 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 acids 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 pkwA was confirmed by sequencing. Overexpression and purification of the fusion protein on an amylase 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 amylase 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/myL-6 plasmocytoma cell line (Harris et al., 1992), hybridoma cloning and production of ascitic 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 XboI 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–XboI fragment carrying *pkwA* was then subcloned in pET24a (+) (Novagen) and named pMPT2-EX (Fig. 1). In this construct there are extra 14 codons from the vector at the N terminus of PkwA.

A plasmid that expresses a His-tag derivative of the WD repeat protein in *Thermomonospora curvata* 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 Smal, 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–Smal 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 Bornmann et al. (1992). RNA–RNA hybridization and disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2-′(5′-chloro)tricyclo[3.3.1.1°]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 × 10⁶ Bq) [γ³²P]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 × 10⁶ Bq) [γ-³²P]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-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
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).

**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, 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.
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 pET28b (+) with purified 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 × 10^9 Bq) $[^{32}P]$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 WDA 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 Mn\(^{2+}\); the reactions carried out in the presence of other cations such as Ca\(^{2+}\) or Mg\(^{2+}\), alone or in combination, were negative. This implies that the putative kinase phosphorylating WDA prefers Mn\(^{2+}\), as in the case of Pkn2 protein kinase of *Mycobacterium 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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