Changes in translatable mRNA species associated with nutrient deprivation and protease synthesis in *Metarhizium anisopliae*

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We have studied the regulation of the extracellular chymoelastase protease (Pr1) of the fungal entomopathogen *Metarhizium anisopliae*. This enzyme is involved in the penetration of insect cuticle by *Metarhizium* and other entomopathogenic fungi. Changes in mRNA sequences during starvation-induced synthesis of chymoelastase were investigated by comparing poly(A⁺)RNAs and their complementary DNA in rapidly growing or nutrient-deprived cultures of *Metarhizium*. Hybrid-selected translation revealed that three novel polypeptides (41, 40·2 and 29·8 kDa) were produced rapidly (<1 h) during nutrient deprivation; the most intensely translated species (41 kDa) was identified as the primary translation product of Pr1 by examination with anti-Pr1-IgG. Measurement of starvation-specific RNA levels by dot-blot hybridization with [³²P]cDNA showed that transcripts were not present in spores but were produced when the formation against a hard surface of infection structures was induced by depletion of endogenous nutrient reserves. Starvation-specific mRNA species failed to appear when new RNA synthesis was blocked with actinomycin D, indicating that control is at the level of transcription. Immunoblot analysis with anti-Pr1-IgG showed that Pr1 protein increased in concert with the appearance of Pr1 transcripts and active enzyme. Two very short lived precursors of Pr1, the primary translation product (41 kDa) and an intermediate (40·8 kDa) in addition to mature Pr1 (30 kDa) were detected by immunoprecipitation of [³⁵S]methionine-labelled cell extracts with anti-Pr1-IgG. Pulse-labelling experiments demonstrated that a time of about 7 min was required for [³⁵S]methionine to be processed into extracellular Pr1. These results suggest that regulation of Pr1 gene expression occurs during starvation which, coupled with fast processing, allows very rapid secretion of Pr1. Isolates of four other entomopathogenic fungi (*Verticillium lecanii*, *Beauveria bassiana*, *Tolypocladium niveum* and *Paecilomyces farinosus*) produced Pr1-type enzymes during nutrient deprivation, suggesting our results may have widespread application in understanding entomopathogenicity.

Introduction

The fungal entomopathogen *Metarhizium anisopliae* penetrates the proteinaceous cuticular barrier of insects by using an extracellular chymoelastase protease (Pr1) that constitutes the major protein product synthesized by its infection structures (Goettel *et al.*, 1989; St Leger *et al.*, 1987*a*, 1989*a*). The purified enzyme has been characterized for substrate specificity and inhibition by typical serine protease inhibitors (St Leger *et al.*, 1987*b*). The molecular mass of Pr1 has been estimated to be 25–31 kDa by various physical means; anomolous estimates may arise from the very basic nature of this protein (pI about 10·5, St Leger *et al.*, 1987*b*). Analogous peptidases

have been resolved in culture filtrates from several other entomopathogenic fungi (St Leger *et al.*, 1987*c*).

Potentially, specialization for a pathogenic lifestyle may operate by way of regulatory controls which allow expression of genes under conditions in which similar genes are not expressed in non-pathogens. Previously, we demonstrated that antisera against *Metarhizium* Pr1 or specific inhibitors of Pr1 block penetration of host cuticles and reduce infection indicating that the level of active Pr1 may determine the capacity of the fungus to cause disease (St Leger *et al.*, 1988*a*). Production of the enzyme without differentiation of infection structures can occur rapidly by nutrient deprivation alone (St Leger *et al.*, 1988*b*) making *Metarhizium* an amenable system for the study of the molecular controls of protein synthesis. In this study we examined changes in the patterns of Pr1 synthesis during nutrient deprivation by immunoprecipitation of Pr1 from ³⁵S-labelled proteins.

Abbreviations: NAG, N-acetylglucosamine; SDA(B), Sabouraud dextrose agar (broth); TLCK, tosyl-lysine chloromethyl ketone; YEM, yeast extract medium.

We also examined the complement of mRNA sequences present during differentiation in two ways. First, we analysed the cell-free translation products of poly(A⁺)-RNA at different times during nutrient deprivation. Second, we performed subtraction hybridization experiments between poly(A⁺)RNAs isolated at different times, and their complementary DNA in order to analyse gross changes in the mRNA population during starvation. We discuss our results in terms of different regulatory strategies, as we were particularly interested in whether PrI, being under a comparatively looser form of control by catabolite repression alone, could be regulated as rapidly as those more tightly regulated enzymes induced specifically by the presence of their substrates.

Methods

Organisms and growth. The fungal isolate (*Metarhizium anisopliae* ME1) and culture media were described by St Leger *et al.* (1986a). Isolates of *Verticillium lecanii* (ARSEF 313), *Paecilomyces farinosus* (ARSEF 1508), *Tolypocladium niveum* (ARSEF 616) and *Beauveria bassiana* (ARSEF 252) were obtained from the USDA-ARS culture collection, Cornell University, Ithaca, NY, USA. The isolates were grown and maintained on Sabouraud dextrose agar (SDA) at 23 °C (*V. lecanii*), 25 °C (*B. bassiana*) or 27.5 °C (other isolates) and stored at 4 °C.

³⁵S incorporation into proteins. Mycelia from 3 d *N*-acetylglucosamine (NAG) cultures (see below) were collected on Whatman no. 1 filter paper and washed extensively with distilled water. Mycelia (1 g wet wt) were then resuspended in 10 ml of chitin medium (0.5% chitin, 0.1% KH₂PO₄, 0.05% MgSO₄, pH 5.8) and shaken at 75 r.p.m. at 27.5 °C. After 15 min cultures were monitored at 10 min intervals for PrI release. For pulse-labelling experiments, 0.5 mCi (18.5 MBq) [³⁵S]-methionine (Trans ³⁵S, a ³⁵S-labelled bacterial hydrosylate from ICN radiochemicals) was added to cultures and, unless otherwise specified, the mycelia were further incubated for 5–20 min. In some experiments tosyl-lysine chloromethyl ketone (TLCK) or tunicamycin were added at the concentrations given in the text 20 min before the radioactive amino acid was added. For pulse-chase experiments, essentially the same procedure was used, except that labelled mycelium was chased with unlabelled methionine (20 mM) plus cysteine (20 mM) at a 4000-fold excess for the times given in the text.

Labelled mycelia were collected on Millipore filters, washed with cold distilled water, and comminuted under liquid nitrogen. The ground mycelium was shaken for 20 min with 5 vols of lysis buffer (1.1% v/v, Triton X-100, 100 mM-Tris/HCl, pH 7.8, 10 mM-EDTA, 1 mM-PMSF, 0.1 mM-aminocaproic acid, 0.02 mM-TLCK and 0.02 mM-leupeptin) at 4 °C. Following clarification (8000 g, 10 min) supernatants were analysed by immunoprecipitation and by SDS-PAGE as described below. Proteins secreted into growth media were precipitated by incubating overnight at –20 °C with 3 vols 0.1 M-ammonium acetate in methanol containing 1 mM-PMSF. Proteins were collected by centrifugation (10000 g, 10 min) and redissolved in water.

Extraction procedures for RNA. Total RNA was extracted from small fungal samples using guanidine.HCl. Infection structures (appressoria) were induced by germinating conidia in yeast extract medium (YEM) (0.0125%) on glass Petri dishes as described previously (St Leger *et al.*, 1989a). RNA was extracted after pouring off the YEM supernatant and adding 2.5 ml of homogenizing buffer (7.5 M-

guanidine.HCl, 0.1% Sarkosyl, 0.1 M-2-mercaptoethanol, 10 mM-EDTA, 50 mM-sodium citrate, pH 7). Adhering germings were scraped off and the buffer was then transferred sequentially to five other dishes. The mycelia from 30 dishes were pooled and homogenized by mixing in a vortex for 5 min with acid-washed glass beads (1 g; <0.3 mm diam.). Following centrifugation, supernatants were heated at 65 °C for 5 min, extracted with phenol/chloroform and the RNA was precipitated with ethanol (Maniatis *et al.*, 1982). After dissolving in water, some samples were treated with RNAase A (overnight) or DNAase I (1 h) at 0.1 mg ml⁻¹ in the presence of 10 mM-MgCl₂. Treated extracts were processed for dot-blot analysis as described below.

Washed conidia (400 mg) harvested from 10 d SDA cultures (St Leger *et al.*, 1989a), acid-washed glass beads (1200 mg) (<0.3 mm diam.) and liquid nitrogen were placed in a 50 ml 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 thawed, the fractured conidia and glass were suspended in 5 ml of homogenization buffer. All subsequent steps were as described for germings.

Preparation and translation of poly(A⁺)RNA. Poly(A⁺)RNA was isolated from finely comminuted frozen mycelium by extracting with phenol/chloroform, precipitating the total RNA with ethanol, and isolating the poly(A⁺)RNA using oligo(dT) chromatography (Maniatis *et al.*, 1982). Poly(A⁺)RNA was dissolved in sterile water, the solution divided into aliquots and stored at –70 °C until used. Poly(A⁺)RNA was translated in a rabbit reticulocyte cell-free translation system. Normally, 1 µg poly(A⁺)RNA was translated in 40 µl of solution containing 35 µl of nuclease-treated rabbit reticulocyte lysate (Promega), 1 unit of RNAasin (Promega) µl⁻¹, a mixture of unlabelled amino acids minus methionine (25 µM final concentration) and 1.5 µCi (55 kBq) [³⁵S]methionine (Amersham) µl⁻¹ for 1 h at 30 °C.

Immunoprecipitations. Translation products were analysed by immunoprecipitation as described by Anderson & Blobel (1983) with modifications. Samples (10 µl) of the translation mixture were added to 20 vols of immunoprecipitation buffer (1% w/v, skimmed milk, 1.1% Triton X-100, 100 mM-Tris/HCl, pH 7.8, 100 mM-NaCl, 10 mM-EDTA, 1 mM-PMSF, 0.1 mM-aminocaproic acid, 0.02 mM-TLCK and 0.02 mM-leupeptin) and incubated sequentially for 1 h each with 10 µl of pre-immune serum and 30 µl of a 1:1 (w/v) suspension of protein A-Sepharose (Sigma) and immunoprecipitation buffer at 4 °C. The samples were spun for 2 min in a microcentrifuge and the supernatants incubated sequentially for 1 h each with 1 µl of rabbit polyclonal antisera specific for PrI (St Leger *et al.*, 1989a) and 30 µl of the protein A-Sepharose suspension. The Sepharose beads were washed extensively in the immunoprecipitation buffer, minus the skimmed milk, followed by a wash in similar buffer, minus detergents. PrI was eluted from the beads by boiling in SDS sample buffer (Laemmli, 1970) for 5 min and analysed by SDS-PAGE and autoradiography (St Leger *et al.*, 1989a). The same procedure was used for immunoprecipitation experiments on labelled mycelial proteins. Samples (10 µl) of cell lysate in lysis buffer or growth media were added to 20 vols of immunoprecipitation buffer before proceeding as above.

Immunoblotting. Proteins, denatured by boiling for 10 min, were placed in 1 µl aliquots onto nitrocellulose pre-wetted with 10 mM-Tris-buffered saline (pH 7.4; 150 mM-NaCl). Blots probed with anti-PrI serum were developed with alkaline phosphatase coupled antibody to rabbit IgG according to the ProtoBlot Western blot AP system technical manual (Promega) except that 1% skimmed milk powder was used as a blocking agent.

Synthesis of subtracted complementary DNA (cDNA) probes. Large scale synthesis of single-stranded cDNA was done using 30 µg of purified poly(A⁺)RNA from 2 h minimal medium cultures (0.01%

KH_2PO_4 , 0.005% MgSO_4 , without carbon or nitrogen source) as template and using random primers as described by Sambrook *et al.* (1989). Radiolabelled cDNAs for probes were synthesized by replacing the unlabelled dCTP with [α - ^{32}P]dCTP [3000 Ci mmol $^{-1}$ (111 TBq mmol $^{-1}$); New England Nuclear] at 2 Ci ml $^{-1}$ (74 kBq ml $^{-1}$). After incubation at 37 °C for 1 h, the reaction was stopped by the addition of 20 μl 0.2 M-EDTA, and the RNA was hydrolysed by the addition of 25 μl of 1 M-NaOH and incubation for 30 min at 70 °C. After neutralization the unincorporated label was removed by centrifugal Sephadex G-50 chromatography (Maniatis *et al.*, 1982). Single-stranded radiolabelled cDNA (13 μg) was subtracted, to obtain unique sequences, with 100 μg poly(A $^+$)RNA from 3 d SDB cultures using a hybridization technique employing photoactivatable biotin and phenol extraction essentially by the method of Sive & St John (1988), except that the volumes of all components in the reaction were increased by a factor of five to match the amount of cDNA. Three similar cycles of hybridization were completed to drive the subtraction hybridization to completion.

Analysis of the subtracted cDNA was by hybridization/selection (Goldberg *et al.*, 1979). The subtracted cDNA was immobilized onto a 0.5 cm 2 disc of freshly diazotized (diazobenzyloxymethyl, DBM) Whatman 540 paper (Williams *et al.*, 1979). The disc was hybridized for 6 h at 37 °C with 0.15 μg poly(A $^+$)RNA from 1 h minimal medium cultures in 50 μl of buffer (50%, v/v, formamide, 0.8 M-NaCl, 0.2% SDS, 2 mM-EDTA, 20 mM-PIPES, pH 6.4). The disc was washed at 37 °C four times for 20 min each with 200 ml 20 mM-NaCl, 8 mM-trisodium citrate, pH 7.0 (1 $\times$ SSC), 50% (v/v) formamide and 0.2% SDS and twice in 200 ml of 2 $\times$ SSC. RNA was eluted from the filters by shaking in 100 μl of extraction buffer (20 mM-PIPES, pH 6.4, 0.5% SDS, 1 mM-EDTA and 90%, v/v, formamide). The eluates from 10 similar hybridization cycles using the same disc (which had been recycled through an alkaloid elution step between hybridizations) were combined and diluted with 2 vols of water, wheatgerm tRNA was added to 20 μg ml $^{-1}$ and the RNA was precipitated with ethanol. Samples were reprecipitated from ethanol and washed in 70% (v/v) ethanol. After dissolving in water, samples were translated using rabbit reticulocyte lysate.

RNA dot-blots. Dot-blots were done by the procedure of Thompson *et al.* (1983). RNA, denatured with 6% (v/v) formalin in 20 mM-sodium

phosphate, pH 6.8, at 55 °C was placed in 2 μl aliquots on pre-wetted (water, then 2 $\times$ SSC) nitrocellulose membranes (BA85; Schleicher and Schuell). Following baking at 80 °C under vacuum, hybridization was done with the subtracted [^{32}P]cDNA probe at 42 °C for 15 h in 50% (v/v) formamide, 3 $\times$ SSC, 1 $\times$ Denhardt's medium, 200 μg yeast RNA ml $^{-1}$ and 20 μg denatured salmon sperm RNA ml $^{-1}$. Blots were washed sequentially in 2 $\times$ SSC (2 $\times$ 10 min, 23 °C), 0.1 $\times$ SSC plus 0.1% SDS (3 $\times$ 20 min, 50 °C) and rinsed rapidly three times in 0.1 $\times$ SSC plus 0.1% SDS at 23 °C, and three times in 0.1 $\times$ SSC before analysis by autoradiography.

Miscellaneous. Pr1 activity toward succinyl-(alanine) $_2$ -proline-phenyl-alanine-*p*-nitroanilide [Suc-(Ala) $_2$ -Pro-Phe-*p*NA] was assayed as described by St Leger *et al.* (1987b). Clean samples of cuticle from 3-d-old fifth instar *Manduca sexta* larvae were prepared as described previously (St Leger *et al.*, 1988b).

Chemicals. Except where indicated in the text, all chemicals were from Sigma.

Results

Production of Pr1 during nutrient deprivation

Mycelia of five species of entomopathogenic fungi, growing rapidly in SDB cultures, were transferred to media in which growth was limited by carbon and nitrogen deprivation (Table 1). In response, these fungi produced activities against the Pr1 substrate (Suc-(Ala) $_2$ -Pro-Phe-*p*NA (Table 1). As reported previously for *Metarhizium* (St Leger *et al.*, 1988b), enzyme levels were frequently enhanced in cultures supplied with *Manduca* cuticle or other insoluble protein or non-protein (cellulose) polymers at levels insufficient to produce catabolite repression. However, in each case, production of protease was repressed in SDB or when *N*-acetylglucosamine (1%, w/v) was added to medium containing cuticle,

Table 1. *Protease production by five species of entomopathogenic fungi 8 h after transfer of growing mycelium to nutrient-rich or nutrient-limiting conditions*

Protease activity was measured spectrophotometrically with Suc-(Ala) $_2$ -Pro-Phe-*p*NA as the substrate. Results represent mean protease activity (nmol *p*NA ml $^{-1}$ min $^{-1}$) for three replicates $\pm$ SD. The results are representative of two similar experiments.

Medium*	<i>M. anisopliae</i>	<i>V. lecanii</i>	<i>B. bassiana</i>	<i>P. farinosus</i>	<i>T. niveum</i>
- CN	32.7 $\pm$ 4.53	24.7 $\pm$ 3.94	20.9 $\pm$ 3.57	6.3 $\pm$ 0.84	21.6 $\pm$ 2.6
- CN + <i>Manduca</i> cuticle (0.5%)	75.2 $\pm$ 6.82	36.2 $\pm$ 3.13	30.3 $\pm$ 2.85	10.41 $\pm$ 1.42	29.4 $\pm$ 1
- CN + chitin (0.5%)	51.1 $\pm$ 4.31	32.8 $\pm$ 4.30	34.1 $\pm$ 4.31	7.9 $\pm$ 0.38	31.8 $\pm$ 3.7
- CN + cellulose (0.5%)†	44.3 $\pm$ 5.62	28.6 $\pm$ 2.62	29.5 $\pm$ 2.65	9.19 $\pm$ 0.83	38.6 $\pm$ 2.4
- CN + <i>Manduca</i> cuticle (0.5%) + NAG (1%)	0.2 $\pm$ 0.01	2.27 $\pm$ 0.13	0	0	3.4 $\pm$ 0.54
- CN + actinomycin D (100 μg ml $^{-1}$)	8.6 $\pm$ 0.81	4.38 $\pm$ 0.44	7.23 $\pm$ 0.23	0	7.38 $\pm$ 0.85
SDB	0	0	0	0	0

* - CN, minimal medium (0.01% KH_2PO_4 , 0.005% MgSO_4) without a carbon or nitrogen source. Except for *V. lecanii* (23 °C) and *B. bassiana* (25 °C) isolates were incubated at 27 °C.

† Insoluble crystalline cellulose.

Fig. 1. Protein coding capacities of poly(A⁺)RNA from *Metarhizium* during production of Pr1. Poly-(A⁺)RNA was extracted from mycelia incubated in minimal medium for (lane 1) 0 h (mycelia taken directly from 1% NAG/basal salts medium), (2) 0.5 h, (3) 1 h and (4) 7 h and translated in the presence of [³⁵S]methionine; lanes 5, 6 and 7, translation products from poly(A⁺)RNA from 1 h minimal medium cultures processed and immunoprecipitated with anti-Pr1-bodies (5) and compared with immunoprecipitated samples of active Pr1 from culture filtrates of 2 h minimal medium cultures (6) and 2 h 0.5% chitin/basal salts medium cultures (7). The arrowhead indicates the position of the primary translation product of Pr1.

showing that repression overrides the enhancing effect of polymeric substrates. The production of extracellular proteases by starved mycelia apparently required *de novo* RNA synthesis as activities were reduced by the transcription inhibitor actinomycin D (Table 1).

Further studies on *Metarhizium* demonstrated that the source of fungal inocula influenced protease (Pr1) release. The use of fungal inocula grown for 3 d on 2% (w/v) NAG/basal salts (St Leger *et al.*, 1986a), a relatively poor growth medium (growth rate 1.5 ± 0.31 mg dry wt ml⁻¹ d⁻¹) but sufficient to repress Pr1 synthesis, resulted in the appearance of Pr1 within 1 h of transfer to minimal medium. By contrast, Pr1 activity was only apparent after 3 h using inocula from 32 h SDB cultures (growth rate 3.97 ± 0.53 mg dry wt ml⁻¹ d⁻¹) or 3 d SDB cultures (4.5 ± 0.43 mg dry wt ml⁻¹ d⁻¹). Pr1 activity 4 h post-transfer from 3 d SDB cultures or 4 h post-transfer from 3 d NAG cultures was 13.64 ± 1.8 nmol pNA ml⁻¹ min⁻¹ and 45.42 ± 3.6 nmol pNA ml⁻¹ min⁻¹, respectively. Pr1 was not detected in *Metarhizium* growth media following transfer of cultures

from SDB to fresh SDB, or after a heat-shock treatment (55 °C, 5 min). Thus the production of Pr1 is not a component of a stereotyped response to stress but part of an adjustment to altered growth conditions.

Quantitative changes in the mRNA production

At various intervals during Pr1 production in nutrient-deprived media (without carbon and nitrogen source), poly(A⁺)RNAs were extracted and their protein-coding capacities were assessed in a cell-free rabbit reticulocyte translation system. The patterns observed with NAG medium and nutrient-deprived medium were very similar until 60 min post-transfer when there was a considerable induction of mRNA for a 41 kDa protein (Fig. 1). The quality of poly(A⁺)RNA had deteriorated in mycelia harvested after several hours in nutrient-deprived conditions. Some of the bands became faint, broad or indistinct. However, the transcript for the 41 kDa protein apparently retained its integrity better than most other transcripts (Fig. 1, lane 4), suggesting

Fig. 2. Changes in the mRNA population during nutrient deprivation. Protein coding capacities of poly(A⁺)RNA isolated from *Metarhizium* from NAG cultures (lane 2) and from cultures transferred from NAG/basal salts medium for 2 h (lane 3). Immunoprecipitation of translation products from 3 d NAG (lane 4) and 1 h minimal medium cultures (lanes 5 and 6). Translation products were processed and immunoprecipitated with anti-Pr1-serum as described in the text with the exception of the sample in lane 6 which was immunoprecipitated after adding 5 µg Pr1. Using the procedure described in Methods, poly(A⁺)RNA isolated from 1 h minimal medium cultures was hybridized to diazotized paper discs bearing cDNA which had been constructed from the same poly(A⁺)RNA and subsequently subtracted with poly(A⁺)RNA isolated from 3 d NAG cultures. RNA was eluted from the discs and translated (lane 7). Lane 1 shows translation products from Brome Mosaic Virus RNA used as a positive control of the translation system.

maintained synthesis and/or that it is a peculiarly stable species. Its protein product comigrated on SDS-polyacrylamide gels with the single protein (41 kDa) precipitated from translation mixtures by anti-Pr1-serum, and which was likewise absent in mycelia grown in nutrient-rich medium (Fig. 2). Pr1 (5 µg) added to the translation mixture before addition of the antibody effectively competed with the 41 kDa translation product, strongly suggesting that this protein is immunologically similar to the mature Pr1 from *Metarhizium* in spite of the 11 kDa difference in molecular mass (Fig. 1).

To clarify further changes in the mRNA population, mRNA enriched for transcripts present only in the nutrient-deprived mycelia was analysed. Fig. 2 (lane 7) shows a typical autoradiogram of the *in vitro* synthesized polypeptides obtained from hybrid selected mRNA. By this method it was possible to detect the *de novo* appearance of three major translatable mRNA species for polypeptides of molecular mass 41, 40.2 and 29.8 kDa. Anti-Pr1 serum selectively precipitated only the 41 kDa species indicating that the 40.2 and 29.8 kDa proteins are immunologically unrelated to Pr1.

To analyse further whether starvation-induced mRNA species involved transcription, dot-blot analysis of total RNA was done using the subtraction [³²P]cDNA probe. Within 1 h of the transfer of mycelium from NAG medium, starvation-specific mRNA transcripts were

detectable and the level increased sharply over the next 4 h (Fig. 3). Treatment of samples with RNAase entirely eliminated binding of the probe further indicating that *de novo* RNA synthesis was involved. Immunoblot analysis confirmed that the corresponding increase in Pr1 activity represented a true increase in the amount of Pr1 protein in the medium (Fig. 3). Actinomycin D was used at a concentration (100 µg ml⁻¹) which massively reduces detectable RNA synthesis in *Metarhizium* (St Leger *et al.*, 1989b). Addition of actinomycin D at the beginning of starvation inhibited the *de novo* formation of unique mRNA and the formation of Pr1 (Fig. 3). These results suggest some regulation of gene expression exerted at transcription during starvation. We wondered, however, whether these RNAs could be packaged into spores, perhaps stored for future use. As shown in Fig. 4, total RNA from fractured spores contained only low levels of mRNA species recognized by the subtraction [³²P]cDNA probe. By contrast, high levels of messenger were present in germlings forming appressoria, which correlated with Pr1 production.

Precursors and processing of Pr1.

[³⁵S]Methionine incorporation into proteins was studied in mycelia transferred to nutrient-deprived medium. Pulse-labelling mycelia for 10 min, 3 h post-transfer to

Fig. 3. Detection of starvation-specific RNA species and Pr1 using dot-blots. Total RNA was extracted from *Metarhizium* mycelia transferred from NAG/basal salts medium to minimal medium for the time indicated. The RNA was denatured with glyoxal and dotted to nitrocellulose paper (0.3 µg in 2 µl aliquots). The dot-blots were hybridized with a [³²P]cDNA probe for starvation-specific mRNA species as reported in the text. A 48 h autoradiograph is shown. Proteins in filtrates from minimal medium cultures were placed in 1 µl aliquots onto nitrocellulose and probed with anti-Pr1 serum; the values underneath the immunoblot represent the corresponding increase in Pr1 activity expressed as nmol pNA ml⁻¹ min⁻¹. Act D, effects of actinomycin D (100 µg ml⁻¹) on RNA and Pr1 synthesis.

Fig. 4. Detection of starvation-specific RNA species and Pr1 during differentiation of appressoria. Total RNA was extracted from germlings differentiating for the time indicated (0, ungerminated conidia), denatured and dotted to nitrocellulose paper (0.2 µg in 4 µl aliquots). The dot-blots were hybridized with a [³²P]cDNA probe for starvation-specific mRNA species. A 72 h autoradiograph is shown. Proteins extracted from conidia or germlings in lysis buffer were placed in 1 µl aliquots on nitrocellulose filters and probed with anti-Pr1 serum.

minimal medium, allowed resolution of more than 30 proteins, and immunoprecipitation of detectable levels of 41 and 40.8 kDa proteins as well as mature extracellular Pr1 (Fig. 5). Pr1 added to the mycelial lysate before addition of the antibody effectively competed with each of these proteins. We assume, therefore, from the identity of molecular masses that the 41 kDa protein represents the primary translation product previously identified by *in vitro* translation. In two out of four replicates, labelling of mycelia for longer than 5 min revealed a faint low molecular mass band (26.5 kDa) that may or may not be related to Pr1.

The 41 and 40.8 kDa precursors were present in very low amounts as compared with the mature protease. This suggested that the precursors are rapidly processed so that the fraction of labelled Pr1 represented by the precursor remains low. Consistent with this, labelling of mycelia for longer than 10 min revealed only the 41 kDa precursor, suggesting that the fraction of total label in the 40.8 kDa precursor was almost zero. This hypothesis was confirmed by a pulse-chase experiment. After a 10 min chase with cold methionine plus cysteine the fraction of labelled Pr1 represented by the precursors was greatly reduced while levels of mature extracellular Pr1 were almost unaffected (Fig. 5). Neither precursor was detectable in growth media.

The time taken for mycelia to process an amino acid nutrient into mature extracellular Pr1 was determined by precipitating Pr1 secreted into the supernatant medium at various times after the addition of [³⁵S]methionine to 3 h minimal media cultures. Labelled extracellular Pr1 appeared first 5–10 min after addition of the labelled amino acid and increased sharply for the 20 min duration of the experiment (Fig. 5). The Pr1 bands were cut-out and their radioactivities were counted in a scintillation counter. The transit time from addition of label to the appearance of extracellular Pr1 was 6.5–7.2 min, as calculated from three similar pulse-chase experiments by plotting the counts incorporated into the Pr1 bands versus time and extrapolating to zero counts.

Production of active Pr1 was reduced by 63% after pre-incubation (3 h) with tunicamycin (100 µg ml⁻¹), indicating that glycosylation reactions are involved in processing of Pr1; the relative molecular mass of the mature protease was unaffected by tunicamycin, showing a low level of Pr1 glycosylation.

The results described above suggest that a precursor of high molecular mass is processed down to the low molecular mass active enzyme, presumably by selective proteolytic cleavage. To test for the involvement of the trypsin-like *Metarhizium* enzyme Pr2 (St Leger *et al.*, 1987b), we pre-incubated mycelia (1 h) with the specific Pr2 inhibitor TLCK. This inhibitor blocks formation of appressoria indicating that it can enter mycelium (unpublished data). Nevertheless, the inhibitor had no effect on production and apparent molecular mass of the Pr1 protein.

Discussion

The appearance of Pr1 is accompanied by a similar change in the prevalence of translatable Pr1 transcripts; thus the formation of translatable mRNA is required for the increased rate of Pr1 synthesis. This information, coupled with the lack of transcripts in ungerminated

Fig. 5. Pulse-chase analysis on the accumulation of Pr1 and Pr1 precursors by *Metarhizium* in 3 h minimal medium cultures. Mycelia were pulse-labelled with [35 S]methionine [$50 \mu\text{Ci ml}^{-1}$ (1.85 MBq ml^{-1})] for 10 min. Lane 1, total labelled proteins extracted from mycelia. Lanes 2–4, radioactivity chased with unlabelled methionine for 0 min (control) (lane 2), 5 min (lane 3) or 10 min (lane 4) and cells processed and immunoprecipitated with anti-Pr1-serum. The arrowheads indicate two bands (41 and 40.8 kDa) in lane 2 and one band in lane 3. Lanes 5–8, time course of secretion of Pr1 following pulse-labelling with [35 S]methionine for 5 min (lane 5), 10 min (lane 6), 15 min (lane 7) or 20 min (lane 8); the culture supernatant was precipitated with ammonium acetate in methanol and re-dissolved precipitates immunoprecipitated with anti-Pr1-serum.

conidia, and our information that inhibition of RNA synthesis inhibits the synthesis of starvation-induced transcripts, strongly suggests that the regulation of synthesis of starvation-induced proteins is at the level of transcription. Nevertheless, since the primary translation product of Pr1 is 11 kDa larger than the mature protease, substantial post-translational processing must be involved in production of active enzyme. Detection of a 40.8 kDa precursor suggests this may involve multiple steps. High molecular mass proenzymes are the norm among the extracellular proteases of bacteria (Kessler & Safrin, 1988). They may also be common to proteases of fungi as attested to by the recent demonstration of a very large precursor (76 kDa) for a vacuolar protease (31 kDa) in *Saccharomyces cerevisiae* (Moehle *et al.*, 1989). This contrasts sharply with the primary translation products

of fungal cutinase and pectinase which are just 2–3 kDa larger than the mature extracellular enzyme (Flurkey & Kolattukudy, 1981; DeLorenzo *et al.*, 1987). The function(s) of the large precursor polypeptides are not known, but presumably they keep the cell-associated enzyme inactive preventing proteolysis of cell constituents. Consistent with this, Pr1 is very active against components of *Metarhizium* mycelium after partial disruption of the cell by heating at 85 °C (unpublished data).

Production of Pr1 was brought about by carbon and nitrogen derepression alone. Rapid growth in nutrient-rich media (e.g. SDB) delayed subsequent Pr1 production, as compared with a poorer growth medium, after transfer to nutrient deprivation, suggesting that depletion of accumulated nutrient reserves linked to reduced

growth rate may be the non-specific signal for Pr1 production. The most rapid initiation of Pr1 synthesis, which occurred within 45–60 min of transfer from NAG medium, is comparable temporally with induction of *Metarhizium* chitinase by chitin degradation products (30–60 min) (St Leger *et al.*, 1986b) and of *Fusarium* cutinase by cutin monomer inducers (30–45 min) (Woloshuck & Kolattukudy, 1986). While cutinase transcripts, however, are detectable within 15 min after addition of the inducers, Pr1 transcripts were not observed until 45 min post-transfer. Pr1 production apparently catches up with cutinase production in the short transit time of 6.5–7.2 min from uptake of labelled amino acid to extracellular release of mature enzyme. Such rapid secretion implies that in spite of the binding of Pr1 to cell wall components (Goettel *et al.*, 1989), a large component of the enzyme secreted through the cell membrane is not retained by the cell wall for a significant length of time. It therefore appears that the 'tighter' form of control for cutinase, involving induction by substrate components (Woloshuck & Kolattukudy, 1986), imparts no significant advantage in terms of rapidity of regulation compared with the 'looser', less precise form of control of Pr1 production by catabolite repression alone. The difference in mode of regulation must relate to the roles of the two enzymes. Cutinase possesses a narrow specificity and, as it is required only for a brief period during penetration of the host plant cell wall, it is energetically conservative for enzyme production to be subject to a high degree of regulatory control mediated by the cutin component of the wall. By contrast, the broad-spectrum proteolytic activity of Pr1 (St Leger *et al.*, 1987b) may have biological functions before and after host cuticle is breached. Its activity will allow utilization of many insoluble substrates during nutrient deprivation, whether the fungus is living as a saprophyte or pathogen.

Pr1 transcript was the major mRNA species novel to starvation-induced cultures, indicating the comparative importance of Pr1 in providing nutrients during starvation conditions as compared with other depolymerases. Other workers have used the two-dimensional electrophoretic technique of O'Farrell (1975) to obtain greater sensitivity of identification of novel proteins. However, the very basic nature of Pr1 (pI about 10.5; it migrates into the region of the cathode), and the sometimes confusing and contradictory results obtained trying to identify novel proteins (Lovett, 1987), led us to use the alternative technique of select-hybridization to identify novel polypeptides. We assume the three transcripts detected represent completely novel mRNA species; even large changes in the abundance of other transcripts should not have been detected because of the exhaustive subtraction hybridization that was done. The two Pr1-unrelated polypeptides were not identified but *Metarhi-*

zium produces a wide range of extracellular hydrolases in nutritionally poor media (St Leger *et al.*, 1986a).

In summary, there appears to be regulation of Pr1 gene expression exerted at transcription during starvation, with production of a high molecular mass precursor which is rapidly processed to active extracellular enzyme. Proteases of several other important entomopathogens are also produced during nutrient deprivation, suggesting our results may have widespread implications in the understanding of fungal pathogenesis of insects.

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