A secreted aspartic proteinase from *Glomerella cingulata*: purification of the enzyme and molecular cloning of the cDNA

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A secreted aspartic proteinase from *Glomerella cingulata* (GcSAP) was purified to homogeneity by ion exchange chromatography. The enzyme has an *M_r* of 36 000 as estimated by SDS-PAGE, optimal activity from pH 3.5 to pH 4.0 and is inhibited by pepstatin. The N-terminal sequence, 23 residues long, was used to design a gene-specific primer. This was used in 3' RACE (rapid amplification of cDNA ends) PCR to amplify a 1.2 kb fragment of the *gcsap* cDNA. A second gene-specific primer was designed and used in 5' RACE PCR to clone the 5' region. This yielded a 600 bp DNA fragment and completed the open reading frame. The *gcsap* open reading frame encodes a protein with a 78 residue prepro-sequence typical of other fungal secreted aspartic proteinases. Based on the deduced sequence, the mature enzyme contains 329 amino acids and shows approximately 40% identity to other fungal aspartic proteinases. Subsequent cloning and sequencing of *gcsap* fragments obtained from PCR with genomic DNA revealed a 73 bp intron beginning at nt 728. Southern analyses at medium and high stringency indicated that *G. cingulata* possesses one gene for the secreted aspartic proteinase, and Northern blots indicated that gene expression was induced by exogenous protein and repressed by ammonium salts. GcSAP is a putative pathogenicity factor of *G. cingulata*, and it will now be possible to create SAP⁻ mutants and assess the role GcSAP plays in pathogenicity.

Keywords: *Glomerella cingulata*, fungi, cDNA cloning, 3' and 5' RACE-PCR, secreted aspartic proteinase

INTRODUCTION

The plant-pathogenic fungus *Glomerella cingulata* (anamorph *Colletotrichum gloeosporioides*) causes anthracnose diseases of a variety of crops world-wide, but particularly in sub-tropical climates (Mordue, 1971; Irwin & Cameron, 1978). Infection involves the attachment of the spore to the host surface, differentiation to form an appressorium and direct penetration of the cuticle and epidermal cell walls. It is widely accepted that penetration of the cuticle results from both the mechanical force generated by a high internal pressure in the appressorium, and the activity of cutinase secreted

by the fungus (Bailey *et al.*, 1993). The subsequent development of the infection depends on the secretion of an array of enzymes, such as endopolygalacturonases, cellulases, pectin lyase, xylanase and β -galactosidase (for reviews see Collmer & Keen, 1986; Bailey *et al.*, 1993; Cooper, 1977) which in concert degrade structural components of plant tissue.

Extracellular proteinases, especially those of animal pathogens, have been widely implicated as pathogenicity factors for several decades (for a review see Ogrydziak, 1993), but remarkably few of these studies have encompassed the proteinases produced by phytopathogenic fungi. Studies of an aspartic proteinase of *Botrytis cinerea* (Movahedi & Heale, 1990) showed that secretion of this enzyme is an early event in infection by *B. cinerea*, occurring before pectic enzyme production. Proteinase activity caused plant cell death and significantly higher levels of activity were secreted by virulent

Abbreviations: GcSAP, secreted aspartic proteinase from *Glomerella cingulata*; RACE, rapid amplification of cDNA ends.

The GenBank accession number for the nucleotide sequence reported in this paper is U43775.

compared with less virulent strains of the fungus. It is also noteworthy that proteinase inhibitors are widespread in the plant kingdom (for reviews see Ryan, 1973; Aviles, 1993) and it has been shown that inhibitor activity in melon seedlings infected with *Colletotrichum lagenarium* increased sharply 3 d after spore inoculation (Roby *et al.*, 1987). Similarly, accumulation of a trypsin inhibitor in tomato plants has been correlated with resistance to *Phytophthora infestans* (Peng & Black, 1976). Collectively, these studies suggest, but do not unequivocally establish, that proteinases have significant roles in pathogenicity.

Production of a proteolytic activity by *G. cingulata* was first detected when sections of apple fruit rotted by *G. cingulata* were assayed for enzyme activity (Kuc & Williams, 1962). More recently, *G. cingulata* has become a particularly suitable organism for the assessment of putative pathogenicity factors with the development of protocols for targeted gene disruptions (Rikkerink *et al.*, 1994; Bowen *et al.*, 1995) and the identification of genes selectively expressed during appressorium formation (Hwang & Kolatukudy, 1995). It should now be possible to assess the roles of the secreted proteinase, and as a first step towards this objective we describe here the purification of a *G. cingulata* secreted aspartic proteinase (GcASP), some properties of the enzyme, and molecular cloning of the cDNA and the gene encoding the enzyme.

METHODS

Organisms and plasmids. *G. cingulata* was obtained from the International Collection of Micro-organisms from Plants (ICMP), Maanaki Whenua Landcare NZ. *Escherichia coli* strain DH5 α was the host for recombinant plasmids. The plasmid pBluescript KS II(+) (Stratagene) was used for cloning experiments.

Media and reagents. *G. cingulata* cultures were maintained on potato dextrose agar. *E. coli* cultures were grown in Luria broth, supplemented with ampicillin as required (Sambrook *et al.*, 1989). BSA (fraction V) was obtained from Boehringer Mannheim, and bovine haemoglobin (type II) was supplied by Sigma. *Taq* DNA polymerase and SUPERScript RNase H⁻ reverse transcriptase were from Gibco BRL. Oligonucleotides were produced by an Applied Biosystems 380B synthesizer using β -cyanoethyl chemistry.

Enzyme purification. Twenty 200 ml cultures of *G. cingulata* were grown in a nitrogen-free salts medium (Wickerham, 1946) containing 0.2% (w/v) BSA and 2% (w/v) glucose (static for 3 d in 1 l Roux flasks at 26 °C). After harvesting the mycelium by centrifugation, the supernatant was adjusted to pH 7.0 with 6 M NaOH and concentrated to approximately 500 ml by ultrafiltration (molecular mass cut-off 10000 Da). After dialysis for 17 h against 10 mM sodium citrate buffer, pH 6.8, the concentrate was applied to a DEAE-Sepharose column (2.5 $\times$ 17 cm) equilibrated with buffer, and the column washed with similar buffer at a flow rate of 1 ml min⁻¹ until the A_{280} returned to zero. Bound protein was eluted with a linear gradient of 10 to 250 mM sodium citrate, pH 6.3 (400 ml) and was collected in 10 ml fractions. Fractions 15–30 contained proteinase activity and were pooled and con-

centrated to approximately 15 ml by ultrafiltration and dialysed against 20 mM Bis-tris buffer, pH 6.0 (fraction 1). Aliquots (0.8 ml) of this concentrate were applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated in the same buffer. The column was eluted with a gradient of 0 to 200 mM NaCl in 20 mM Bis-tris, pH 6.3, over 30 min at a flow rate of 0.75 ml min⁻¹. Fractions containing the enzyme were pooled and stored at -20 °C (fraction 2).

Proteinase enzyme assays. Proteinase activity was estimated by measuring the hydrolysis of (a) BSA or (b) haemoglobin. Enzyme activity was also detected in the growth medium with Azocoll (Calbiochem).

BSA assay. This was based on the assay of MacDonald & Odds (1980). Each assay mixture contained 0.6 ml 1% BSA in 50 mM sodium citrate buffer, pH 3.2, and 0.2 ml enzyme solution. After 2 h at 37 °C, 0.4 ml 10% (w/v) trichloroacetic acid was added, the tubes were stored on ice for 10 min and then centrifuged (13000 r.p.m. for 10 min). The absorbance of the supernatant was read at 280 nm and corrected for background using a zero-time control in which trichloroacetic acid was added prior to the enzyme. One unit of enzyme was defined as that amount which catalysed a change in A_{280} of 1.0 min⁻¹.

Haemoglobin assay. Each assay mixture contained 0.2 ml enzyme solution and 1 ml acid-denatured haemoglobin [2% (w/v) in 0.2 M glycine/HCl, pH 3.5]. After incubation at 30 °C for 30 min, a 300 μ l aliquot was mixed with 200 μ l 10% trichloroacetic acid and incubated on ice for 10 min before being centrifuged at 3600 r.p.m. for 10 min. Zero-time controls were obtained by taking a 300 μ l aliquot of the assay immediately after the enzyme was added. Samples (75 μ l) of the supernatant were neutralized with 75 μ l 0.5 M NaOH and assayed for soluble peptides by the Lowry method using tyrosine as a standard. One unit of enzyme activity was defined as that amount which released 1 μ mol tyrosine equivalents min⁻¹.

Protein estimations. These were performed using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985).

SDS-PAGE and electrotransfer. SDS-PAGE was carried out with 10% (w/v) acrylamide gels (Laemmli & Favre, 1973) and the following proteins were used as molecular mass standards: β -galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.5 kDa). Electrotransfer to a PVDF membrane (Problott) was done in buffer consisting of 96 mM glycine, 12.5 mM Tris/HCl, pH 8.3 and 20% (v/v) methanol. After 2 h at 75 V and 250 mA, the transfer was visualized with 0.1% Ponceau S in 1% acetic acid. The membrane was destained in 1% acetic acid, washed with distilled water and blotted dry between filter paper.

N-terminal sequencing. The proteinase was sequenced on an Applied Biosystems (ABI) 470A/170A on-line gas-phase sequencer using standard ABI protocols. Stained protein bands on the Problott membrane were excised and directly applied to the sequencer. A total of approximately 200 pmol protein was applied for analysis.

DNA and RNA manipulations. Restriction endonucleases and DNA-modifying enzymes were incubated under the conditions recommended by the suppliers. Standard conditions for molecular cloning, hybridization, transformation and electrophoresis were used (Sambrook *et al.*, 1989). Molecular mass markers were *Hind*III fragments of bacteriophage I (Gibco

BRL). Purification of DNA fragments from agarose gels was done as described by Vogelstein & Gillespie (1979). DNA sequencing was performed on an ABI 373A automated sequencer using standard ABI protocols. *G. cingulata* DNA was isolated as described by Raeder & Broda (1985). *G. cingulata* RNA for RACE was extracted from either 48 h stationary cultures or 36 h shake cultures by the methods of Carlson & Botstein (1982) and Teeri *et al.* (1987), respectively. These cultures were grown in the BSA medium described above. The method of Teeri *et al.* (1987) was also used to extract RNA for Northern analysis. Poly(A⁺) RNA was purified from 1 mg total RNA in a batchwise manner using 30 mg microcrystalline oligo (dT)-cellulose (New England Biolabs). Binding and elution buffers used were those recommended by the manufacturer. For Southern blotting, the membranes were hybridized at 56 °C and then washed as follows: twice with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS at room temperature for 10 min, and then 2 × SSC, 0.5% SDS at 63 °C for 1 h (medium stringency). This was followed by a higher stringency wash of 0.1 × SSC, 0.5% SDS at 68 °C for 1 h. Northern membranes were hybridized at 65 °C and then washed twice with 2 × SSC, 0.5% SDS at room temperature for 15 min, and then twice at 65 °C for 10 min.

Rapid amplification of cDNA ends (RACE)

3' RACE. The basic protocol for 3' RACE was that described by Ausubel *et al.* (1992). Three primers were required. A gene-specific oligonucleotide (SAP-primer1) [5'-GGGGTACCAGGA(C/T)TA(C/T)GA(C/T)GT(A/G)AG-(G/A/C/T)TA] was based on the sequence of amino acid residues QDYDVEY in the determined N-terminal sequence of mature GcSAP. SAP-primer1 also contained a *KpnI* site. A poly T primer [5'-GGGGTACCGTCGACATCGA(T₁₂)] that contained an adaptor sequence was used in first strand cDNA synthesis. The adaptor sequence contains restriction sites for *KpnI*, *Sall* and *Clal*, and an adaptor primer containing this sequence (5'-GGGGTACCGTCGACATCGA) was made for use in PCR with SAP-primer1. Total RNA was reverse-transcribed under conditions recommended by Gibco BRL. For 20 µl PCR reactions, 2 µl undiluted cDNA was used. Optimized PCR conditions included 5 cycles with an annealing temperature of 56 °C and a further 35 cycles with annealing at 55 °C. The MgCl₂ concentration was 2.5 mM.

5' RACE. This was performed using the 5'-AmpliFINDER RACE kit from Clontech. Two gene-specific primers were synthesized. Oligonucleotide SAP-primer2 (5'-GCTCTAGAAAAAGTCTTCTG) contained sequence complementary to the region from nt 652 to 666 as well as a restriction site for *XbaI* and was used to make first strand cDNA. Oligonucleotide SAP-primer4 (5'-GGGGTACCGTAGGAGATGGACCA) contained sequence complementary to the region from nt 454 to 470 and a restriction site for *KpnI*. After first strand cDNA had been made, AmpliFINDER anchors were ligated to the 3' ends of the cDNAs under conditions recommended by Clontech. The anchored cDNA was used in PCR with SAP-primer4 and an anchor primer provided in the 5' RACE kit. The anchor primer contained an *EcoRI* site. Optimized PCR conditions included an annealing temperature of 60 °C and a MgCl₂ concentration of 2.5 mM in a reaction volume of 50 µl.

PCR of a genomic sap fragment. *G. cingulata* genomic DNA was used as the template in a PCR reaction with primers SAP-primer1 and SAP-primer3 (5'-GGGGTACCATCGAAGACAACGAA). SAP-primer3 contained sequence complementary to the region from nt 1238 to

1255. Annealing was done at 57 °C for 5 cycles, then at 56 °C for 30 cycles. The MgCl₂ concentration was 2.5 mM.

Computer programs. Editing, analysis and alignment of sequences was carried out using the ABI SeqEd Macintosh program, and PILEUP, LINEUP and SEQED programs from the Genetics Computer Group (Wisconsin Sequence Analysis Package, version 8). Autoradiograms were scanned with a Bio-Rad GS-670 Imaging densitometer and the digital images were processed with Adobe Photoshop 3.0 and Canvas 3.5.1 from Denaba systems.

RESULTS AND DISCUSSION

Induction and purification of GcSAP

Providing protein as the sole nitrogen source in fungal cultures has been widely documented as a system for induction of proteinase production (Ross *et al.*, 1990, and references cited therein) and this strategy was used for *G. cingulata* proteinase induction. *G. cingulata* grew well when cultured in salts medium containing either (NH₄)₂SO₄ or BSA as the sole nitrogen source. Proteinase activity in the culture medium was monitored for 5 d and detected in the BSA cultures in assays with either BSA or Azocoll as the substrate. Maximum proteinase activity was observed on day 3 in the BSA medium and there was no proteinase activity in the medium in which (NH₄)₂SO₄ was the nitrogen source.

For proteinase enzyme purification (see Methods for details), *G. cingulata* was cultured in BSA medium for 3 d. Following concentration by ultrafiltration, the growth medium was dialysed against 10 mM sodium citrate buffer, pH 6.8, and the crude enzyme was applied

Fig. 1. SDS-PAGE of GcSAP. Lane 1, purified GcSAP (10 µg of fraction 2, as described in Table 1); lane 2, molecular mass standards as indicated on the right.

Table 1. Purification of GcSAP

Fraction*	Volume (ml)	Activity (U)†	Protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)
F _{0-c}	544	10.5	6800	0.0015	100
F ₁	14.5	6.3	8.7	0.7	60
F ₂	10.5	1.5	2.0	0.8	14

*F_{0-c}, concentrated growth medium; F₁, concentrated dialysed fraction from DEAE-Sepharose chromatography; F₂, pooled active fractions (peak 3) from Mono Q chromatography.

† Activity was measured in the BSA assay (see Methods).

to a DEAE-Sepharose column. Most of the protein, as judged by the A₂₈₀ profile, was eluted during loading, washing with buffer, and in the 10–100 mM sodium citrate gradient. The proteinase was recovered in fractions 15–30 from 100–200 mM sodium citrate, pH 6.3. These fractions were pooled, concentrated and dialysed against 20 mM Bis-tris buffer, pH 6.0 (fraction 1). Separation of proteinase from other proteins was achieved by chromatography on a Mono Q column with a gradient of 0–200 mM NaCl in Bis-tris buffer, pH 6.3. The third and final peak contained the activity and a single protein of 36 kDa as judged by SDS-PAGE electrophoresis (Fig. 1). No other proteinase activity was detected during the fractionation. Table 1 summarizes the purification of GcSAP.

Properties of GcSAP

GcSAP exhibited properties typical of a fungal secreted aspartic proteinase. In assays with haemoglobin as substrate maximum activity was at pH 3.5–4.0. However, in contrast with other secreted aspartic proteinases, significant activity was observed over a wide range (pH 2.0–7.0) and at pH 7.0 was 40% of the maximum value (data not shown). The activity was completely inhibited by pepstatin, a specific aspartic proteinase inhibitor. The purified GcSAP also degraded BSA, keratin and casein, but haemoglobin was the best substrate. The specific activity of purified GcSAP in the BSA assay was 0.8 U mg⁻¹ which is low compared to SAP2 from *Candida albicans*, which has an activity of 6.8 U mg⁻¹ (Wright *et al.*, 1992). However, the specific activity of GcSAP in the haemoglobin assay (4.1 U mg⁻¹) is comparable to the reported value for the secreted aspartic proteinase from *B. cinerea* (3.1 U mg⁻¹) assayed under the same conditions (Movahedi & Heale, 1990).

N-terminal sequence of GcSAP

GcSAP was electroblotted onto a PVDF membrane and subjected to automated Edman degradation. This yielded a sequence of 23 residues (ATGSVAATPQDYDVEYLSPV?IG) and was subsequently matched with amino acids 79–102 of the deduced sequence (Fig. 2). This experimentally determined N-terminus has 74% similarity and 57% identity when aligned with the N-terminal sequences of

Rhizopus chinensis rhizopuspepsin II and *Rhizopus niveus* aspartic proteinase 1, as shown in Fig. 3.

Cloning and sequencing of the *gcsap* cDNA

3' RACE. Total RNA was extracted from *G. cingulata* grown in BSA medium and used to make cDNA templates for PCR. Based on the size of the GcSAP protein, the lengths of typical 3' untranslated sequences and poly(A⁺) tails associated with eukaryotic mRNA transcripts, it was anticipated that the PCR product would be approximately 1.2 kb. PCR with the adaptor primer and gene-specific primer SAP-primer1 yielded two products close to the expected size (1.2 kb and 990 bp). Control PCR reactions were performed with only one primer (the adaptor primer) and cDNA was derived from either (NH₄)₂SO₄ or BSA cultures. These eliminated the 990 bp PCR product as a *gcsap* fragment, and the 1.2 kb product was reamplified from a band stab. The amplified fragment was digested with *Kpn*I, gel-purified and ligated into the *Kpn*I site of pBluescript KS II(+), yielding pSJ1. Sequencing of pSJ1 confirmed that the PCR product coded for the *gcsap* cDNA because the deduced amino acid sequence matched exactly with the N-terminus of GcSAP determined by the Edman degradation. The *gcsap* cDNA fragment consisted of 960 bp of coding sequence, which represented 97% of the mature protein, and contained 200 bp of downstream sequence (Fig. 2).

5' RACE. Poly(A⁺) RNA was purified from RNA enriched with the transcript of GcSAP and used to make the cDNA template for PCR. Following the ligation of an anchor oligonucleotide to the 3' ends of the cDNA preparation, PCR was performed using the anchor primer and the gene-specific primer SAP-primer4. One product of 600 bp was observed on agarose gels and this was subsequently cloned between the *Kpn*I and *Eco*RI sites of pBluescript KS II(+). Two clones were sequenced and this confirmed that the PCR product was the 5' region of the *gcsap* cDNA. The 5' cDNA fragment contains 104 bp of upstream noncoding sequence and 234 bp of sequence downstream of the initiation ATG that encodes a typical prepro-sequence of 78 amino acids, and this is followed by sequence coding for the mature protein. This sequence matched exactly that previously determined by 3' RACE.

	ATCGGCTCACTCCCGTCTCGATCCTCATCCTCATCTTCTCATCTCCTGCCTCTCCCGGT	-45
	CCTCTTCCTCCTCAGGTCTCTCGCTCCGCCTTGTCTGTCAAGATGACTCTCATCACCG	16
		M T L I T A
	CCCTCACCGCCGGTCTGGCTCTGGCCTCCTCCGTCAATGGAGCGCCCAACAACGCCA	76
7	L T A G L A L A S S V I G A P T N N A N	
	ACGAGAAGCGCTTACCGTCGACCAGATCAAGAACCCTCGCTACATCCGCAACGGTCCCC	136
27	E K R F T V D Q I K N P R Y I R N G P L	
	TGGCCCTCGCCAAGGCTACCGCAAGTACGGCAAGGCTCTCCCCGAGGACCTCTCCCGCG	196
47	A L A K A Y R K Y G K A L P E D L S R V	
	TCGTCGCCAACATCACCTCCACCGGTGCCACCAAGCGCGCTACCGGCAGCGTCGCCGCCA	256
67	V A N I T S T G A T K R A T G S V A A T	
	CCCCCAGGACTACGACGTCGAGTACCTGAGCCCCGTCCAGATCGGCACCCCGGCCAGA	316
87	P Q D Y D V E Y L S P V Q I G T P A Q T	
	CCCTCACCTCGACTTCGACACCGGATCTTCCGATCTCTGGGTCTTCTCCACCTCCACCC	376
107	L T L D F D T G S S D L W V F S T S T P	
	CGTCCAGCCAGCGCAACGGCCAGACCGTCTACGACCCAGCAAGTCCAGCACCGCCTCCC	436
127	S S Q R N G T V Y D P S K S S T A S R	
	GCCTCACCGCGGCCACCTGGTCCATCTCCTACGGCGACGGCTCCAGTCCAGCGGTATCG	496
147	L T G A T W S I S Y G D G S S S S G I V	
	TCTACAAGGACACCGTCTCCGTCCGGCAGCCTGTCCGTACCGGCCAGGCTGTCGAGGCCG	556
167	Y K D T V S V G S L S V T G Q A V E A A	
	CCTCCAAGGTTCTCTCTCTCTCCGAGGAGAGCGACCTGGACGGTCTCTGGGTCTCG	616
187	S K V S S S F S E S D L D G L L G L G	
	GCTTCAGCTCCATCAACACCGTCTCCCCACCCAGCAGAAGACCTTCTTCGAGACTGCCA	676
207	F S S I N T V S P T Q Q K T F F E T A K	
	AGTCCAAGTTGGATGCTTACCTCTTACTGTGACTTGAAACACAACACCCGtaagatt	736
227	S K L D A Y L F T A D L K H N T P	
	tcacttcccggtctcatatcacactcgcgagtgagacagtaaacatatagtgcacatccaca	796
	acagccggcaagtacaaacttcggctacatcgactcctctgcctacacggcgccatcacc	856
244	G K Y N F G Y I D S S A Y T G A I T	
	TACGTCAAGATCGACAACCTCCGACGGCTGGTGGCAGTTCACCTCCTCCGGCTACTCTGTC	916
262	Y V S I D N S D G W W Q F T S S G Y S V	
	GGCTCCGCCAGCTTACCTCCACCTCCCTCAACGGCATCGCTGACACCGGAACACCTC	976
282	G S A S F T S T S L N G I A D T G T T L	
	CTCCTCCTCCCCAGTCTGTCTGCTACTGCCTACTACGCCAAGATCTCCGGCGCCAAGTAC	1036
302	L L L P Q S V V T A Y Y A K I S G A K Y	
	GACTCTTCCAGGGAGGCTACACCTTCCCCCTGCTCCGCCACCGTCCCCCTCCTTACCTTC	1096
322	D S S Q G G Y T F P C S A T V P S F T F	
	GGCGTCGGCTCCGCCCGCTCACCATCCCCCGCAGCTACATGAACTACGCTCCCGTCAGC	1156
342	G V G S A R V T I P A S Y M N Y A P V S	
	ACCAGCACTTGCTTCGGTGGCCTGCAGAGCTCCTCCGGCATTGGCATCAACATCTTCGGT	1216
362	T S T C F G G L Q S S S G I G I N I F G	
	GATGTCGCCCTCAAGGCTGCCTTGTGCTTCGATGGTGCCTCCAACCGCCTTGGCTGG	1276
382	D V A L K A A F V V F D G A S N R L G W	
	GCCGCCAAGACCTCTCTTAAACACTCTTATAGAGACACAGAGAGAGAGAACTTAGAG	1336
402	A A K T L S *	
	AGGGATCTGCCCATTAGAGAGATGGCCTGAGGGATTTCGGAATGAGGACCTTGAGGACGG	1396
	AAGATCGAGGGCGCATCTGATGGGCAACTCCCTGTTTATGACAATCCATGTGATGAGA	1456
	CGCTCGAGTTTACCAATCACTGCTTCTTATACATATTTTCATATATCT	1504

Fig. 2. Nucleotide sequence of *gcsap* and derived amino acid sequence. These data were obtained from cDNA and genomic clones. The predicted signal peptidase and KEX2 processing sites in the prepro-sequence are indicated by (▼) and underlined text (KR) respectively. The N-terminal sequence data determined by Edman degradation are double-underlined. The conserved and repeated nucleotide sequences in untranslated regions, as discussed in the text, are underlined. The intron (nt 728–800) is in lower case letters.

Intervening sequence

Genomic sequence of the *gcsap* gene was obtained by performing PCR with SAP-primer1 and SAP-primer3 using genomic DNA as the template. A single product of 950 bp was produced and cloned into the *Kpn*I site of pBluescript KS II(+). Sequencing of the resulting clone revealed a 73 bp intron beginning at nt 728 (Fig. 2). The intron/exon splice junctions (GTAAG and CAG) and putative lariat structure (TAGTGACA) fit the consensus

sequences found in other filamentous fungi (Gurr *et al.*, 1987).

The untranslated sequences

The 5' untranslated region contains a triple repeat of the motif CTCATC between nt –80 and –61 (Fig. 2). Similar repeats have been detected in the same region of many *Aspergillus nidulans* genes (Ward & Turner, 1986)

	1		70
GcSAP	..MTLITALT AGLALASSVI GAPTNNAN..	..EKRFTVDQ IKNPRYIRNG PLALAKAYRK	YGKALPEDLS
RcaspII	MKFTLISSCI AIAALAVAVD AAP.....	..GEKKISIP L AKNPYKPSA KNAIQKAIK	YNK.....
Rnaspl	MKFTLISSCV ALAAMTLAVE AAP.....	..NGKKINIP L AKNNSYKPSA KNALNKALAK	YNR.....
Casap2	...MFLKNIF IALAIALLVD ATPTTT....	...KR.....	...SAG FVALDFSUVK TPKAFP....
Mmasp	MLFSQITSAL LLTAASLSLT TARPVSKQSE	SKDKLLALPL TSVSRKFSQT	KFGQQQLAEK LAGLKPFF...
	71		140
GcSAP	RVVANITSTG ATKRAATGSAV ATPQDYDVEY	LSPVQIGTPA QTLTLDFDTG	SSDLWV..FS TSTPS.....
RcaspII	HKINTSTGGI VPDAGVGTVP MTDYGNQDVEY	YGQVTIGTPG KKFNLDFDTG	SSDLWI..AS TLQTN.....
Rnaspl	RKV..GSGGI TTEAS.GSVP MVDYENDVEY	YGEVTVGTPG IKLKLDFDTG	SSDMWF..AS TLQSS.....
Casap2	..VTNGQEGK TSKR..QAVP VTLHNEQVTV	AADITVGSNN QKLNVIIVDTG	SSDLWVPDVN VDCQVTVSDQ
Mmasp	SEA AADGSVDTPG YYDFDLE.EY	AIPVSIPTG QDFLLLFDTG	SSDTWVPH.. KGGTKSEG..
	141		210
GcSAP	...SQRNGQ. TVYDPSKSSST ASRLTGATWS	ISYGDGSSSS GIVYKDTVSV	GSLSVTGQAV EAASKVSSSF
RcaspII	...CGSRQ. TKYDPKQSSST YQ.ADGRWTS	ISYGDGSSAS GILAKDNVNL	GGLLIKQQT I ELAKREAAFS
Rnaspl	...CSNSH. TKYDPKQSSST YA.ADGRWTS	ISYGDGSSAS GILATDNVNL	GGLLIKQQT I ELAKRESSAF
Casap2	TADFCK.QKG T.YDPSGSSA SQ.DLNTPFK	IGYGDGSSSQ GTLYKDTVGF	GGVSIKNQVL ADVDTSTSID.
Mmasp	...CV..GS RFFDPSASST F.KATNYNLN	ITYGTG.GAN GLYFEDSIAI	GDITVTKQIL AYVDNVRGPT
	211		280
GcSAP	SEESD.... LDGLLGLGFS SINT.....V	SPTQ...QKT FFETAKSKLD	AYLFTADLKH NTPGKYNFGY
RcaspII	ANGPN.... DGLLGLGFD TITT.....V	RGVKTMDNL ISQGLISRPI	FGVYLGKASN GGGGEYIFGG
Rnaspl	ATDVI.... DGLLGLGFD TITT.....V	RGVKTMDNL ISQGLISRPI	FGVYLGKASN GGGGEYIFGG
Casap2	QGILGVGYK TNEA.....G	GSYDNVPVTL KKQGVIAKNA	YSLYLNSPD. AATGQIIFGG
Mmasp	AEQSPNADIF LDGLFGAAYP DNTAMEAEG	STYNTVHVN L YKQGLISSPL	FSVYMN..TN SGTGEVVFEG
	281		350
GcSAP	IDSSAYTGAI TYVSIDNSDG ...WWQFTSS	GYSVGSASFT ..STSLNGIA	DTGTTLLLLP QSVVTAYYAK
RcaspII	YDSTKFKGSL TTVPIDNSRG ...WWGITVD	RATVG.TSTV ..ASSFDGIL	DTGTTLLILP NN.VAASVAR
Rnaspl	YDSSKFKGSL TTVPIDNSRG ...FWGVTVK	STKIG.GTTV ..SASFDAIL	DTGTTLLLLP DD.VAAKVAR
Casap2	VDNAKYSGSL IALPVTSD.R ...ELRISLG	SVEVS.GKTI N.TDNVDVLL	DSGTTITYLQ QD.LADQIIK
Mmasp	VNNTLLGGDI AYTDVMSRYG GYFWDAPVT	GITVDGSAV RFSRPQAFIT	DTGTNFFIMP SS.AASKIVK
	351		420
GcSAP	ISGAKYDSSQ GG...YTFF QSAT.VPSFT	FGV..... GSARVTIPAS	YMN..YAPVS TS...TCFGG
RcaspII	AYGAS.DNGD GT...YTIS CDTSRFKPLV	FSI..... NGASFQVSPD	SLV..FEEYQ .G...QCIAG
Rnaspl	SYGAS.DNGD GT...YSIT CDTSKLQPLV	FTL..... GSSTFEVPSD	SLI..FEKDG .N...KCIAG
Casap2	AFNGK.LTQD SNGNSFYEDV CNLSGD.VVF	NFS..... KNAKISVPAS	EFAASLQGDQ GQPYDKCQLL
Mmasp	AALPDATETQ QG...WVVP CASYQNSKST	ISIVMQKSGS SSDTIEISVP	VSKMLLPVDQ SNE..TCMFI
	421		469
GcSAP	LQSSSGIGIN IFGDVALKAA FVVFDGASNR	LGWAAKTL*	
RcaspII	F.GYGNFDFA IIGDTFLKNN YVFNQGVPE	VQIAPVAQ*	
Rnaspl	F.AAGG.DLA ILGDVFLKNN YVFNQGVPE	VQIAPVAN*	
Casap2	F.DVNDA..N ILGDNFLRSA YIVYDLDDNE	ISLAQVKYTS	ASSISALT*
Mmasp	I.LPDGGNQY IVGNLFLRF VNVYDFGNR	IGFAPLASAY	ENE*.....

Fig. 3. Alignment of fungal aspartic proteinases. GcSAP, secreted aspartic proteinase of *G. cingulata* (GenBank accession number U43775); RcaspII, *R. chinensis* rhizopuspepsin II (L33857); Rnaspl, *R. niveus* aspartic proteinase 1 (Horiuchi *et al.*, 1988, M19100); Casap2, *C. albicans* SAP2 (Wright *et al.*, 1992, M83663); Mmasp, *M. miehei* aspartic proteinase (Gray *et al.*, 1986, M15267). Paired basic amino acids in the prepro-sequence are underlined and the N-terminal residues of the mature enzymes are in bold type and underlined. The catalytic aspartic residues are in bold type, and conserved cysteine residues are indicated by shading.

and also 5' of the initiation ATG of the *gpdA* gene of *G. cingulata* (Templeton *et al.*, 1992). The sequence directly upstream of the start codon (CAAGATGAC) closely resembles the consensus sequence (CA^C/_A^A/cCATGGC) for filamentous fungi, although a G in the -1 position is rare (Gurr *et al.*, 1987).

The 3' region of the *gcsap* cDNA was cloned using a poly(T) primer which presumably annealed to the poly(A⁺) region of the mRNA. However, there is no consensus eukaryotic polyadenylation signal (AAUAAA) in the 3' untranslated region. But this region (nt 1309–1504) contains a number of intriguing features: the sequence from nt 1309 to 1328 contains eight GA repeats; there are two copies of a GAGGAC motif between nt 1380 and 1394; and a palindrome of the

motif TATAC is located 5–20 nt before the end of the sequence. The significance of these features is not known.

Analysis of genomic DNA

Southern analysis was used to determine the copy number and to detect any closely homologous genes in the genome. Genomic DNA from *G. cingulata* was digested with *EcoRI*, *BamHI* and *KpnI*, which do not cut within the cloned sequence, and with *SalI*, *PstI*, *SacI* and *BglII*, which each cut once within the sequence. Hybridization with a 950 bp probe and washing at both medium and high stringency revealed one band in each digest, except for the *BglII* digest which had a major

Fig. 4. Southern analysis of *gcsap*. A Southern blot of restriction-enzyme-digested genomic DNA was probed with a 950 bp gene fragment. The region of the gene covered by the probe is shown in Fig. 5. Lanes: 1, *EcoRI*; 2, *BamHI*; 3, *PstI*; 4, *SacI*; 5, *KpnI*; 6, *SalI*; 7, *BglII*. (a) Membrane washed at medium stringency; (b) membrane washed at high stringency (see Methods).

Fig. 5. Restriction map of the *gcsap* gene sequence. The PCR product amplified from genomic DNA using SAP-primer1 and SAP-primer3 (see Methods) was cloned and used as a probe, and this is represented by the solid line below the map. The coding regions are represented by the black boxes. The intron and other untranslated regions are shown as a narrow line.

band at 10.6 kb and a faint band at 1.7 kb (Fig. 4a, b). These results are consistent with the restriction map of the *gcsap* gene and the region covered by the probe (Fig. 5). The *SalI* site is upstream from the region which hybridized to the probe and the second *PstI* and *SacI* fragments only overlap the probe by 70 bp and were not detected. These results indicate that the genome does not contain other genes that are closely related to *gcsap*.

Expression of *gcsap*

The secretion of GcSAP is induced by exogenous protein and repressed by ammonium salts. A Northern analysis indicated that this regulation is at the level of transcription. As shown in Fig. 6, a transcript of 1.7 kb was detected when BSA was the sole nitrogen source but was absent when NH_4Cl was the sole nitrogen source. These results are consistent with the pattern of proteinase secretion under these growth conditions. There was also no *gcsap* transcript detected when the growth media contained both BSA and NH_4Cl , indicating that ammonium salts repressed expression of the gene.

The amino acid sequence

The deduced amino acid sequence of GcSAP is 38 % and 51 % identical with rhizopuspepsin (Delany *et al.*, 1987) and penicillopepsin (James & Sielecki, 1983) respectively.

Fig. 6. Northern analysis of *gcsap*. *G. cingulata* was grown in media where the nitrogen source was either BSA (lane 1), NH_4Cl (lane 2) or BSA + NH_4Cl (lane 3). RNA was extracted after 48 h growth and analysed with the probe illustrated in Fig. 5. All lanes contained approximately the same amount of total RNA.

ively. The alignment in Fig. 3 indicates in particular that the regions surrounding the two catalytic aspartic acid residues are absolutely conserved.

The N-terminus of the mature enzyme starts at position 79 (Fig. 2), as determined by Edman sequencing of pure GcSAP. Most aspartic proteinases possess a pro-sequence of 50 residues (Davies, 1990) and a prepro-sequence of 70–80 residues and the predicted prepro-sequence in GcSAP (78 residues) is typical. It contains a hydrophobic core signal sequence from position 3 to 12

and there is a putative signal peptidase cleavage site between residues 16 and 17 (Fig. 2). Cleavage here would yield a protein with a pro-sequence of 62 amino acids. The pro-sequence contains multiple basic residues including three pairs of basic residues; basic residues are common in pro-sequences of aspartic proteinases (Davies, 1990). The last of the paired basic residues (Lys₇₇-Arg₇₈) is adjacent to the mature N-terminus and is probably the cleavage site of a KEX2-type processing enzyme (Bussey, 1988). The other basic amino acid residues in the pro-sequence may bind to the active site aspartic acid residues, as proposed for pepsinogen (James & Sielecki, 1986), thus giving the pro-sequence a possible role in self-inhibition of pro-GcSAP. Based on comparisons with pepsinogen, probable candidates are Arg₃₉, Arg₅₃ and Lys₅₄ as they are each adjacent to a tyrosine residue, which in pepsinogen stabilizes the interaction by hydrogen-bonding to the active site aspartic acids.

There are no Asn-Xxx-Ser/Thr motifs in the sequence and hence no potential sites for N-glycosylation. The SDS-PAGE-estimated M_r of 36000 is consistent with a deduced M_r of 34200.

GcSAP contains only two Cys residues, at positions 332 and 365 (Fig. 2). These residues align with conserved Cys residues in other aspartic proteinases (Fig. 3) which are known to form a disulphide bond (Cutfield *et al.*, 1995). Interestingly, GcSAP does not possess the first pair of conserved Cys residues (positions 133 and 145, Fig. 3) beyond the first active site aspartic residue. This disulphide bond loop is common in many aspartic proteinases, but it is clearly not essential for activity because it is not present in some other aspartic proteinases, for example endothiopepsin and penicillopepsin (Cutfield *et al.*, 1995).

Many filamentous fungi and some yeasts secrete aspartic proteinases; for example, *C. albicans*, the common human fungal pathogen, possesses at least eight genes encoding enzymes of this type (Hube *et al.*, 1994) and *Rhizopus* species such as *R. niveus* also have a similar family (Delaney *et al.*, 1987). Other examples include *Aspergillus awamori*, *Mucor miehei* and *Endothia parasitica* (for a review see Ward & Kodama, 1991). The properties of the GcSAP as determined in this study, for example M_r , conserved regions of amino acid sequence, inhibition by pepstatin and optimum activity at acidic pH values, are consistent with those for other fungal aspartic proteinases.

Although the sequence identity of aspartic proteinases from diverse sources is often no greater than 20–27%, the crystal structures of numerous members of the family have revealed common features such as a predominance of β structure and organization of the polypeptide into two domains with the two DTG motifs in the extended active site cleft (Cutfield *et al.*, 1995). GcSAP will undoubtedly have these general features, together with unique detailed features. With the molecular cloning of the cDNA for GcSAP it will now be

possible to assess the role of the enzyme in pathogenicity by a targeted gene disruption.

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