Protein kinases in the entomopathogenic fungus *Metarhizium anisopliae*

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(Received 17 October 1989; revised 21 February 1990; accepted 23 March 1990)

Cyclic AMP (cAMP)- and Ca^{2+} -dependent protein kinase activities of the fungus *Metarhizium anisopliae* were sought in extracts of ungerminated conidia, germinating conidia and mycelium, as well as in purified plasma membranes from mycelium. Ungerminated conidia contained a Ca^{2+} /calmodulin-dependent protein kinase capable of phosphorylating multiple endogenous proteins and involved in triggering germination. cAMP-dependent protein kinase activity was not detected in ungerminated conidia in spite of the presence of cAMP in these conidia and the pre-germination synthesis of two cAMP-binding proteins. Most phosphorylation events in crude mycelial extracts were Ca^{2+} -dependent but H-series inhibitors of cAMP-dependent kinases selectively repressed phosphorylation of a 27 kDa protein. Plasma membranes from mycelium contained a Ca^{2+} -independent but H-8-sensitive protein kinase with multiple endogenous substrates for phosphorylation. 8-Azido[^{32}P]cAMP bound selectively to a 52 kDa membrane protein indicative of a single cAMP-binding protein. Plasma membranes contained a phosphatase which rapidly (< 1 min) and selectively dephosphorylated a polypeptide of 15.5 kDa, thus being suited to cause rapid and reversible changes in membrane function. Membranes also contained an adenylate cyclase apparently involved in transmembrane signalling reactions, since mechanical or chemical treatments which stress the fungus caused rapid increases in intracellular levels of cAMP. Reconstitution experiments with a homogenate from a *crisp-1* mutant of *Neurospora crassa* suggested G-protein regulation of *Metarhizium* plasmalemma adenylate cyclase.

Introduction

Conidia of the entomopathogenic fungus *Metarhizium anisopliae* attach, swell and form a germ-tube upon contact with an insect host. The germling then develops an appressorium from which an infection peg eventually penetrates the cuticle. A recent study has demonstrated that while water and a variety of nutrients allow germination, more specific thigmotropic and chemical stimuli are required for appressorium formation (St Leger *et al.*, 1989a). The pathway of signal transduction has not been defined.

The biochemical basis of transduction of extracellular signals has been extensively studied in animal cells, where signalling systems use second messengers such as cAMP and Ca^{2+} to mediate protein phosphorylation (Cohen, 1982; Nestler *et al.*, 1984). Considerable indirect evidence exists in the literature indicating that cAMP has a parallel role as a second messenger in fungi (Pall,

1981; Hoch & Staples, 1984; Marques *et al.*, 1989). The importance of Ca^{2+} in fungal growth and development has been known for many years. Also, the occurrence of CaM in fungi has been well documented (Muthukumar *et al.*, 1987) and studies with CaM inhibitors suggest a role for CaM in growth and oosporogenesis of *Lagenidium giganteum* (Kerwin & Washino, 1986). Only recently, however, with the isolation of Ca^{2+} - and calmodulin (CaM)-dependent protein kinases from *Neurospora crassa* (Van Tuinen *et al.*, 1984) and *Aspergillus nidulans* (Bartelt *et al.*, 1988) has more direct evidence been produced to suggest that Ca^{2+} has a regulatory role in protein phosphorylation in fungi. In a previous study using intact conidia, we reported decreased incorporation of [^{32}P]orthophosphate and [^{35}S]methionine into proteins in response to Ca^{2+} depletion or CaM antagonists (St Leger *et al.*, 1989b). The intact cell phosphorylation approach has many advantages (reviewed by Garrison, 1983) but also the disadvantages that a prolonged incubation with [^{32}P]orthophosphate is required to achieve a constant specific activity of [γ - ^{32}P]ATP, and that the drugs we used may have had a marked effect on the permeability of the plasma membranes to [^{32}P]orthophosphate or on

Abbreviations: CaM, calmodulin; Con A, concanavalin A; Gpp(NH)p, guanylyl 5'-imidodiphosphate; SDB(A), Sabouraud dextrose broth (agar); TPA, 12-O-tetradecanoylphorbol-13-acetate; YEM, yeast extract medium.

the turnover of ATP. At least one CaM antagonist, *N*-(6-aminohexyl)-1-naphthalenesulphonamide (W-5), is not membrane permeable in *M. anisopliae* (unpublished result) and therefore inhibition of nutrient uptake or germination may have been due, at least in part, to membrane stabilization, a side effect of CaM antagonists which may or may not be mediated by CaM inhibition (Landry *et al.*, 1981).

In this report we have directly assayed kinase activity in broken cell preparations to confirm that CaM-dependent phosphorylation plays a significant role in the Ca^{2+} responses associated with germination. Since germination and germ-tube growth are Ca^{2+} -sensitive processes (St Leger *et al.*, 1989b), it is tempting to speculate that transient increases in the concentration of intracellular messengers (e.g. Ca^{2+} and cAMP) and the activation of protein kinases may be involved in signal transduction between germlings and an inductive surface for appressorial formation. We have used *in vitro* assay techniques to measure the effects of external stimuli on levels of cAMP and cAMP-binding proteins, and to demonstrate Ca^{2+} -dependent kinase activity in germling cytoplasm. The plasma membrane contains several GTP-binding proteins suited to be coupling factors for transmembrane signal transduction (St Leger *et al.*, 1989c). As critical events in signal transduction probably occur at the plasma membrane, we have investigated phosphorylation of membrane proteins. Data are presented on a Ca^{2+} -independent membrane kinase and its rapid phosphorylation of approximately 40 membrane proteins.

Methods

Organisms and growth. The fungal isolate (*Metarhizium anisopliae* ME1), culture media and germination experiments were as described previously (St Leger *et al.*, 1989a, d). The *crisp-1* mutant of *Neurospora crassa* (C-Ex-11-67) was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, USA.

Chemicals. [α - ^{32}P]ATP, [γ - ^{32}P]ATP and ^{125}I -labelled CaM were obtained from NEN. 8-Azido[^{32}P]cAMP was from ICN. H-8 was from Seikagaku America Inc. YEM and SDB were from Difco. Other chemicals and 8-hydroxyethylthio-cAMP-beaded agarose were from Sigma.

Preparation of membranes. Plasma membranes were prepared from mycelium by the β -glucuronidase procedure of Bowman & Bowman (1988) as described previously (St Leger *et al.*, 1989b). The procedure includes centrifugal washes through EGTA to remove bound Ca^{2+} .

Cyclic AMP content. Cultures were sampled using a 10 ml dispenser, vacuum filtered on 2.1 cm Whatman GF/A glassfibre filters and dropped into 5 ml ice-cold 5% (w/v) trichloroacetic acid (TCA). This process took about 5 s. After 10 min mycelia were homogenized (1 min) using a motor-driven (~1100 r.p.m.) Teflon pestle and extracted for a further 30 min at 0 °C. The homogenate was centrifuged (20000 g, 10 min, 2 °C) and TCA removed from the supernatant solution by

extraction with water-saturated diethylether. The ether was removed by heating the solution (100 °C, 1 min) and cAMP measured in 50 μl of the supernatant with the cyclic AMP assay kit (Amersham).

Washed conidia (200 mg) harvested from 10-d-old SDA cultures (St Leger *et al.*, 1989b), acid-washed glass beads (600 mg) (<0.3 mm diam.) and liquid nitrogen were placed in a 50 ml screwtop centrifuge tube and vortexed for five periods of 5 min each, with renewal of liquid nitrogen as needed. Rapidly, before the contents of the tube were thawed, the fractured conidia and glass were suspended in 5 ml ice-cold 5% TCA. All subsequent steps were as described for mycelia.

In control experiments samples of fractured mycelia or conidia treated with phosphodiesterase (beef heart, Sigma) before assay yielded zero values. Addition of cAMP to samples yielded recoveries near 100%.

Photoaffinity labelling with 8-azido[^{32}P]cAMP. Membranes (0.1 mg) were added to a reaction mixture containing (final concentration) sodium acetate buffer (50 mM, pH 4.5), 3-isobutyl-1-methyl xanthine (1 mM), BSA (100 $\mu\text{g ml}^{-1}$), PMSF (50 μM) and 0.2 μM 8-azido[^{32}P]cAMP [specific activity 67.5 Ci mol $^{-1}$ (2.5 TBq mol $^{-1}$)]. After incubating at 4 °C for 1 h in the dark the samples were exposed to UV light for 7 min. The reaction was terminated by a 100-fold dilution with acetate buffer (50 mM, pH 4.5) containing BSA (100 $\mu\text{g ml}^{-1}$) and cAMP (1 mM) and the mixture centrifuged (40 min, 108000 g). The pellet was suspended in Laemmli buffer (Laemmli, 1970) by heating at 100 °C for 3 min and proteins were separated by SDS-PAGE.

Adenylate cyclase assay. The reaction mixture (final volume 0.1 ml) contained 100 mM-PIPES, pH 6, 1 mM-cAMP, 50 mM-KCl, 0.2 mM-3-isobutyl-1-methylxanthine, 5 mM-phosphoenolpyruvate, 0.02 mg pyruvate kinase, 3×10^6 c.p.m. [α - ^{32}P]ATP plus the indicated enzyme fractions (0.05 ml) and additions [0.5 mM-MnCl $_2$, 4 mM-MgCl $_2$ or 10 μM -Gpp(NH)p]. Incubations were at 25 °C for 30 min and [α - ^{32}P]cAMP was purified and assayed according to Salomon *et al.* (1974).

Reconstitution of plasma-membrane adenylate cyclase activity. A crude homogenate of a *crisp-1* (Cr-1) mutant of *Neurospora crassa* was prepared in extraction buffer (5 mM-PIPES, sodium salt, pH 6.9, 1 mM MgCl $_2$, 0.25 mM-EDTA, 1 mM-PMSF, 0.1 mM-leupeptin) by the method of Rosenberg & Pall (1983). *Neurospora* homogenate (4 mg protein ml $^{-1}$) was mixed with an equal volume (total volume 50 μl) of purified *Metarhizium* plasma membrane (1 mg protein ml $^{-1}$) and pre-incubated at 25 °C for 15 min before assaying for adenylate cyclase activity.

***In vitro* phosphorylation of proteins.** Non-differentiated germlings from 20 h yeast extract cultures (St Leger *et al.*, 1989a) were pulverized under liquid nitrogen. Conidia were vortexed under liquid nitrogen with acid-washed glass beads as described above. Proteins from fractured conidia or germlings were extracted in 50 mM-Tris/HCl (pH 7.5) containing CHAPS (10 mM), 2-mercaptoethanol (5 mM), EGTA (0.5 mM), EDTA (0.5 mM) and PMSF (1 mM). Proteins were washed by ultrafiltration (cut-off 5 kDa) in 50 mM-Tris/HCl (pH 7.5) containing 1 mM-PMSF. The reaction mixture for assaying protein phosphorylation (0.1 ml) contained 0.2 mg protein, 50 mM-Tris/HCl (pH 7.5), 5 mM-MgCl $_2$, 0.5 mM-DTT, 1 mM-EGTA, 0–3.0 mM-CaCl $_2$ and 0.5 nmol [γ - ^{32}P]ATP [3000 Ci mmol $^{-1}$ (111 TBq mmol $^{-1}$)]. Membrane proteins were phosphorylated under similar conditions except that the assay buffer was supplemented with 0.25 M-sucrose and in most experiments Triton X-100 (0.1%, w/v) was added to the reaction mixture 15 min before starting the assay (Salimoth & Marme, 1983). Preliminary experiments showed that pre-incubation with Triton X-100 massively enhanced total phosphorylation of membranes without changing the profile of bands. For phosphorylation, the reaction mixture was pre-incubated for 1 min at 30 °C and phosphoryl-

ation was initiated by adding [γ - 32 P]ATP. Incubation was for 3 min at 30 °C and the reaction was terminated by adding electrophoresis sample buffer. Phosphorylated polypeptides were analysed by SDS-PAGE and autoradiography as described previously (St Leger *et al.*, 1989*d*). Unless stated otherwise, acrylamide concentrations of 8 or 15% (w/v) were used to gain maximum resolution for molecular mass estimates of high and low molecular mass polypeptides, respectively.

8-Hydroxyethylthio-cAMP-agarose adsorption. Affinity chromatography was done as described by Marques *et al.* (1989) and by Weber *et al.* (1979) with modifications. Conidia germinating in 0.0125% YEM or YEM supplemented with 2% sucrose were labelled with [35 S]methionine as previously described (St Leger *et al.*, 1989*d*). After 6 h, conidia (3% germination frequency) were collected on a glassfibre disc by vacuum filtration, frozen under liquid nitrogen and lyophilized. Cell extracts were prepared by fracturing conidia as described above and sonicating fragments in 200 mM-MES buffer, pH 6.2, containing 0.13 M-NaF, 20 mM-EDTA, 0.3 mg BSA ml⁻¹ and 1 mM-PMSF. The extracts were centrifuged (2 min in a Beckman microfuge) and the supernatants incubated with 0.5 ml 8-hydroxyethylthio-cAMP-agarose beads for 45 min at 4 °C in a final volume of 2 ml. The agarose beads were then collected by centrifugation and washed five times, 15 min each with 1 ml 10 mM-MES buffer, pH 6.2, containing 0.1 mM-EDTA, 10 mM-2-mercaptoethanol, 2 M-NaCl and 1 mM-5'-AMP to remove non-specifically bound proteins, and washed once for 30 min with 1 ml 5 mM-MES buffer (pH 6.2), containing 1 mM-5'-AMP and 1 mM-cAMP to remove low-affinity binding proteins. Residual high-affinity proteins were eluted by heating the beads at 100 °C for 5 min in the presence of 100 μ l Laemmli buffer. Following centrifugation, eluted proteins were separated by SDS-PAGE.

Miscellaneous. CaM levels were determined with a CaM-specific radioimmunoassay (RIA) (St Leger *et al.*, 1989*b*). Proteins were processed for and subjected to SDS-PAGE and Western blots were done as described previously (St Leger *et al.*, 1989*d*). 125 I-labelled CaM binding was done by an overlay method after transfer to nitrocellulose as described by Hubbard & Klee (1987) except that skimmed milk was used as the blocking agent. Staining of glycoproteins with Con A/ peroxidase was done after transfer to nitrocellulose (Faye & Chrispeels, 1985). Protein concentration was determined by the method of Bradford (1976).

Results

In vitro phosphorylation of proteins in ungerminated conidia

Approximately 21 radiolabelled proteins were detected when a crude preparation of fractured conidia was incubated in the presence of Ca²⁺ and [γ - 32 P]ATP. Only two phosphoproteins [14.4 kDa (Fig. 1) and 96 kDa (calculated from an 8% SDS-polyacrylamide gel, data not shown)] were major components of the conidia as determined by Coomassie blue staining. However, five of the more abundant phosphoproteins [34, 39.5, 46 (Fig. 1), 62 and 83 kDa (from an 8% SDS-polyacrylamide gel, data not shown)] comigrated with Con A/peroxidase stained bands indicating that they might be glycoproteins.

Removal of free Ca²⁺ by inclusion of EGTA slightly

promoted phosphorylation of a low incidence (as determined by Coomassie blue staining) polypeptide (15.2 kDa) but severely repressed phosphorylation of other proteins (Fig. 1). The addition of bovine CaM did not promote phosphorylation over the Ca²⁺-promoted levels or allow phosphorylation in the absence of Ca²⁺. The CaM antagonists, R24571 and trifluoroperazine were used to investigate the role of endogenous CaM. R24571 (Fig. 1) and trifluoroperazine (not shown) almost duplicated the effects of EGTA, reducing phosphorylation events except at 15.2 kDa. The addition of exogenous bovine CaM (15 μ g) to the assay mixture reversed the inhibition caused by R24571 (Fig. 1).

To assess whether other signalling agents might operate in addition to, or substitute for, the Ca²⁺/CaM-promoted phosphorylation, a variety of agents were tested for their ability to influence the phosphorylation profile. The addition to the reaction mixtures of TPA (1–100 μ M; an activator of protein kinase C), cAMP (5–100 μ M), cGMP (5–100 μ M), H-7 [10–100 μ M; the H-series inhibitor with the highest affinity for protein kinase C (Hidaka & Tanaka, 1987)] and H-8 [10–100 μ M; the H-series inhibitor with the highest affinity for cyclic-nucleotide-dependent kinases (Hidaka & Tanaka, 1987)], did not affect phosphorylation of conidial proteins either in the presence or absence of Ca²⁺ (data not shown).

In vitro phosphorylation of proteins extracted from pre-differentiated germlings

Approximately 12 and 32 radiolabelled proteins were detected when proteins from 12 h pre-differentiated germlings were incubated for 1 and 3 min, respectively, in the presence of Ca²⁺ and [γ - 32 P]ATP (Figs 2 and 3). Two phosphoproteins (25.8 and 42 kDa) were major protein components of the extract. Three of the seven more abundant phosphoproteins (30, 33.7 and 42 kDa) co-migrated with glycoproteins (Fig. 2). A Ca²⁺-free medium (+EGTA) promoted phosphorylation of a 27 kDa protein but severely repressed phosphorylation of other proteins (Fig. 3). Identical effects by EGTA in the presence of the phosphatase inhibitors sodium molybdate (5 or 10 mM) and NaF (5 or 10 mM) are indicative of an inhibition by EGTA of Ca²⁺-dependent protein kinase(s) rather than activation of a phosphatase. While cAMP was without effect on total phosphorylation, H-7 and H-8 selectively repressed phosphorylation of the 27 kDa protein (Fig. 3). The addition of TPA, bovine CaM, compound R24571 or cGMP did not affect the profile of protein phosphorylation either in the presence or absence of Ca²⁺ (Fig. 3 or not shown).

Fig. 1. SDS-PAGE analysis of *Metarhizium* conidial proteins. Lane 1, conidial proteins stained with Coomassie blue dye; lane 2, glycoproteins detected by binding of Con A to proteins transferred to nitrocellulose membranes. The other lanes are autoradiographs of conidial proteins phosphorylated in the presence of [γ - 32 P]ATP for 1 min (lane 3); for 3 min in the presence of 0.2 mM- Ca^{2+} (+ Ca^{2+}) (lane 4); in the presence of 2 mM-EGTA ($-\text{Ca}^{2+}$) (lane 5); of 0.2 mM- Ca^{2+} + 5 μM -R24571 (lane 6); of 0.2 mM- Ca^{2+} + 5 μM -R24571 + 15 μg CaM (lane 7); or of 0.2 mM- Ca^{2+} + 20 μM H-8 (lane 8).

In vitro phosphorylation of membrane proteins

Plasma membranes contained both kinase activity and at least 40 endogenous substrates. The predominant 52 kDa band is a major component of the membrane as determined by Coomassie blue staining (Fig. 4). The 52 kDa band and four other phosphoproteins (36, 38, 40 and 41.5 kDa) comigrated with glycoproteins (Fig. 4).

Proteins in the plasma membrane were phosphorylated in Ca^{2+} and CaM-independent reactions (Fig. 4). Although cAMP (1–50 μM) did not increase phosphorylation, millimolar levels of cAMP strongly inhibited total phosphorylation. Also, H-7 and H-8 inhibited the bulk of membrane phosphorylation at 10 μM and 5 μM concen-

trations, respectively. H-8 (5 μM) was sometimes effective at suppressing phosphorylation of the major 52 kDa band; 15 μM was effective on each occasion (five replicates). H-8 was without effect on a minor (as determined by Coomassie blue staining) polypeptide (15.5 kDa) (Fig. 5).

The ^{32}P incorporation observed in Figs 4 and 5 may represent net phosphorylation resulting from protein kinase and phosphatase activity. To demonstrate phosphatase activity membranes were incubated with [γ - ^{32}P]ATP for 1 min and then chased with nonradioactive ATP in the presence of 25 μM -H-8 (Fig. 6). The 15.5 kDa phosphoprotein was rapidly (<1 min) and specifically dephosphorylated indicative of a selective membrane-bound phosphoprotein phosphatase.

Fig. 2. SDS-PAGE analysis of soluble proteins extracted from germlings of *Metarhizium*. Lane 1, germling proteins stained with Coomassie blue dye; lane 2, glycoproteins detected by binding of Con A to proteins transferred to nitrocellulose membranes; lane 3, autoradiograph of germling proteins phosphorylated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 1 min. Arrows indicate phosphoproteins which comigrated with glycoproteins.

8-Azido ^{32}P cAMP labelling of a cAMP-binding protein in plasma membranes

Use of the photoaffinity analogue of cAMP, 8-azido- ^{32}P cAMP, demonstrated one major high-affinity cAMP-binding protein in the plasma membrane accompanied by several additional faint bands on either side. The major band had the same molecular mass

(52 kDa) as the protein which was phosphorylated most intensively with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 7). The 8-azido- ^{32}P cAMP-labelled band represents a specific cAMP-binding protein since adding unlabelled cAMP (0.1 mM) to the reaction mixture prior to irradiation prevented incorporation of radioactivity (Fig. 7), whereas 0.2 mM-cGMP, AMP, ATP or GTP did not prevent labelling (Fig. 7 or data not shown).

Adenylate cyclase activity in *Metarhizium* membranes

Metarhizium membranes had adenylate cyclase activity in assay mixtures containing Mn^{2+} but not in mixtures containing Mg^{2+} (Table 1). Activities were not influenced by the stable GTP analogue Gpp(NH)p. Mg^{2+} -dependent and Gpp(NH)p-sensitive adenylate cyclase activity were observed when *Metarhizium* membranes were reconstituted with a homogenate of Cr-1 *Neurospora crassa* defective in the adenylate cyclase catalytic component but not in a GTP-sensitive regulatory component (Rosenberg & Pall, 1983). Thus, the regulatory component in the Cr-1 extract can impart Mg^{2+} and GTP sensitivity to the catalytic unit in *Metarhizium* plasma membranes.

Quantification of cAMP and demonstration of second messenger target proteins in *Metarhizium*

Using biotinylated CaM we previously demonstrated the presence of CaM-binding proteins in conidia and mycelia (St Leger *et al.*, 1989b). ^{125}I -labelled CaM binding confirmed the presence of CaM-target proteins in the preparations of conidia and mycelia used for phosphorylation assays. The membrane preparation, however, possessed only trace levels of one ^{125}I -labelled CaM-binding protein (Fig. 8). CaM levels in membranes and conidial and mycelial preparations were <0.05 , 2.5 ± 0.31 and $1.8 \pm 0.30 \mu\text{g}$ (mg total protein) $^{-1}$, respectively ($n = 5$ independent measurements).

cAMP levels in extracts from conidia and mycelia were 0.4 ± 0.08 and $1.1 \pm 0.06 \text{ pmol}$ (mg dry wt fungus) $^{-1}$, respectively. Levels of cAMP in plasma membranes were low [about $0.4 \pm 0.02 \text{ pmol}$ (mg dry wt) $^{-1}$] compared with mycelia. Similar levels of cAMP were found in mycelia grown for periods up to 2 h in nutrient-limiting conditions (0.005% YEM) or in nutrient-rich media (YEM supplemented with 2% sucrose). Stressing the fungus, however, by drying mycelia from 30 h SDB cultures for 5 min in a laminar flow cupboard or chilling for 1 min by adding ice-cold media (final temperature 12°C) increased levels of cAMP from 1.1 ± 0.12 to 2.3 ± 0.43 and $3.2 \pm 0.47 \text{ pmol}$ (mg dry wt) $^{-1}$, respectively ($n = 5$ independent measure-

Fig. 3. Cofactor dependence of phosphorylation of soluble proteins extracted from germlings of *Metarhizium*. Proteins were phosphorylated for 3 min with [γ - 32 P]ATP in the presence of: 0.2 mM- Ca^{2+} (+ Ca^{2+}) (lanes 1 and 5); 2 mM-EGTA ($-\text{Ca}^{2+}$) (lane 2); 0.1 mM-TPA (lane 3); 10 mM-NaF + 10 mM-sodium molybdate (lane 4); 0.2 mM- CaCl_2 + 5 μM -H-8 (lane 6); 0.2 mM- CaCl_2 + 15 μM -H-7 (lane 7); 0.2 mM- CaCl_2 + 20 μM -cAMP (lane 8). Proteins were subjected to SDS-PAGE followed by autoradiography.

ments). The elevation of cAMP levels induced by chilling was transitory, normal levels returning within 15 min. Drying, however, produced a more prolonged increase in cAMP [2.8 ± 0.17 pmol (mg dry wt) $^{-1}$ after 15 min]. Adding the membrane-depolarizing agent polymyxin B (50 $\mu\text{l ml}^{-1}$) to 30 h SDB cultures elevated cAMP 2.3-fold [2.5 ± 0.26 pmol (mg dry wt) $^{-1}$, $n = 5$] which declined to normal levels within 15 min.

The synthesis of high-affinity cAMP-binding proteins (16.2 and 36.3 kDa) during germination was demonstrated by SDS-PAGE of labelled conidial extracts absorbed to the affinity substrate 8-hydroxyethylthio-

cAMP-agarose (Fig. 9). The data also show that production of these proteins was constitutive as it occurred irrespective of levels of sucrose in media capable of repressing catabolite-controlled enzymes (St Leger *et al.*, 1988). Stressing the fungus by adding polymyxin B did not affect the profile of labelled cAMP-binding proteins in mycelia (data not shown).

Discussion

We report the occurrence of a Ca^{2+} /CaM-dependent protein kinase capable of multiple phosphoryl-

Fig. 4. SDS-PAGE (12%, w/v, acrylamide) analysis of *Metarhizium* plasma membrane proteins. Lane 1, membrane proteins stained with Coomassie blue dye; lane 2, glycoproteins detected by binding of Con A to proteins transferred to nitrocellulose membranes. The other lanes are autoradiographs of membrane proteins phosphorylated with [γ - 32 P]ATP for 3 min in the presence of: 0.2 mM- Ca^{2+} (+ Ca^{2+}) (lane 3); 2 mM-EGTA ($-\text{Ca}^{2+}$) + 5 μM -R24571 (lane 4); 0.2 mM- Ca^{2+} + 2 mM-cAMP (lane 5); 2 mM-EGTA + 2 mM-cAMP (lane 6); 0.2 mM- Ca^{2+} + 30 μM -cAMP (lane 7); 0.2 mM- Ca^{2+} + 15 μM -H-7 (lane 8); 0.2 mM- Ca^{2+} + 5 μM -H-8 (lane 9).

ation events in ungerminated conidia of *Metarhizium anisopliae*. These results corroborate previous findings from *in vivo* labelling experiments (St Leger *et al.*, 1989*b*), and provide strong evidence that a Ca^{2+} /CaM-dependent kinase serves to modulate the effects of Ca^{2+} on the system which triggers germination. Phosphorylation occurred in the absence of exogenously added CaM but was inhibited by R24571 and trifluoperazine, indicating that endogenous levels of CaM (about 0.3% of total protein; St Leger *et al.*, 1989*b*) are not limiting to conidial phosphorylation events. The presence of CaM-target proteins in conidia also implies that at least some elements required for Ca^{2+} activation of germination events are constitutive.

Although Ca^{2+} has been implicated in a variety of responses in other fungi (Jackson & Heath, 1989) the present results provide the first direct evidence of a role for Ca^{2+} /CaM-dependent phosphorylation in a filamentous fungus.

The apparent absence of any active cAMP-dependent protein phosphorylation distinguishes *Metarhizium*

conidia from conidia of several other fungal species in which germination is triggered by a glucose-induced cAMP-dependent activation of trehalase (Thevelein, 1984). In contrast to glucose, however, nitrogen-source-induced trehalase activation is not mediated by cAMP in the yeast *Saccharomyces cerevisiae* (Thevelein & Beullens, 1985), which suggests there may be a connection between the regulatory mechanism observed in *Metarhizium* conidia and their poor germination on glucose as compared with germination on nitrogen sources (St Leger *et al.*, 1989*a*). The presence of cAMP in ungerminated conidia and the pre-germination synthesis of cAMP-target proteins suggests some role for cAMP after germination is triggered. Apparently, this function is not critical; while the cAMP-dependent kinase inhibitor H-8 specifically repressed phosphorylation of a 27 kDa protein, it had no effect on protein synthesis or germination in YEM (St Leger *et al.*, 1989*b*).

The phosphorylation of *Metarhizium* membrane proteins is Ca^{2+} -independent, but Ca^{2+} -dependent protein kinase activity is nevertheless a substantial proportion of

Fig. 5. Phosphorylation of membrane proteins. Proteins were phosphorylated for 2 min with [γ - 32 P]ATP in the presence of 0.2 mM- Ca^{2+} (lane 1) and 0.2 mM- Ca^{2+} + 50 μM -H-8 (lane 2). Proteins were subjected to SDS-PAGE (15%, w/v, acrylamide) followed by autoradiography.

total activity as assessed using a crude mycelial preparation. A similar situation has been found in some plant systems (Polya *et al.*, 1984). Ca^{2+} promoted the phosphorylation of polypeptides in crude extract from *Metarhizium* even in the presence of CaM antagonists. This suggests that endogenous CaM was not mediating Ca^{2+} -promoted phosphorylation even though CaM and CaM-target proteins are present in *M. anisopliae* germ-lings. The single Ca^{2+} -independent protein phosphorylation event inhibited by H-series inhibitors is presum-

Fig. 6. Phosphatase activity on isolated plasma membranes. Membranes were incubated with [γ - 32 P]ATP for 1 min (time 0) and then chased with cold ATP in the presence of 25 μM -H-8 for 1 to 7 min.

ably the same 27 kDa phosphorylated protein identified *in vivo* and by its sensitivity to H-7 and H-8 (St Leger *et al.*, 1989b).

The Ca^{2+} -dependent protein kinase activity was eliminated with the cytoplasmic fraction during isolation of plasma membranes. Membrane phosphorylation occurred in the absence of any effector (e.g. cAMP or Ca^{2+}). It is conceivable that the membrane-associated protein kinase was activated prior to or during membrane isolation – in which case cAMP may be the mediator as it is present in the plasma membrane preparation. Inhibition by millimolar levels of cAMP is a

Fig. 7. Phosphorylation of membrane proteins and 8-azido[^{32}P]cAMP labelling of a cAMP-binding protein in plasma membranes. Lane 1, autoradiograph of membrane proteins phosphorylated with [γ - ^{32}P]ATP for 3 min. Lanes 2 and 3, 8-Azido[^{32}P]cAMP labelling of a 52 kDa protein in the presence of 0.2 mM-ATP (lane 2) and 0.1 mM-cAMP (lane 3).

Table 1. Adenylate cyclase activity in plasma-membranes of *Metarhizium* assayed alone or reconstituted with homogenate of the *Cr-1* mutant of *Neurospora*

The experiment was repeated three times using different preparations of plasmalemma and homogenate, with very similar results.

Compound	Total activity [pmol cAMP min ⁻¹ (mg membrane protein) ⁻¹ ± SD]	
	Plasma membrane	Plasma membrane + homogenate
No addition	0	<0.01
Mn ²⁺ (0.5 mM)	4.5 ± 1.1	5.2 ± 1.2
Mg ²⁺ (4 mM)	<0.01	1.8 ± 0.2
Mg ²⁺ (4 mM) + Gpp(NH)p (10 µM)	<0.01	5.6 ± 0.7

* Activity in *Neurospora* homogenate plus Mg²⁺ (4 mM) and Mn²⁺ (0.5 mM) was <0.01 pmol cAMP min⁻¹ (mg membrane protein)⁻¹.

characteristic of cAMP-dependent kinases from plasma membranes of animals (Maeno *et al.*, 1974; Müller *et al.*, 1988). The rank order of effectiveness of H-series inhibitors of cAMP-dependent phosphorylation ap-

Fig. 8. Detection of CaM-binding proteins by binding of ^{125}I -labelled CaM to proteins transferred to nitrocellulose membranes. Lane 1, soluble proteins extracted from conidia; lane 2, total proteins extracted from conidia; lane 3, soluble proteins extracted from mycelia; lane 4, membrane proteins.

proximated that of their reported binding affinity to cAMP-dependent kinases from other systems [K_i (µM) values for H-7 and H-8 compared with mammalian cAMP-dependent kinase are 3.0 and 1.2 respectively (Hidaka & Tanaka, 1987)]. H-7 is also an inhibitor of C-kinase [K_i (µM) = 6 (Hidaka & Tanaka, 1987)] but the Ca²⁺-independent nature of the reaction and the lack of any effect by TPA makes it very unlikely that a C-kinase is involved.

The major phosphorylated protein in *Metarhizium* plasma membranes had the same molecular mass (52 kDa) as the putative regulatory subunit of the cAMP-dependent kinase which readily bound 8-azido-[^{32}P]cAMP indicative of autophosphorylation. The 52 kDa band, and several others, co-migrated with glyco-

Fig. 9. SDS-PAGE analysis of [^{35}S]methionine-labelled conidial extracts adsorbed to 8-hydroxyethylthio-cAMP-agarose. Lane 1, low affinity binding proteins extracted from conidia germinated for 6 h on 0.0125% YEM; proteins were released from the gel by washing in 5 mM-MES buffer (pH 6.2) containing 1 mM-5'-AMP and 1 mM-cAMP. Lanes 2 and 3, high-affinity binding proteins from conidia germinated for 6 h in 0.0125% YEM (lane 2) or YEM supplemented with 2% sucrose (lane 3). High-affinity binding proteins were released by boiling the agarose beads (5 min) in Laemmli buffer (Laemmli, 1970).

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proteins. In mammalian cells, membrane glycoproteins have been implicated in communication networks involving polar signals and Ca^{2+} mobilization (Bowles, 1987). Little is known about the occurrence of such systems in fungal cell plasma membranes.

To function as an efficient mechanism for signal transduction, protein phosphorylation should be both rapid and reversible. Phosphorylation of membrane proteins was evidently rapid as it was initiated within 1 min of incubation with [γ - ^{32}P]ATP and was not affected by phosphatase inhibitors (sodium molybdate and NaF). Loss of radioactivity from the labelled 15.5 kDa polypeptide following incubation for 1 min in unlabelled ATP provides supporting evidence for the existence of a selective dephosphorylating mechanism in the membrane suited to cause rapid and reversible changes of membrane functions which depend on this phosphorylation event.

Metarhizium plasma membranes contain high levels of bound adenylate cyclase suggesting that the membrane plays an integral role in regulating intracellular levels of cAMP. Localization on the plasma membrane implies an involvement in the transfer of extracellular signals to intracellular components. Consistent with this, treatments which can depolarize the plasma membrane or cause mechanical stress produced rapid, usually transient, increases in intracellular cAMP levels, presumably by stimulating the membrane-bound adenylate cyclase(s). *Metarhizium* membranes contain several GTP-binding proteins suited to be coupling factors for transmembrane signal transduction (St Leger *et al.*, 1989c). Isolated *Metarhizium* membranes, however, lacked a functional G-component being Mn^{2+} -dependent and insensitive to a stable guanyl nucleotide. The G-component in *Neurospora* homogenates is highly labile but its activity can be restored by reconstitution with a carefully prepared extract from Cr-1 mutants (Rosenberg & Pall, 1983). Likewise, adenylate cyclase activity in *Metarhizium* membranes recognized the regulatory component of a Cr-1 mutant suggesting very similar control of adenylate cyclase activity in the ascomycete *Neurospora* and the deuteromycete *Metarhizium*.

This work was supported in part by a grant (89-37263-4463) from the USDA Competitive Research Grants Office.

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