Regulation of restrictocin production in *Aspergillus restrictus*

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The production of restrictocin (a cytotoxin that specifically cleaves ribosomal RNA) by cultures of *Aspergillus restrictus* grown in liquid medium was investigated. The function of restrictocin, the method of its accumulation and the mode of resistance to restrictocin in *A. restrictus* are unknown. Previous studies have indicated that restrictocin accumulates in the medium with culture age. These observations have been extended in this study by cloning the cDNA of the *res* gene and using this cDNA clone to probe the onset of messenger RNA synthesis in the cells. The results of the Northern analysis were compared to the production and accumulation of restrictocin and morphological differentiation of the cells in culture. Restrictocin was found in the medium at the same time that mRNA was detected in the cells. This suggests that the leader sequence encoded by the cDNA provides an efficient secretion system for the protein. Both the protein and the mRNA were detected coincident with the formation of differentiated cell structures. These structures develop into conidiophores with one layer of sterigmata and conidia forming from the sterigmata. These results suggest that restrictocin is either involved in the process of conidiation or is coordinately regulated with differentiation leading to conidiation.

Introduction

Restrictocin is a small basic protein (M_r 17000), produced by *Aspergillus restrictus*, that inactivates ribosomes (Olson *et al.*, 1963). This protein, along with an almost identical protein, mitogillin (from *A. restrictus*), and another similar protein, α -sarcin (from *Aspergillus giganteus*), have anti-tumour activity (Olson *et al.*, 1965). This anti-tumour activity appears to be due to the ability of restrictocin to enter cells and inactivate ribosomes. While these proteins showed promise in canine cancer tests (Roga *et al.*, 1971) they were not effective in human cancer trials due to non-specific toxicity (Wool, 1984; Goldin *et al.*, 1966).

Restrictocin, mitogillin and α -sarcin act as ribonucleases which cleave a single specific phosphodiester bond 393 nucleotides from the 3' end of the 28S rRNA of eukaryotic ribosomes (Fando *et al.*, 1985; Schindler & Davies, 1977). The cleavage site is located in a highly conserved region of 28S rRNA and this region is also the target site of some other ribosome-inactivating proteins such as ricin (Sperti *et al.*, 1989).

α -Sarcin acts *in vitro* on the ribosomes from *A.*

giganteus which produces α -sarcin (Miller & Bodley, 1988), raising the question of how the protein is formed. Several mechanisms could be employed by the fungus to produce a ribosome-inactivating protein. A protein or other substance could bind restrictocin to inactivate it, as is the case for colicin E3 and the immunity protein in *Escherichia coli* (Jakes & Zinder, 1974), or the synthesis of restrictocin might be via an inactive proprotein. An efficient translocation signal peptide could also remove the protein away from the site of its synthesis. A recent report comparing the genomic sequence of restrictocin to the cDNA sequence of α -sarcin suggested that the immature restrictocin does not have activity (Lamy & Davies, 1991). How these ribosome-inactivating proteins are produced by *Aspergillus* and why they are produced are questions that remain unanswered.

A recent study showed that a restrictocin-like protein could be the cause of inflammation and local cell death in Aspergillosis infections (Arruda *et al.*, 1990). If the function of restrictocin is to aid in the proliferation of the fungus in the lungs, it is important to know when and where the protein is made. A role in pathogenesis might be suggested for this protein if it were present on conidia or formed early upon germination. Alternative hypotheses are that restrictocin may bestow some survival advantage or be involved in the differentiation of cells.

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We report here that the cDNA sequence which encodes restrictocin does encode a putative translocation signal sequence. We find that the simultaneous appearance of mRNA in the cells and protein accumulation in the medium limits any possible protein storage and interactions at a later stage in the fungal life cycle. Accumulation of the protein in the medium and the presence of mRNA in the cells is correlated with the appearance in liquid medium of differentiated cells involved in conidiation. The timing of restrictocin production appears to be similar to that of other secondary metabolites by filamentous fungi.

Methods

Culture of organism. *A. restrictus* (strain NRRL 2869) was maintained on complete medium agar plates (Cove, 1966). Conidia from the agar plates were used to inoculate liquid media. Liquid cultures were grown at 30 °C with constant shaking.

Purification of restrictocin and antibody production. *A. restrictus* was grown in 30 litres of medium C according to Olson *et al.* (1965). After 60 h incubation, the culture supernatant was used for purification of restrictocin with an IRC-50 ion-exchange column, also as described by Olson *et al.* (1965). Further purification of the protein was accomplished (for the generation of antibodies) by passing the column fractions through a 30 kDa Millipore Pellicon filter and concentrating on a 10 kDa filter; the concentrate was lyophilized and stored at -20 °C. During purification, the protein was characterized by 12% (w/v) polyacrylamide SDS gel electrophoresis (Laemmli, 1970) followed by silver staining (Wray *et al.*, 1981).

Anti-restrictocin rabbit serum was produced at Promega. Purified protein (0.5 mg) in complete Freund's adjuvant was injected initially and the same amount of antigen in incomplete Freund's adjuvant was used for the second and final boosts at 3 and 5.5 weeks. The antibody was characterized by its ability to bind to antigen on Microtiter plates (ELISA) and immunoblots. In the ELISA, 100 µl [2.5 µg protein ml⁻¹ in phosphate-buffered saline (10 mM-sodium phosphate, 150 mM-NaCl), pH 7.4] of antigen were used to coat each well at 4 °C overnight. The wells were blocked with 1% (v/v) goat serum in PBST (phosphate-buffered saline, 0.05% Tween 20, pH 7.4) after which 100 µl of diluted anti-restrictocin serum were added to each well and incubated for 1 h at room temperature. Incubation with the alkaline phosphatase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) was done under the same conditions and the colour developed for 30 min at room temperature using *o*-nitrophenyl phosphate as substrate. The absorbance at 405 nm was measured on a microplate reader (Bio-Tek).

cDNA and subgenomic DNA library construction and screening. Poly-A-rich mRNA from *A. restrictus* was prepared by total nucleic acid extraction followed by oligo-dT affinity column purification (Ausubel *et al.*, 1987). The BRL cDNA synthesis system was used to synthesize double stranded cDNA from the mRNA and recombinant λ gt11 phages were produced by *in vitro* packaging and then plated on *E. coli* Y1088 to establish the cDNA library (BRL manual).

About 5% of the original cDNA library (5000 plaques/4 plates) was screened using plaque lifts, anti-restrictocin antibody and alkaline-phosphatase-labelled goat anti-rabbit second antibody (Ausubel *et al.*, 1987). Positive clones were confirmed by their ability to hybridize with a synthetic 32-fold degenerate probe which was derived from amino acids 46 to 51 of the reported protein sequence (Lopez-Otin *et al.*, 1984).

Positive clones were plaque-purified twice and the insert fragment was subcloned into the *Eco*RI site of M13mp18.

A subgenomic DNA library was also constructed by ligating 5 kb fragments of *Eco*RI-digested DNA and *Eco*RI-cut λ gt11 and the recombinant phages were introduced into *E. coli* Y1088 as described above to generate a subgenomic library. The whole library (about 40000 plaques) was screened using the synthetic probe as described above. Three positive clones were observed and plaque-purified. The insert fragment was regenerated from phage DNA and proved to contain the restrictocin gene by Southern analysis using restrictocin cDNA as a probe.

Sequence analysis of the clones containing the restrictocin gene. Two M13 clones with the cDNA inserted in opposite orientations were used along with an M13 primer to sequence the insert DNA as described by Sanger *et al.* (1977). The sequence reported here was the result of at least two sequencing reactions for each subclone. The sequences of restrictocin and α -sarcin were compared using the GAP program from the Genetics Computing Group (Madison, WI).

The cloned genomic restrictocin gene was regenerated, digested with several different restriction enzymes and analysed on agarose gel to obtain a partial restriction map. The *Eco*RI-*Xma*I and the *Xma*I-*Hind*III fragments, which contain the entire coding region of the restrictocin gene, were subcloned into both M13mp18 and M13mp19. The upstream section of the transcription initiation region was cloned by screening a subgenomic library of 4.5 kb *Sac*I fragments (in plasmid pUC19) and the *Sac*I-*Eco*RI fragment containing the upstream region was subcloned into M13mp18 and M13mp19. Sequence analysis on both strands was done as described above.

Northern analysis of mRNA. Total RNA was extracted from *A. restrictus* at different times of growth in complete fungal medium. The same amount of RNA (determined as A_{260}) from each sample was loaded onto a 1.2% (w/v) denaturing formaldehyde agarose gel and the relative amount of total RNA in each lane was estimated from a photograph of the ethidium-bromide-stained gel by densitometric analysis. Northern analysis was done by using the purified cloned gene fragment as a nick-translated probe (Promega manual). Prehybridization and hybridization were performed at 40 °C in the presence of 50% (v/v) formamide (Ausubel *et al.*, 1987) and the blot was exposed to Kodak X-OMAT AR film.

Immunoblot analysis of restrictocin. Culture samples were taken throughout incubation. The medium was separated from the cells by centrifugation. Supernatant was mixed with SDS sample buffer (0.125 M-Tris, 2% w/v, SDS, 10% v/v, glycerol, 5% v/v, β -mercaptoethanol) in a 1:1 ratio. The cells were washed with water and extracted with SDS sample buffer (0.1 mg cell per ml of buffer). All samples were placed in a boiling water bath for 10 minutes followed by centrifugation and application of the supernatant to the gel. After gel electrophoresis, an immunoblot was made by using a semi-dry electrophoretic transfer unit (Gelman). The blot was stained with amido black and blocked in 5% (w/v) powdered dry milk in phosphate-buffered saline. Anti-restrictocin antibody and alkaline-phosphatase-labelled goat anti-rabbit second antibody were used for detection of restrictocin (Ausubel *et al.*, 1987).

Microscopy. Samples were taken from the cultures throughout the incubation period for observation by phase contrast and differential interference contrast microscopy. Photomicrographs were taken with a Zeiss Axiophot photomicroscope using wet mounts of the hyphal material.

Nucleotide sequence accession numbers. The cDNA and genomic DNA sequences have been assigned GenBank accession numbers M55508 and M65257.

Results and Discussion

Protein identification and antibody generation

The protein purified according to the method of Olson *et al.* (1965) from 30 liters of medium (estimated >98% pure from silver-stained polyacrylamide gels) (Fig. 1) was tested for its ability to inhibit an *in vitro* protein translation system (Promega; rabbit reticulocyte incorporation of [³⁵S]methionine into acid-precipitable counts). The purified protein preparation was similar in its ability to inhibit protein synthesis to the restrictocin preparation reported by Fando *et al.* (1985). Since the protein was of the correct size and activity, we concluded that the protein purified was restrictocin and proceeded to use the protein for antibody generation.

Antibodies generated in rabbit against this protein were present 4 weeks after the first injection. The serum at that time reacted with M_r 17000 and M_r 30000 proteins on immunoblots and this reaction was not present in the pre-immune serum. The M_r 30000 protein was identified as a dimer by Fando *et al.* (1985). Using a serum dilution of 1:400, as little as 30 ng of restrictocin could be detected with a dot-blot protocol. The antibody produced has an ELISA titre of 1:4800, as determined by an antibody dilution series and 50% of maximal reaction.

Sequence of restrictocin

The sequence of the restrictocin cDNA contained the coding region of the mature protein; it and our genomic sequence are in agreement with the genomic sequence reported by Lamy & Davies (1991). The 6.4 kb fragment of genomic DNA that we cloned contained the entire restrictocin gene between the internal *Eco*RI site at 1.4 kb and the *Hind*III site at 2.5 kb. Other restriction sites identified were *Xma*I at 2.0 kb and 5.5 kb; *Hind*III, 3.0 kb; *Bam*HI, 3.2 kb; and *Sac*I, 4.8 kb. The cDNA sequence of restrictocin was compared with the cDNA sequence of α -sarcin (Oka *et al.*, 1990) and 79% identity was found in the coding region. The non-coding regions have 39.4% similarity (gap weight 5.0, length weight 0.3).

Fig. 1. Silver-stained SDS gel showing purified restrictocin. Lane 1, restrictocin purified by IRC-50 cation-exchange resin; lane 2, restrictocin further purified by membrane filtration. Migration of M_r markers is indicated at the left.

Table 1. Comparison of the leader sequences of *A. awamori* glucoamylase and *A. restrictus* restrictocin

Protein	Signal peptide	pro Region	First amino acid -mature
Glucoamylase	MSFRSLLALSGLVCTGLA	NVISKR	A
Restrictocin	MVAIKNFLLAATAVSVLA	APSPLDAR	A

Fig. 2. Northern analysis of the total RNA from *A. restrictus* at different stages of growth. The cDNA fragment was used as a probe. Different amounts of total RNA were loaded: lane 1, 12 h growth; lane 2, 24 h growth; lane 3, 36 h growth; lane 4, 48 h growth. Standards are indicated at the left. Ethidium bromide staining and densitometric analysis showed that the ratio of total ribosomal RNA in the four lanes was 4.3:2.7:2.1:1. Densitometric analysis of the hybridization bands in the four lanes of the autoradiograph gave a ratio of 1:8.6:11.8:22.

The manner by which restrictocin is made (and the ribosome making it not being inactivated) is suggested from the cDNA and genomic sequences. A leader sequence (27 amino acids N-terminal to the form found in the medium) is indicated by the genomic and cDNA sequences. The secretion of restrictocin by transformed *Aspergillus nidulans* (Lamy & Davies, 1991) indicates that other species of *Aspergillus* are able to recognize this signal sequence. Efficient secretion of the protein away from the ribosome may protect the ribosome from the action of restrictocin.

Table 1 compares the leader sequence of restrictocin with the leader sequence of glucoamylase from *Aspergillus awamori* which also contains a potential 'pro' region (Innis *et al.*, 1985). A signal peptidase site was demonstrated in the processing of glucoamylase when it

was expressed in the heterologous system of *Saccharomyces cerevisiae* (Innis *et al.*, 1985). Further processing in *S. cerevisiae* was confirmed when the protein was expressed in a KEX2 mutant that produced glucoamylase with an N-terminal extension of six amino acids. The amino acid sequence before alanine-18 of glucoamylase contains a hydrophobic region of 13 residues and a basic residue which is directly comparable to that of restrictocin (Table 1). A signal cleavage site after either alanine-19 or -20 of restrictocin would leave an N-terminal extension of seven to eight amino acids that would have to be removed in further processing (Table 1).

While the signal peptidase and further endoproteolytic processing have never been demonstrated for either of these proteins in the original host, these protein

Fig. 3. Immunoblot analysis of proteins secreted and cell-associated at different stages of growth. Lane 1, 25 h medium; lane 2, 25 h cell; lane 3, 35 h medium; lane 4, 35 h cell; lane 5, 47 h medium; lane 6, 47 h cell; lane 7, 60 h medium; lane 8, 60 h cell; lane 9, 70 h medium; lane 10, 70 h cell. The samples loaded for cells and medium represented 500 and 50 μ l of culture respectively.

extensions, if present, could serve a variety of purposes. The final destination of a protein in a filamentous host can be quite distal to its point of synthesis. These peptides could serve as a targeting system for the proteins, or in the case of restrictocin it is possible that N-terminal extensions could inactivate the cytotoxin as suggested by Lamy & Davies (1991). We are conducting further studies to detect N-terminal extensions and their role in protein targeting in *A. restrictus*.

Expression of mRNA and protein production

Fig. 2 is a representative Northern analysis where only a trace amount of the *res* mRNA could be detected at 12 h. The *res* mRNA increased dramatically between 12 and 24 h and in this experiment reached its highest level at our last time point of 48 h. Densitometric measurements of the photographs of the ethidium-bromide-stained RNA gel and the Northern analysis (Fig. 2) showed that the amount of probe hybridizing increased 14-, 24- and 95-fold at 24, 36 and 48 h respectively. Significant growth had taken place by 12 h with germ tubes extending from the germinated conidia.

Immunoblot analysis indicated restrictocin was first detected in both the cell and medium when the *res*

mRNA was present at detectable levels. In the experiment represented by Fig. 3, the mRNA was already present in the first sample. The amount of detectable restrictocin was higher in the supernatant. However, this is not shown in this immunoblot where the cell sample represented 10-fold more of the culture than the supernatant. The cell material always contained more restrictocin dimer than did the supernatant. Dissolution of the dimer by SDS might not have been complete in the cell material due to the greater amount of protein in the sample.

The Northern and immunoblot analysis indicated that the mRNA and the protein are made after significant growth has accumulated in the liquid culture and the messenger is probably used and not stored. Most of the protein is secreted into medium. The protein associated with the cells increases with time but not as much as that secreted into the medium.

Microscopy

Liquid cultures of *A. restrictus* were observed during the entire growth and production curve of restrictocin. In medium containing glucose the germinating conidia aggregated into large balls soon after growth had

Fig. 4. Differential interference contrast photomicrograph of *A. restrictus* at 24 (a) and 35 h (b) of growth. Bars, 5 μ m.

commenced. In medium containing starch this aggregation was much less evident. Hyphal growth was observed following germination and after 25 to 35 h an enlargement of the hyphal tip, with branching near the tip, could be observed on some of the hyphae in the culture (Fig. 4). Often these would also contain a second layer of cells, also as shown in Fig. 4. Differentiated structures that are similar to conidiophores, sterigmata and conidia (Thom & Raper, 1945) were observed in submerged cultures of *A. restrictus*. These structures can be observed in the hyphae that remain free in the medium and are not just associated with hyphae that have stuck to the sides of the vessel.

In the experiment reported in Fig. 3, enlarged hyphal tips that had started to branch were observed at 25 h and complete differentiation was first observed in the 35 h sample. Upon continued incubation, some of these extensions would undergo further growth and result in a slightly longer hyphal filament that died and lysed after this extension. When differentiation first started the differentiated structures were rare in the culture. By the time restrictocin was very evident as detected by immunoblot, as many as 50% of the observed hyphal tips on the edges of clumps had differentiated.

In more than 10 growth experiments analysed, the presence of differentiated structures was observed in the samples when restrictocin accumulated in the medium. We have also observed similar structures and timing of α -sarcosine appearance in cultures of *A. giganteus* (data not shown). In the four experiments where mRNA was analysed, along with protein production, the first evidence of *res* mRNA was when these differentiated structures could be observed. The mRNA from cultures that did not yet contain these structures did not have

RNA bands that hybridized significantly with the probe, and maximum mRNA and protein accumulation correlated with the highest frequencies of differentiated structures.

Conclusions

The results from our studies show that restrictocin is produced only after significant growth of *A. restrictus* has taken place. This is common with many toxic secondary metabolites produced by filamentous fungi. The accumulation of restrictocin in the medium is coincident with the appearance of both mRNA and differentiated structures in the culture. The results suggest that restrictocin is coordinately regulated with, or involved in, the process of conidiation.

The question of the function of restrictocin in *Aspergillus* remains unanswered. The appearance of the protein in the medium coordinate with the synthesis of mRNA precludes a hypothesis of storage of the protein in some inactive form. Restrictocin is made in cultures undergoing differentiation in liquid medium. It is possible that these structures would have restrictocin associated with them if they were formed by growth on solid medium. Also the protein itself could be involved in the selective inactivation of fungal cells in the culture.

A survival advantage might be conferred on the fungus by the production of restrictocin during the process of conidiation. If the conidiophore or associated cell structures have restrictocin associated with them then insect predation might be deterred. The protein might also kill an insect by inactivating cells in the digestive system and the conidia that were present could germinate and grow on the insect.

An alternative function can be proposed for restrictocin. A restrictocin-like protein could cause inflammation and local cell death in Aspergillosis infections (Arruda *et al.*, 1990). If restrictocin is involved in the invasion of the lungs, the protein should already be present on the initial particles deposited in the lung. Our results indicate that initial growth of the fungus in the lung cannot be assisted by the inflammation provided by restrictocin unless the protein is formed quickly in the lungs. Arruda *et al.* (1991) have indicated that a restrictocin-like protein in *Aspergillus fumigatus* cultures requires growth of the fungus and is not localized on the conidia.

Experiments to determine the cellular location of restrictocin in cells from liquid or solid medium and the conditions that regulate restrictocin production should provide important information on the function of this protein. The accompanying paper (Brandhorst & Kenealy, 1992) reports experiments on the cellular location of restrictocin.

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