Characterization and properties of protein kinase C from the filamentous fungus *Trichoderma reesei*

Thomas LENDENFELD and Christian P. KUBICEK[1]

Abteilung für Mikrobielle Biochemie, Institut für Biochemische Technologie und Mikrobiologie, TU Wien, Getreidemarkt 9-172.5, A-1060 Wien, Austria

The *Trichoderma reesei pkc1* gene encodes a fungal homologue of the protein kinase C (PKC) family. Using antibodies directed against the nt-sequence-deduced pseudosubstrate domain for identification, Pkc1p was purified by dye–ligand affinity chromatography and Mono Q anion-exchange chromatography. Both the denatured as well as the native enzyme showed an M_r of 116–118 kDa, indicating that Pkc1p is a monomer. The enzyme phosphorylates the mutated (A → S) pseudosubstrate peptide and myelin basic protein, but not histone. Replacing three of the five basic amino acids around the serine acceptor residue resulted in a 25-fold increase in the K_m. Pkc1p activity was stimulated by phospholipids, but this stimulation was counteracted by micromolar concentrations of Ca^{2+}. Three proteins (85, 48 and 45 kDa) were identified as preferred endogenous substrates of Pkc1p *in vitro*. The enzyme was capable of autophosphorylation, and neither phosphorylation nor dephosphorylation *in vitro* affected the activity of the enzyme. A 116 kDa protein of *T. reesei* was demonstrated to bind to the N-terminal C2-region of Pkc1p *in vitro*. These data define Pkc1p as a unique member of the PKC family.

INTRODUCTION

Ca^{2+}/phospholipid-dependent serine/threonine protein kinases (protein kinase C, PKC), acting as transmitters and amplifiers in signal transduction, are key components in the phosphoinositide cascade, which evokes a wide variety of responses in various cell types, such as cell proliferation, regulation of gene expression, membrane transport and organization of the cytoskeleton. Molecular cloning and sequence analysis of a family of at least eight PKC-encoding genes from mammalian sources have revealed that PKC molecules constitute a protein family, which – based on their structure and enzymic properties – can be classified into two major groups, conventional PKC and novel PKC [1]. The latter also differ by their Ca^{2+}-insensitivity. Homologous genes have been also be cloned from such non-mammalian sources as *Drosophila melanogaster* [2,3] and *Caenorhabditis elegans* [4]. With respect to fungi, genes predicted to encode PKCs have been isolated from yeasts (e.g. *Saccharomyces cerevisiae* [5], *Schizosaccharomyces pombe* [6,7] and *Candida albicans* [8]) and the filamentous fungi *Trichoderma reesei* and *Aspergillus niger* [9]. A common property of all the fungal PKC proteins is the presence of a 150 amino acid-long extended N-terminus whose function is not known. The protein encoded by the *S. cerevisiae PKC1* gene has been investigated in some detail, and shown to be independent of Ca^{2+} and phospholipids, but regulated by (auto)phosphorylation [10,11]. Consistent data have been obtained for the enzyme from *C. albicans* [8]. Preliminary data on the *pkc1*-encoded proteins of *Sc. pombe* and *T. reesei* indicate that their activity is phospholipid-dependent, but Ca^{2+}-independent [6,9]. In contrast, partially purified PKC preparations from *S. cerevisiae* and *Neurospora crassa* were stimulated by Ca^{2+} as well as phospholipids [12,13], whereas a partially purified PKC from the basidiomycete *Pleurotus ostreatus* was not influenced by either component [14], but was activated by phorbol esters.

We have previously presented preliminary evidence that the activity of Pkc1p, obtained by overexpression of *pkc1* in a baculovirus, shows stimulation by phospholipids but not by Ca^{2+} [9], which would be in accordance with the properties of the enzyme from *Sc. pombe*. In the present paper, we have purified the native *pkc1* gene product from *T. reesei* using an antibody raised against its pseudosubstrate domain to monitor specifically Pkc1p. The highly purified preparation that was obtained was used to investigate the biochemical properties of *T. reesei* Pkc1p.

MATERIALS AND METHODS

Organism and cultivation

T. reesei QM 9414 was used throughout these studies. Its maintenance has been described recently [9]. To obtain mycelia for enzyme purification, the fungus was grown in 1 l wide-mouthed Erlenmeyer flasks containing 250 ml of medium [10 g/l glucose/1 g/l peptone/1.4 g/l $(NH_4)_2SO_4$/10.5 g/l KH_2PO_4/0.3 g/l $MgSO_4.7H_2O$/7.6 g/l sodium citrate/2.5 mg/l $FeSO_4.7H_2O$/0.8 mg/l $MnSO_4.H_2O$/0.75 mg/l $ZnSO_4.7H_2O$/1 mg/l $CaCl_2.2H_2O$; pH adjusted to 5.0 with Na_2HPO_4] on a rotary shaker (250 r.p.m.) at 30 °C for 24 h.

Preparation of cell-free extract

Mycelia were harvested, washed with cold tap water and then distilled water, and excess water was removed by blotting between sheets of filter paper. The mycelia were then suspended in ice-cold buffer A [20 mM Hepes (pH 6.9)/1 mM EDTA/20 % (w/v) glycerol/10 μM leupeptin/1 μM pepstatin/1 μM PMSF/1 mM dithiothreitol (DTT)] at a ratio of 10 ml of buffer per g of mycelium, and homogenized in a Bead Beater (Biospec Products, Bartelsville, OK, U.S.A.) using an equal volume of glass beads (0.5 mm diameter). Homogenization was carried out by six treatments of 20 s, interrupted by cooling-down periods of 2 min. The homogenate was then centrifuged (5 min, 5000 rpm; Beckmann JA-20 fixed-angle rotor). The protein concentration

Abbreviations used: PKC, protein kinase C; Pkc1p, protein encoded by the *T. reesei pkc1* gene; DTT, dithiothreitol; GS, glutathione-S; GST, glutathione-S-transferase; MARCKS, myristoylated alanine-rich kinase C substrate; PMA, phorbol ester.

[1] To whom correspondence should be addressed.

of the supernatant was determined and adjusted to 10–15 mg of protein/ml by dilution with buffer A, and was either used immediately for purification of PKC1 or frozen in liquid nitrogen and stored at −70 °C until use.

Purification of PKC1

All subsequent steps were carried out at a temperature between 0 and 4 °C: 70 ml of cell-free extract was applied to a column (26 × 130 mm) of Procion MX-GR Yellow [15], covalently bound to Sepharose CL-6B (Pharmacia–LKB, Bromma, Sweden), previously equilibrated with 2 vols of buffer A. Flow rate was 2 ml/min. The column was washed with buffer A until no more protein (A_{280}) eluted from the column. Pkc1p was then eluted by applying a linear gradient 0–0.6 M NaCl in a total volume 150 ml of buffer A. Fractions of 4 ml were collected and assayed for PKC by immunoblotting (see below) and activity assay. Fractions containing high amounts of Pkc1p were pooled, diluted with buffer A to decrease the NaCl concentration below 0.2 M and applied on to a MonoQ HR 5/5 (Pharmacia–LKB) ion-exchange column, equilibrated in buffer A and operating at a flow rate of 1 ml/min. The column was again rinsed with buffer A until no more protein was eluted, and Pkc1p was then eluted by a stepwise gradient of NaCl in buffer A (0–0.25 M in a total volume of 12 ml buffer A; 3 ml buffer A plus 0.25 M NaCl; 0.25–0.40 M NaCl in a total volume of 42 ml buffer A; and 15 ml buffer A plus 1 M NaCl). Fractions of 1 ml were collected and assayed for the presence of Pkc1p as described above. Pkc1p was eluted at between 0.3 and 0.35 M NaCl. Fractions containing the highest amounts of Pkc1p were frozen individually in liquid nitrogen and stored at −70 °C until use.

Gel-permeation chromatography

Gel-permeation chromatography was carried out on a Superose 12 HR 16/60 (Pharmacia–LKB) connected to a FPLC system, at a flow rate of 1 ml/min. The column was equilibrated in buffer B [which is identical to buffer A except that the glycerol concentration had been reduced to 5 % (w/v)]. To determine the M_r of Pkc1p, the column was calibrated with a Bio–Rad molecular-weight calibration kit. Fractions of 4 ml were collected and assayed for presence of Pkc1p as described above.

Assay of PKC activity

PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into substrates in a total volume of 20 μl, as described previously [9]. The concentration of the PKC1 in all assays (except when assaying individual fractions during purification) was 20 μg/ml. To assay the effect of phorbol ester PMA on PKC activity, a stock solution of 1 mg/ml in DMSO (Sigma, St. Louis, MO, U.S.A.) was diluted with double-distilled water to give the desired concentration in the total volume of the standard assay (20 μl). To assay the effect of phospholipids, the lyophilized preparations (all obtained from Sigma) were dissolved in DMSO, aliquoted to quantities of 1 μmol, lyophilized again and stored at −20 °C until use. For use in the assay, the preparations were suspended in 1 ml of 1 × kinase buffer [9] and sonicated on ice twice for 5 s each.

Autophosphorylation and protein phosphorylation

For autophosphorylation and phosphorylation of endogenous substrates, the PKC assay as described above was used with a total radioactivity of 18.5 kBq [γ-^{32}P]ATP (111 mBq/mol) per assay, omitting the substrate peptide. For autophosphorylation, a final concentration of 40–50 μg/ml of purified Pkc1p were used. For phosphorylation of endogenous proteins, 20–30 μg of purified Pkc1p, and 100–250 μg of cell-free extract were used per ml of assay. In both cases, the reaction was stopped after 20 min by adding 10 μl 2 × electrophoresis loading buffer [60 mM Tris/HCl (pH 6.8)/3 % (w/v) SDS/10 % (v/v) β-mercaptoethanol/ 0.005 % (w/v) Bromophenol Blue], boiled for 10 min, and then applied to SDS/PAGE using 10 % polyacrylamide gels. Proteins were blotted on to nitrocellulose and exposed overnight at −70 °C.

Construction and expression of glutathione-S-transferase (GST)::C2 fusion protein

Standard protocols were used for all molecular biological techniques reported [16,17]. All clones obtained were tested for correct insertion of the fragment by restriction analysis.

A 751 bp fragment of the C2 region [9] was amplified from *pkc1* cDNA by PCR using primers C2 (5′-ACCTGGTGCCA-GAATTCTGCGGCA-3′) and $C2_{rev}$ (5′-GAAGTTGAAGTG-CTCGAGACCAAT-3′), annealing at nt +1697/+1720 and +2425/+2448 in the *pkc1* sequence, respectively. Both primers were modified in a single base with respect to the original sequence to incorporate restriction sites (underlined) for *Eco*RI (C2) and *Xho*I ($C2_{rev}$). The modified codons resulted in a change from D to E in both sequences, which is unlikely to affect the properties of the encoded polypeptide. The resulting 751 bp PCR amplicon was partially digested with *Eco*RI/*Xho*I, separated on a 1 % agarose gel, excised, extracted using a Quiaex II extraction kit (Quiagen, Hilden, Germany) and ligated into pGEX-4T-1 (Pharmacia–LKB), previously cut with *Eco*RI/*Xho*I, to yield pTLC2 containing a fusion of the *T. reesei* Pkc1C2-domain-encoding gene fragment with the *Escherichia coli GST* (GST-encoding) gene.

For the expression of the gene fusion, *E. coli* LC137 cells (Pharmacia) were transformed with pTLC2. A single colony was transferred into 4 ml of Luria broth/ampicillin medium and incubated on a rotary shaker (250 r.p.m.) at 37 °C for 6 h. The whole culture was then transferred to a 100 ml Erlenmeyer flask containing 50 ml of Luria broth/ampicillin medium and further incubated under the same conditions for 16 h. This culture was finally transferred into a 1 l Erlenmeyer flask containing 450 ml medium and incubated until an A_{600} of 0.6. Then a final concentration of 0.1 mM isopropyl β-D-thiogalactoside was added to induce the expression of the fusion protein and incubation continued for a further 3–5 h. All subsequent steps were carried out between 0 and 4 °C: *E. coli* were harvested by centrifugation (10 min, 10000 ***g***), and the bacterial pellet resuspended in 20 ml of lysis buffer [20 mM Hepes (pH 6.9)/1 mM EDTA/10 % (w/v) glycerol/10 μM leupeptin/1 μM pepstatin/1 μM PMSF/1 mM DTT/2 % (w/v) Triton-X-100]. The cells were broken by sonification (2 min, twice), the homogenate was centrifuged (10 min, 20000 ***g***) and the supernatant filtered through a 0.45 μm filter.

Identification of proteins binding to Pkc1pC2 domain

The C2–GST fusion protein was bound to glutathione-S-Sepharose (GS-Sepharose) by the standard protocol recommended by the manufacturers (Pharmacia–LKB). For identification of proteins binding to the C2 domain, 10 ml of a cell-free extract of *T. reesei* (containing 10 mg of protein/ml) were applied to the column at a flow rate of 2 ml/min, and the eluent reapplied twice. The column was then washed with buffer A until no more

protein was eluted. Bound proteins were eluted by a stepwise gradient of NaCl in buffer A (i.e. 3 ml of 50 mM, 3 ml of 200 mM and 3 ml of 1 M NaCl). Control experiments, with (i) *T. reesei* cell-free extracts and GS-Sepharose, (ii) the overexpressed GST protein (without C2 domain) bound to GS-Sepharose and *T. reesei* extract, and (iii) the C2::GST fusion protein without *T. reesei* cell-free extract, were carried out in the same way. Fractions were analyzed by SDS/PAGE on 10 % polyacrylamide gels.

RESULTS

Purification of Pkc1p

To aid purification of Pkc1p from *T. reesei*, we originally attempted to overexpress *pkc1* by introduction of multiple gene copies into the fungus. However, while this strategy led to a 3–4-fold increase in Pkc1p activity, the corresponding transformants grew only very slowly and the final biomass concentration was less than 10 % of the wild-type. Microscopic examination showed that the hyphae were irregularly shaped, swollen and laterally branching (data not shown).

Pkc1p was therefore purified from *T. reesei* wild-type mycelia. Upon homogenization, roughly 40 % of the total Pkc1p activity was detected in the cell debris (data not shown) indicating that part of the enzyme was particulate. Purification was achieved by a combination of dye–ligand affinity chromatography and FPLC anion-exchange chromatography, which yielded a final specific activity of 60000–65000 units/mg. The degree of purification and recovery of activity could not be determined because of the presence of Pkc1p inhibitors in the cell-free extract whose removal was apparent by a 260-fold increase in the total activity after the dye–ligand affinity chromatography (data not shown). From a total of 1 g of cell-free extract protein, 0.4 mg of purified Pkc1p were finally obtained. SDS/PAGE of the fraction containing the highest specific activity revealed a major protein of 114 ± 6 kDa, which was still accompanied by a few other faint bands (Figure 1, lane 2). A polyclonal antiserum, raised against the pseudosubstrate domain of Pkc1p [9], specifically recognized this band in Western blots (Figure 1B), thereby proving that this band represents Pkc1p. Gel filtration of purified Pkc1p determined a relative M_r of 117 ± 10 kDa, which is in good agreement with the value obtained by SDS/PAGE, thus indicating that the enzyme is a monomer. The enzyme was fairly stable at this stage. However, it was strongly susceptible to proteolysis during earlier steps: immunostaining of partially purified samples, which were kept at 4 °C, showed that Pkc1p was first truncated to a 105–110 kDa protein which later appeared to be processed to a 47–50 kDa form (Figure 1B).

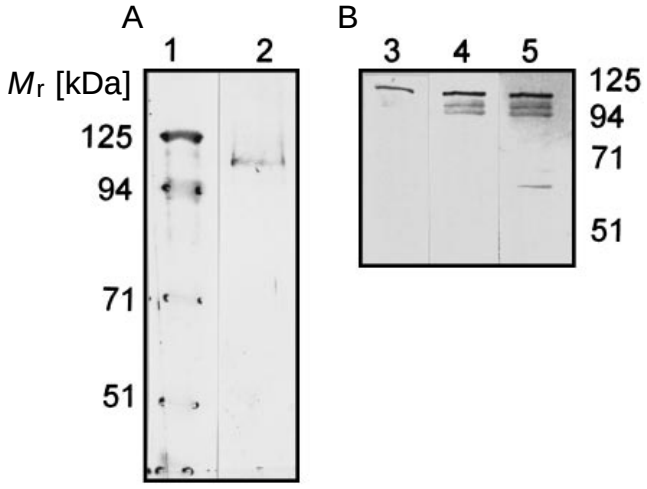

Figure 1 SDS/PAGE of purified *T. reesei* Pkc1p and its proteolysis during storage

(**A**) 20 μl of the fraction with highest purity were applied to SDS/PAGE, blotted onto nitrocellulose and stained with Ponceau Red. (1) Marker proteins, marked with a pencil (M_r given on the left in kDa), (2) purified Pkc1p. (**B**) Proteolysis of Pkc1p during storage at 4 °C: (3) 18 h; (4) 36 h; (5) 14 days. 10 μl of purified sample were subjected to SDS/PAGE, blotted onto nitrocellulose and immunostained. The relative M_r of prestained marker proteins is given on the right.

Table 1 Relative affinities of Pkc1p for modified pseudosubstrate peptides

Pkc1p was assayed as described in Materials and methods using different derivatives of the PKC1 pseudosubstrate peptide (peptides 1–5). Amino acid residues in bold indicate changes in the modified substrate peptides. Numbers given as $\pm$ indicate the standard deviation ($n = 5$).

Peptide	Sequence	K_m (μM)
Pseudosubstrate	RKGAVRQRKE	–
Peptide 1	RKG**S**VRQRKE	1.8 ± 3
Peptide 2	LG**A**KG**S**VRQ**AA**E	50 ± 10
Peptide 3	LGRKG**S**VRQ**AA**E	3 ± 6
Peptide 4	LG**A**KG**S**VRQRKE	6 ± 3
Peptide 5	LGR**H**G**S**V**I**QRKE	2 ± 7
S. cerevisiae PKC1	LHRHGSIINRKE	9 ± 5

Biochemical properties of Pkc1p

In agreement with the substrate preference of mammalian PKCδ and PKCϵ [18] and PKC1 from yeast [10], Pkc1p had a high affinity for the modified (A → S) pseudosubstrate peptide (K_m = 1.8 μM), and high activity was also obtained with myelin basic protein. In contrast, histone H1 and BSA were only poor substrates (data not shown). The reaction was optimal at a pH of 6.9 and a Mg^{2+} concentration of 8 mM. The K_m for ATP was 80 μM. To define the requirements for Pkc1p substrate-binding in detail, several modified pseudosubstrate peptides were synthesized, and relative affinities for Pkc1p were determined (Table 1). When three of the five both N- and C-terminal basic amino acids flanking the acceptor serine were removed, the K_m of the enzyme was increased 25-fold. Removing the two C-terminal basic amino acids, or the substitution of K by H, had only a slight effect on the activity. Also, removing one of the two N-terminal basic amino acids increased the K_m only two-fold.

Pkc1p activity was markedly inhibited by the general kinase inhibitor staurosporin and the specific PKC inhibitor bisindolylmaleimide, with IC_{50} values of 3×10^{-11} and 6×10^{-7} M, respectively (Figure 2). The K_i for bisindolylmaleimide was 1.4 nM.

To identify potential intracellular substrates of Pkc1p, the purified enzyme was used to phosphorylate proteins from crude cell-free extracts, or partially purified fractions thereof (Figure 3). SDS/PAGE fluorography identified five proteins in cell-free extracts of *T. reesei* that were phosphorylated by Pkc1p, among which three (exhibiting M_rs of 85, 48 and 45 kDa) were predominant. Controls, using bisindolylmaleimide to inhibit Pkc1p activity, abolished the incorporation of labelled phosphate into these two bands, hence proving their phosphorylation by Pkc1p. It is therefore possible that these three proteins are major cellular targets of Pkc1p phosphorylation.

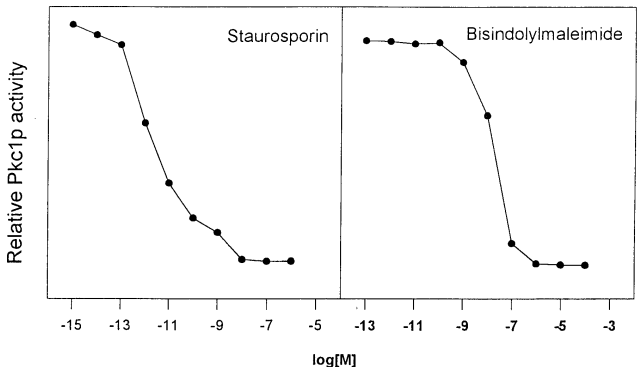

Figure 2 Inhibition of Pkc1p by staurosporine and bisindolylmaleimide

Pkc1p was assayed as described under Materials and methods with the pseudosubstrate peptide as substrate in the presence of increasing concentrations of staurosporine. The IC_{50}s were determined for staurosporine and bisindolylmaleimide as 3×10^{-11} and 6×10^{-7} M, respectively.

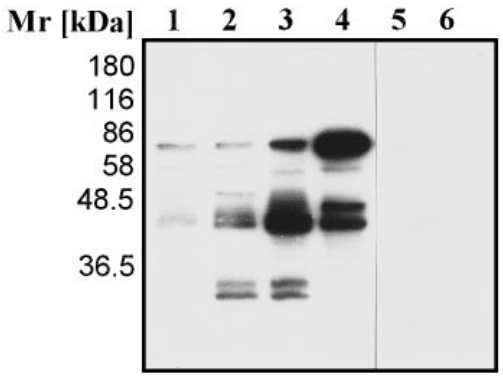

Figure 3 Phosphorylation of proteins present in cell-free extracts by Pkc1p

The experiment was carried out as described in Materials and methods and the phosphorylated proteins identified by SDS/PAGE and autoradiography. (1) Cell-free extract (1 mg of protein/ml), no addition of purified Pkc1p; (2) buffer eluate from Procion MX-GR Yellow containing low Pkc1p activity; (3) fraction eluting from Procion MX-GR Yellow containing highest Pkc1p activity; (4) as (1), but with addition of 10 μl of purified Pkc1p; (5) 10 μl of purified Pkc1p alone; (6) as (4), but with addition of 10^{-6} M bisindolylmaleimide. Numbers on the left indicate the respective position of M_r markers.

Table 2 Stimulation of Pkc1p by phospholipids and phorbol ester

Values are given as percentage of the control without addition of effector; numbers given in brackets indicate the standard deviation ($n = 4$). ND, not determinable.

Phospholipid	V_{max} (%)	K_a (μM)
Phosphatidylserine	374 (±30)	3.4 (±1.8)
Phosphatidylethanolamine	428 (±45)	5.6 (±2.0)
Cardiolipin	389 (±65)	87 (±18)
Phosphatidylinositol	115 (±20)	ND
Phosphatidylcholine	76 (±15)	ND
PMA	281 (±32)	1.4 (±0.6)

Table 3 Effect of Ca^{2+} ions on the activation of Pkc1p by phospholipids

The activity obtained in the presence of 50 μM concentrations of either phospholipid was taken to be 100%.

$[Ca^{2+}]$ (μM)	Phosphatidylserine (%)	Phosphatidylethanolamine (%)
–	100 (±15)	100 (±17)
1	180 (±25)	275 (±30)
10	180 (±23)	238 (±26)
17	142 (±20)	180 (±15)
170	110 (±14)	120 (±18)

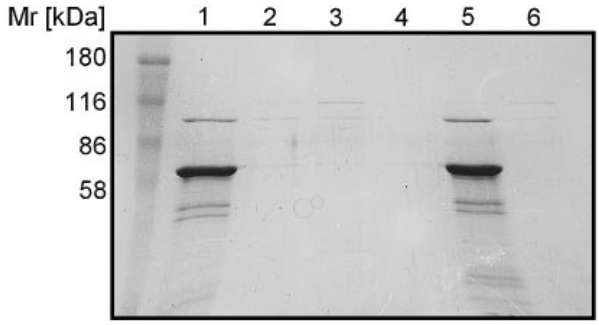

Figure 4 Purification of a protein binding to the 'C2'-domain of *T. reesei* Pkc1p

Purification was carried out as described in Materials and methods and various fractions analyzed by SDS/PAGE. (1) Control (GST–C2 fusion bound to GS-Sepharose); (2) the eluate with 50 mM NaCl; (3) the eluate with 200 mM NaCl; (4) the eluate with 1.0 M NaCl; (5) the eluate with 10 mM glutathione after the 1.0 M NaCl elution; (6) as (3), but from a separate experiment.

Activation of PKC1 by PMA and cofactors

As reported earlier, PMA activated Pkc1p by approximately 200% [9]. Similar results were obtained with diolein (data not shown). The enzyme was also activated by phosphatidylserine and phosphatidylethanolamine, whereas cardiolipin showed activation only at concentrations > 50 μM, and PtdIns showed no effect. Phosphatidylcholine alone seems to have a slight inhibitory effect on PKC1 activity (Table 2).

Ca^{2+} alone had no effect on Pkc1p. However, when Ca^{2+} and individual phospholipids were tested simultaneously at different concentrations, Ca^{2+} counteracted the activation by phosphatidylserine and phosphatidylethanolamine (Table 3), whereas Ca^{2+} inhibited the activity of Pkc1p in the absence of phospholipids only at concentrations > 0.5 mM (data not shown). No precipitation of phospholipids by Ca^{2+} was observed under the conditions employed.

Autophosphorylation of Pkc1p

PKC from all organisms studied so far is activated by autophosphorylation. To determine the capability of Pkc1p to autophosphorylate *in vitro*, the purified protein was incubated in the presence of phosphatidylethanolamine with radiolabelled ATP without external substrate. At the end of the incubation period, the catalytic fragment of protein phosphatase 2A was added to dephosphorylate Pkc1p. After further incubation, the protein phosphatase 2A inhibitor okadaic acid was added and

the enzyme reincubated with radiolabelled ATP. Labelling of Pkc1p could not be detected on autoradiographs (see above, Figure 3), yet spotting the putatively phosphorylated protein on filter paper with trichloroacetic acid revealed the incorporation of a low level of ^{32}P over the control (1896 c.p.m.), which was somewhat lower upon dephosphorylation (1313 c.p.m.), and correspondingly increased again after blockage of the phosphatase by okadaic acid (2366 c.p.m.). The significance of these low values is uncertain; however, the activity of Pkc1p remained practically unchanged (96–82 % [$\pm$12–13 %, S.D.] of the control; $n = 4$) during all these treatments (data not shown).

Binding of cellular proteins by the C2 domain

PKC proteins characteristically contain three distinguishable domains, C1, C3 and C4 [9]. The C2 domain of fungal PKCs has remarkably little similarity to that of mammalian PKC proteins, yet contains a characteristic conservation of PXXP sequences typical of SH3 domains [19]. SH3 domains have been found to serve various functions, including protein–protein interactions. To test this, and to identify possible proteins binding to the *T. reesei* C2 domain, a cDNA fragment encoding the C2 domain was amplified by PCR, overexpressed in *E. coli* as a 3′-fusion to the *GST* gene and specifically bound to GS-Sepharose. When cell-free extracts of *T. reesei* were loaded on this column, a protein of 116 kDa was specifically bound and eluted only by 200 mM NaCl (Figure 4). Binding of the 116 kDa protein was specifically dependent on the *T. reesei* C2 domain, as: (i) elution of the bound GST fusion with 10 mM glutathione yielded essentially the same protein pattern as the fusion-protein preparation originally bound to the column; and (ii) the 116 kDa protein was not obtained when cell-free extracts were directly loaded on GS-Sepharose, or on GS-Sepharose binding GST not fused to C2. It should be noted that the 116 kDa protein was not stained by the antibody against Pkc1p (data not shown).

DISCUSSION

Based on the high homology between *T. reesei* Pkc1p and mammalian and yeast PKCs, particularly in the regions that are unique to PKC, we previously suggested that *pkc1* encodes a member of the PKC family [9]. Several of the data obtained here show that *T. reesei* Pkc1p is most closely related to the Ca^{2+}-independent PKC family among the PKC family, rather than the PKC-related protein family [1]. The enzymic properties of *T. reesei* Pkc1p are consistent with the preliminary data reported for PKC1 of *Sc. pombe* [6], yet quite different from the purified PKCs of *S. cerevisiae* [10,11] and *C. albicans* [8]. As *T. reesei* Pkc1p is the first PKC purified from 'non-yeast' fungi, the deviating properties of the enzymes from *N. crassa* [12] and *P. ostreatus* [14] should be reassessed.

PKCs are known to be phosphoproteins. In accordance with PKCs from mammalian tissues and yeast [5,20], Pkc1p contains several target sequences for autophosphorylation [9]. Unfortunately, autophosphorylation experiments *in vitro* yielded a ^{32}P-incorporation into the purified protein which was too low to detect the phosphoprotein by autoradiography. However, a measurement of the radioactivity introduced into protein and its decrease by protein phosphatase 2A treatment suggests that the enzyme was phosphorylated *in vitro*. The fact that the dephosphorylation occurs without loss of activity coincides with the behaviour of *S. cerevisiae* Pkc1p [10,11], but contrasts with the behaviour of mammalian PKCs, which become inactivated [21]. Antonsson et al. [10] have suggested that the phosphate residues in yeast PKC that are important for activity may not be susceptible to dephosphorylation, and that these may be due to phosphorylation by an 'activating PKC kinase' [21]. Our data would be compatible with such an explanation as well.

Several proteins have been proposed to be Pkc1p substrates *in vivo* (see [22]). We showed that several proteins present in cell-free extracts of *T. reesei* are specifically phosphorylated by Pkc1p and that three proteins, 85, 48 and 45 kDa in size, are most prominent. Phosphorylation of an 85 kDa protein was also observed in *N. crassa* PKC [23], and a related experiment with cell-free extracts from *Sc. pombe* revealed major substrate proteins with apparent M_rs of 100, 40, 33 and 26 kDa [6]. While the identity of these proteins is not yet known, it is intriguing that one of the major substrates of mammalian PKCs, the myristoylated alanine-rich kinase C substrate (MARCKS; [24]), migrates at a position compatible with approx. 80 kDa in SDS/PAGE [25]. MARCKS-like proteins have not yet been reported in fungi or yeast.

Phospholipids such as phosphatidylserine are obligatory for a wide range of PKCs to obtain full activity [26–28]. The *S. cerevisiae* and *C. albicans* PKC proteins behave differently: PKC1p of *S. cerevisiae* is activated by neither phospholipids nor phorbol esters [8,10]. On the other hand, Favre and Turian [12] purified a PKC homologue of *N. crassa*, which was dependent on Ca^{2+} and phospholipid but not activated by phorbol esters, a similar behaviour to that determined for *S. cerevisiae* PKC by Ogita et al. [13]. In contrast, Magae [14] purified a PKC of *P. ostreatus* with an M_r of approx. 68 kDa, which was activated by phosphatidylserine only in presence of low levels of Ca^{2+} and was activated by phorbol esters. It is important to note that the M_r of the *N. crassa* PKC had not been given, and that that quoted in [13] and [14] was considerably less than that of the PKCs described for yeasts and *T. reesei*, and it is hence unclear whether the enzyme properties described by these authors reflect those of the native enzyme. The activation of Pkc1p of *T. reesei* by phospholipids and TPA but not by Ca^{2+} ions is in accordance with preliminary data on PKC1 from *Sc. pombe* [6]. The lack of Ca^{2+} requirement and the only modest degree of activation by phospholipids and PMA (3–4-fold, in comparison with approximately 10-fold in PKCα; see [29]) is also in accordance with the absence of a C2 domain and the absence of an N in the second cysteine-rich repeat of Pkc1p as discussed previously [9]. Interestingly, activation by phospholipids was inversely dependent on the Ca^{2+} concentration, as optimal activation by phosphatidylserine or phosphatidylethanolamine occurred in the complete absence of Ca^{2+}, and a marked reduction in activation was observed between 1 and 10 μM Ca^{2+}, which would be comparable with the concentration range of Ca^{2+} occurring in fungi *in vivo* [30]. Ca^{2+} concentrations exceeding 100 μM practically abolished activation by phospholipids. Inhibition by Ca^{2+} ions has been reported of the PKC1 activity in cell-free extracts of *Sc. pombe* PKC1 [6] and of purified PKCϵ [31], yet at higher concentrations only.

Proteins described as interacting with PKC without being substrates have been described in mammalian systems and yeast [32–36]. In these cases, binding was suggested to occur by the pseudosubstrate domain. Our data show that the C2 region of *T. reesei* Pkc1p, which contains a disproportionally high number of PxxP motifs – conserved among all fungal PKCs – that are typical for SH3 domains [19], is capable of specifically binding an as-yet-unknown protein from *T. reesei* cell-free extracts.

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