Stimuli that induce production of *Candida albicans* extracellular aspartyl proteinase

CLAUDE G. LERNER and ROBERT C. GOLDMAN*

Abbott Laboratories, Anti-infective Research Division of Pharmaceutical Discovery, D47M-AP9A, 1 Abbott Park Road, Abbott Park, IL 60064-3500, USA

(Received 16 November 1992; revised 19 February 1993; accepted 4 March 1993)

Several species of the opportunistic fungal pathogen *Candida* produce an extracellular aspartyl proteinase that may assist the organism to invade and colonize host tissues, evade the host immune response and assimilate nitrogen from proteinaceous sources. Although addition of exogenous proteins, such as bovine serum albumin (BSA), to cultures of *C. albicans* is known to elicit proteinase production, the precise molecular mechanisms controlling regulation of proteinase induction are unknown. We have examined the ability of a variety of macromolecules to induce proteinase production using a chemically-defined nitrogen-limited growth medium and a rapid, sensitive microtitre fluorescent assay for proteinase activity in culture supernatants. BSA and the extracellular matrix protein collagen induced proteinase production. Homopolymers of both poly-L- and poly-D-glutamate also induced proteinase activity, whereas polyglycine, heparin sulphate and dextran sulphate did not. Thus, molecular recognition of proteinase-inducing stimuli is not highly stereospecific, but apparently requires both main- and side-chain interactions. Peptides 8 or more residues in length generally induced proteinase production while most shorter peptides did not. These data reveal that internalization of small peptides with less than 7 residues by peptide transport was not the inducing signal for proteinase production, since *Candida* dipeptide and oligopeptide permeases do not efficiently transport peptides of more than 6–7 residues. In addition a tight-binding synthetic inhibitor of *Candida* proteinase ($K_i = 0.17$ nM) prevented growth of *C. albicans* on BSA as a sole nitrogen source by blocking protein degradation. Immunodetection of proteinase in these culture supernatants suggests that fully intact proteins, in addition to peptide fragments of sufficient size, are capable of inducing proteinase production. A model involving stimulation of a plasma membrane signal transduction event by extracellular protein and/or polypeptide ligands of more than seven residues is compatible with these data.

Introduction

The clinically important opportunistic fungal pathogens of the genus *Candida* produce an extracellular acid (aspartyl) proteinase *in vivo* during infection (MacDonald & Odds, 1980; Ruchel *et al.*, 1983, 1991; De Bernardis *et al.*, 1990), as well as *in vitro* when cultured in the presence of exogenous protein as a nitrogen source (Staib, 1965; Remold *et al.*, 1968; Ruchel, 1981; Hattori *et al.*, 1984; Kaminishi *et al.*, 1986; Ross *et al.*, 1990; Ray & Payne 1990; Banerjee *et al.*, 1991). While some debate exists as to the importance

of the proteinase in virulence, proteinase production is considered to enhance the organism's ability to colonize and penetrate host tissues, and to evade the host immune system (Ruchel *et al.*, 1983; MacDonald, 1984; Odds, 1985; Ruchel, 1983, 1986; Douglas, 1988; Borg & Ruchel, 1990; Cutler, 1991). Correlation between virulence and levels of proteinase production in both clinical isolates of *C. albicans* (Ghannoum & Abu Elteen, 1986; De Bernardis *et al.*, 1990) and laboratory isolates with altered proteinase levels (MacDonald & Odds, 1983; Kwon-Chung *et al.*, 1985; Kondoh *et al.*, 1987; Ross *et al.*, 1990) generally supports the notion of its role in virulence. The proteinase hydrolyses a variety of extracellular substrates, including albumin (Remold *et al.*, 1968), immunoglobulins, coagulation factor X, angiotensinogen analogues, and haemoglobin (Ruchel, 1986), keratin (Hattori *et al.*, 1984) and collagen (Kaminishi *et al.*, 1986, 1988).

* Author for correspondence. Tel. (708) 937 4477; fax (708) 938 6603; e-mail: goldmanr@randb.abbott.com.

Abbreviation: EDANS, [5-(2-aminoethyl)-amino]-naphthalene-1-sulphonic acid.

Although proteinase production is induced by a variety of protein substrates, the precise molecular mechanisms controlling regulation of proteinase induction are unknown. In addition to bovine serum albumin (BSA) (Remold *et al.*, 1968), proteinase production is induced *in vitro* by haemoglobin (Ruchel, 1981), histones and ovalbumin (Banerjee *et al.*, 1991), keratin (Hattori *et al.*, 1984), collagen (Kaminishi *et al.*, 1986, 1988) and human serum (Capobianco *et al.*, 1992). Complex mixtures of peptides (peptone and tryptone) can also stimulate proteinase production *in vitro* (Banerjee *et al.*, 1991). Although induction of proteinase synthesis is tightly coupled to secretion and proteinase does not accumulate in cells (Ross *et al.*, 1990; Banerjee *et al.*, 1991), intracellular precursors do exist (Homma *et al.*, 1992). Thus exogenous protein induces the synthesis and secretion of newly synthesized proteinase rather than stimulating exocytotic secretory vesicles containing stored proteinase. Mechanisms by which induction of proteinase synthesis and secretion could occur include: (i) transmembrane signal transduction in which cell surface receptors bind proteinaceous ligand molecules, thus signalling induction of proteinase synthesis; (ii) peptides released from protein in the medium due to the basal level of proteinase production are transported via *Candida* peptide permeases (Payne & Shallow, 1985; McCarthy *et al.*, 1985; Milewski *et al.*, 1988; Naider & Becker, 1988; Payne *et al.*, 1991; Shallow *et al.*, 1991), thus allowing accumulated intracellular peptides to trigger an increase in proteinase synthesis and secretion; and (iii) internalization of protein and/or peptides by fluid phase (Basrai *et al.*, 1990), or receptor-mediated endocytosis, with subsequent signalling of proteinase induction by engulfed material.

We used several improvements over existing methodologies to elucidate further the molecular mechanism of induction of proteinase synthesis and secretion, and to determine the general characteristics of compounds which induce proteinase production. An extremely sensitive, rapid fluorescent microtitre assay for *Candida* aspartyl proteinase activity (Capobianco *et al.*, 1992), previously developed to discover inhibitors of enzyme activity, was used to monitor extracellular proteinase production. This assay detected extracellular proteinase activity induced by BSA or human serum in all strains of *C. albicans*, *C. tropicalis*, *C. stellatoidea*, *C. kefyr*, *C. lusitaniae* and *C. krusei* tested (Capobianco *et al.*, 1992). The level of proteinase followed the order *C. albicans/stellatoidea* > *C. tropicalis* > *C. kefyr* > *C. lusitaniae* > *C. krusei*. In addition, a completely defined medium was developed which allowed precise examination of the chemical nature of the inducing stimulus, including factors such as peptide size and amino acid side-chain stereochemistry. Finally, the availability of a

fast-tight binding inhibitor (Capobianco *et al.*, 1992) of the proteinase allowed examination of whether fully intact proteins, in the absence of peptides, could induce proteinase production.

Methods

Materials. The fluorogenic peptide substrate, 4-(4-dimethylamino-phenylazo)benzoyl- γ -aminobutyryl-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-[5-(2-aminoethyl)-amino]naphthalene-1-sulphonic acid, was synthesized at Abbott Laboratories as previously described for other substrates of this type (Capobianco *et al.*, 1992). This substrate is commercially available from Molecular Probes Inc. (Eugene, OR, USA; catalogue no. R-2931). The proteinase inhibitor, (2*S*)-2-[(3*R*)-3-benzyl-4-*N*-(4-methylpiperazin-1-yl-carbonyl)-2-keto-piperazin-1-yl]-hexanoic acid amide hydrochloride (A-70450), was prepared at Abbott Laboratories according to the methods described in US Patent 5120718. Dextran, bovine serum albumin and collagen were obtained from Sigma. Peptides and protein homopolymers including Lys-Val, Arg-Gly, β -lipotropin fragment (KKDSGPY), angiotensin I (DRVYIHPFHL), angiotensin II (DRVYIHPF), bradykinin (RPPGFSPFR), bradykinin fragment 1-7 (RPPGFSP), laminin fragment 929-933 (YIGSR), neurotensin fragment 1-8 (ELYENKPR), neurotensin fragment 1-11 (ELYENKPRRPY), neurotensin (ELYENKPRRPYL), polyglycine, poly-D-glutamate, poly-L-glutamate, poly-D-lysine and poly-L-lysine were obtained from Sigma. Angiotensin III (RVYIHPF), cholecystokinin heptapeptide 27-33 (YMGWMDF), cholecystokinin hexapeptide 28-33 (MGWMDF), [Met⁵]-enkephalin (YGGFM), and [Met⁵]-enkephalin-Lys-Gly-Leu (YGGFMRGL) were obtained from Clontech Laboratories. Heparin sulphate was obtained from ICN Biochemicals and dextran sulphate from Pharmacia.

Organism and growth conditions. *C. albicans* strains ATCC 10261 and 10231 (originally obtained from the American Type Culture Collection, Rockville, MD, USA) were from our culture collection and were grown at 37 °C. The chemically defined basic medium consisted of yeast nitrogen base (Difco), lacking amino acids and (NH₄)₂SO₄, but supplemented with 2.0% (w/v) glucose (YNB/glucose) and other nutrients as indicated. Complete amino acid supplementation (Ausubel *et al.*, 1990) consisted of (μ g ml⁻¹ final concn): L-arginine (20), L-aspartic acid (100), L-glutamic acid (100), L-histidine (20), L-leucine (60), L-lysine (30), L-methionine (20), L-phenylalanine (50), L-serine (375), L-threonine (200), L-tryptophan (40), L-tyrosine (30) and L-valine (150). Additional supplementation with adenine to 40 μ g ml⁻¹ and uracil (20 μ g ml⁻¹) was included with the amino acids. Nutritional supplements were obtained from Sigma. All liquid media were filter-sterilized. Media for plates contained YNB/glucose, complete amino acid supplementation, adenine, uracil, (NH₄)₂SO₄ (5 mg ml⁻¹) and 1.4% (w/v) agar. Liquid cultures of nitrogen-limited *C. albicans* were prepared by inoculating single colonies from plates into YNB/glucose medium containing (NH₄)₂SO₄ (0.2 mg ml⁻¹), but lacking amino acids. After overnight incubation, cultures were diluted (about 1:50) to an OD₄₂₀ of 0.15 with fresh nitrogen-limited medium (NL-YNB/glucose) which contained YNB/glucose and (NH₄)₂SO₄ (0.025 mg ml⁻¹), with or without additional nitrogen sources as indicated. Standard induction of proteinase production was achieved by addition of filter-sterilized BSA. Other putative inducers were prepared as 20-40% (w/v) stocks in dimethylsulphoxide (DMSO), and were added to media at final concentrations of 0.2-0.4 mg ml⁻¹. Addition of DMSO up to 10% (v/v) did not interfere with growth or proteinase induction by BSA.

Proteinase assay. Secreted aspartyl proteinase production in cultures of *C. albicans* was assayed by the fluorescent method of Capobianco *et*

al. (1992) in which proteolysis is detected by cleavage of a peptide fluorogenic substrate (see *Materials*), yielding an increase in relative fluorescence intensity. The primary cleavage site is between His and Thr (Capobianco *et al.*, 1992). Cell-free supernatants were prepared by centrifugation of culture samples for 5 min at 15000 *g* in a TOMY microcentrifuge. Aliquots were assayed for proteinase activity in microfluor plates (Dynatech) using the fluorescent assay. Reaction mixtures contained 16 μ M-fluorogenic substrate in 50 mM-sodium citrate buffer pH 4.5 plus 20 μ l culture supernatant in a final volume of 200 μ l. The reaction was initiated by addition of substrate (in 100 μ l citrate buffer, pH 4.5) to culture supernatants in 100 μ l 8 mM-sodium citrate buffer pH 6.8. The increase in relative fluorescence intensity (RFI) was measured over time at 22 °C in a luminescence spectrophotometer (Perkin-Elmer LS-50) equipped with a 96-well microplate reader. The excitation wavelength was 340 nm and the emission was monitored at 490 nm (430 nm filter employed). Reaction rates were determined by linear regression analysis of plots of the increase in relative fluorescence versus time. Analysis was performed on at least four data points defining line fits with r^2 values of greater than 0.98. An estimate of the mass concentration of proteinase was calculated as the product of the rate of change in fluorescence units min^{-1} multiplied by the constant $14.42 \mu\text{g ml}^{-1} \Delta\text{RFI min}^{-1}$. This constant was calculated from the reaction volumes, specific activity of highly purified proteinase ($15300 \text{ nmol h}^{-1} \text{ mg}^{-1}$) and the RFI of pure EDANS (2.72 RFI at $1 \mu\text{M}$), which was corrected for the inner filter effect as described (Capobianco *et al.*, 1992). The RFI was converted to concentration of product released as described previously (Capobianco *et al.*, 1992). Several methods are used in the literature to estimate secreted protease activity, making comparisons difficult. We present the following conversion factors: 1.0 haemoglobin unit = 0.88 BSA units = 0.90 nmol Thr-EDANS released from the fluorogenic substrate, where the specific activity is $15300 \text{ nmol Thr-EDANS released h}^{-1} \text{ mg}^{-1}$ for protease purified from *C. albicans* strain ATCC 10231. Although the specific activity of purified protease from *C. albicans* strain ATCC 10261 used in the present study was not determined, data were converted to allow an estimate of mass based on the specific activity of protease from strain 10231.

SDS-PAGE, electrotransfer, and immunoblotting. Samples of culture supernatants were examined for the presence of proteinase by immunodetection of antigen transferred from 12% (w/v) SDS-PAGE gels to nitrocellulose by semi-dry electroblotting. Proteins were concentrated from the cell-free culture supernatant by acetone precipitation. Ice-cold acetone (0.9 ml) was added to 0.1 ml chilled culture supernatant, and the sample was mixed and kept on ice for 45 min. The precipitate was collected by centrifugation for 15 min in a microcentrifuge at 4 °C. The supernatant was carefully decanted and the pellet gently dried under a stream of air. The pellet was resuspended in 16.5 μ l H_2O . After addition of an equal volume of $2 \times$ sample buffer (Ross *et al.*, 1990) the sample was heated at 100 °C for 10 min and 12.5 μ l applied to a 12% gel (Mini-Protean II, Bio-Rad). The samples were electrophoresed at 80 V for 2 h in 49 mM-Tris, 384 mM-glycine, 0.1% (w/v) SDS. Following electrophoresis the gel was soaked in semi-dry transfer buffer (48 mM-Tris base, 38 mM-glycine, 0.037% SDS, 20%, v/v, methanol). After 15 min a piece of wet nitrocellulose was laid on top of the gel and the two layers were assembled into a sandwich between six sheets of semi-dry transfer buffer soaked Whatmann 3MM chromatography paper. The gel was placed in a semi-dry transfer apparatus (Gelman) and the proteins were transferred to the nitrocellulose by electrophoresis at 0.8 mA cm^{-2} for 1.5 h. Following transfer the filter was incubated in 2% BSA, TBST (20 mM-Tris, 500 mM-NaCl, 0.05% Tween-20) overnight. The next day the filter was probed for 45 min with a 1:3000 dilution (in TBST) of rabbit anti-proteinase antiserum raised against purified secreted aspartyl proteinase from strain ATCC 10261 (kindly provided by P. A. Sullivan, University of Otago,

New Zealand). Following 3×10 min washes with TBST the blot was incubated with a 1:3000 dilution (in TBST) of secondary alkaline-phosphatase-conjugated goat anti-rabbit IgG. After 45 min incubation the filter was washed as above and the blot developed with alkaline phosphatase colour development reagents (5-bromo-4-chloro-indolyl phosphate, nitrotetrazolium blue; Bio-Rad).

Results and Discussion

Media conditions for testing C. albicans proteinase induction

Limiting the supply of inorganic nitrogen is important for maximal induction of proteinase production (Ross *et al.*, 1990; Banerjee *et al.*, 1991). To evaluate the ability of various compounds to induce proteinase production, we developed appropriate media conditions to obtain nitrogen-limited cultures. This was achieved by preparing nitrogen-starved overnight cultures which were subcultured in chemically defined medium containing $(\text{NH}_4)_2\text{SO}_4$ as the sole limiting nitrogen source. We first determined the level of $(\text{NH}_4)_2\text{SO}_4$ required in overnight cultures to effectively yield a nitrogen-starved culture in the morning. Nitrogen starvation was not observed when overnight cultures of *C. albicans* ATCC 10261, grown on YNB/glucose containing the usual level of $(\text{NH}_4)_2\text{SO}_4$ (5 mg ml^{-1}), were used to inoculate fresh YNB/glucose medium completely lacking a source of nitrogen, i.e. such cells exhibited transient growth. Nitrogen starvation was obtained when overnight cultures were grown in YNB/glucose with 0.2 mg $(\text{NH}_4)_2\text{SO}_4 \text{ ml}^{-1}$, since these cultures did not grow when transferred into fresh YNB/glucose lacking a nitrogen source. We next determined the amount of growth obtained when such nitrogen-starved cultures were transferred into YNB/glucose containing various amounts of $(\text{NH}_4)_2\text{SO}_4$. Starved cultures grew to levels dependent on the amount of $(\text{NH}_4)_2\text{SO}_4$ present in the YNB/glucose into which they were subcultured. Transfer of overnight cultures grown at 37 °C in YNB/glucose with 0.2 mg $(\text{NH}_4)_2\text{SO}_4 \text{ ml}^{-1}$ into YNB/glucose medium containing 0.025 mg $(\text{NH}_4)_2\text{SO}_4 \text{ ml}^{-1}$ yielded apparent nitrogen exhaustion after 7 h growth, reaching a maximal OD_{420} of 0.75. These culture conditions were used to test induction of proteinase production since it appeared obvious that proteinase synthesis would initially require a non-protein source of nitrogen to support cellular protein synthesis until such time that a proteinaceous inducer could provide an adequate nitrogen source for further growth.

Growth on BSA as a sole nitrogen source requires proteinase production. We evaluated the amount of cell growth in NL-YNB/glucose media containing various concentrations of BSA, in order to estimate the minimal concentration of putative inducer required for expression

Fig. 1. Growth yield in various concentrations of BSA. *C. albicans* strain ATCC 10261 was grown overnight in NL-YNB/glucose to induce nitrogen starvation. Cells were subcultured into YNB/glucose containing 0.025 mg $(\text{NH}_4)_2\text{SO}_4$ ml^{-1} , and various amounts of BSA (mg ml^{-1}): ●, 2.0; ▲, 0.2; ■, 0.02; ▼, 0.002; ○, none. Growth was monitored at 420 nm.

of significant levels of proteinase (Fig. 1). Full growth was supported by 2.0 mg BSA ml^{-1} , while little or no growth occurred with 0.002 mg ml^{-1} . Growth yield was 15 and 50 % of maximum at 0.02 and 0.2 mg BSA ml^{-1} , respectively. Due to limited supplies of certain putative inducers, a concentration of 0.2 mg ml^{-1} was tested for the ability to induce proteinase production. The rapid fluorescent assay was used to monitor the kinetics of proteinase produced during growth in the presence of BSA (Fig. 2). Proteinase activity was first detected in cultures at early-exponential phase, i.e. at OD_{420} 0.8, which coincides with the OD_{420} at which cultures lacking BSA exhibit a shift in growth rate due to nitrogen limitation (Fig. 1). Maximal proteinase production (Table 1) and final culture turbidity (Fig. 1) increased with increasing amount of BSA. Furthermore, the level of proteinase produced per OD_{420} unit was relatively constant (within twofold) and independent of BSA concentration, indicating that induced cells produce a constant amount of proteinase. Thus, the maximal amount of proteinase produced is dependent upon the amount of total cell growth rather than on an increase in the amount of proteinase each cell is producing at higher BSA concentrations. A 2 h delay in addition of BSA did

not affect the timing or maximal level of proteinase production. This further supports the idea that induction of proteinase production is coupled to the onset of nitrogen limitation.

While the nitrogen-limiting NL-YNB/glucose medium was suitable for observing proteinase induction with good nitrogen sources such as BSA, it became apparent that proteinase production might not be observed with putative inducers that were poor nitrogen sources. For example, when collagen was added to the medium as sole nitrogen source, there was little cell growth and only low levels of proteinase activity were detected (data not shown). Since continued cell growth is required for detecting high levels of proteinase production, additional nitrogen supplements were evaluated to find appropriate culture conditions that would allow clear detection of proteinase induction by compounds that were poor nitrogen sources.

Excess $(\text{NH}_4)_2\text{SO}_4$ inhibits induction by proteinase by BSA (Crandall & Edwards, 1987; Ross *et al.*, 1990; Banerjee *et al.*, 1991); thus we examined the effect of $(\text{NH}_4)_2\text{SO}_4$ and supplementation with amino acids on repression of BSA induction in order to determine the appropriate type of additional nitrogen supplementation (Table 1). Levels of proteinase activity detected in cultures containing 2 mg BSA ml^{-1} were inversely related to the amount of $(\text{NH}_4)_2\text{SO}_4$ present. Proteinase activity was still detected in cultures supplemented with 5 mg $(\text{NH}_4)_2\text{SO}_4$ ml^{-1} , while no proteinase activity was detected ($< 0.0014 \mu\text{g ml}^{-1}$) in the presence of 50 mg $(\text{NH}_4)_2\text{SO}_4$ ml^{-1} (< 0.03 % of the maximal control proteinase level). The culture containing 5 mg $(\text{NH}_4)_2\text{SO}_4$ ml^{-1} had not exhausted the supply of $(\text{NH}_4)_2\text{SO}_4$, because (i) the spent, filter-sterilized medium of a test culture grown in YNB/glucose plus 5 mg $(\text{NH}_4)_2\text{SO}_4$ ml^{-1} still supported full growth when re-inoculated, and (ii) it only requires 0.5–1 mg $(\text{NH}_4)_2\text{SO}_4$ ml^{-1} to support maximum growth yields. These data indicate that induction of proteinase occurred while $(\text{NH}_4)_2\text{SO}_4$ was still present. Addition of a mixture of amino acids to media containing BSA and limiting (0.025 mg ml^{-1}) or excess (5.0 mg ml^{-1}) $(\text{NH}_4)_2\text{SO}_4$ caused a slight reduction (about 30 %) in the amount of proteinase produced. Medium containing the amino acid mixture as the sole nitrogen source supported growth of the culture to high turbidity, indicating that these supplements provided an adequate nitrogen supply for growth. NL-YNB/glucose containing the amino acid mixture and limiting (0.025 mg ml^{-1}) $(\text{NH}_4)_2\text{SO}_4$ was therefore used to test proteinase induction by compounds which might not support further growth following exhaustion of limiting $(\text{NH}_4)_2\text{SO}_4$. Putative inducers were added to a final concentration of 0.2–0.4 mg ml^{-1} , which was adequate for induction of proteinase by BSA.

Fig. 2. Kinetics of proteinase production during growth of *C. albicans* strain ATCC 10261 with BSA as the sole nitrogen source. Nitrogen-starved cells were inoculated into YNB/glucose containing 0.025 mg $(\text{NH}_4)_2\text{SO}_4 \text{ ml}^{-1}$ and 0.2 mg BSA ml^{-1} . Culture supernatants were collected and assayed for secreted proteinase using the fluorogenic substrate. The estimate of proteinase in the culture medium was calculated as given in Methods [○, proteinase ($\mu\text{g ml}^{-1}$); ●, OD_{420}].

Table 1. Effect of nitrogen supplements on induction of *C. albicans* aspartyl proteinase

Cells were grown in YNB/glucose with nitrogen supplements as indicated. Proteinase activity was monitored in culture supernatants during growth, and peak activities were detected using the fluorogenic peptide substrate.

Nitrogen supplements			Proteinase production†	
BSA (mg ml^{-1})	$(\text{NH}_4)_2\text{SO}_4$ (mg ml^{-1})	Amino acid mixture*	$\mu\text{g ml}^{-1}$	% of control
2.0	0.025	—	5.33	100
2.0	0.025	+	3.61	67
2.0	0.2	—	1.87	35
2.0	5	—	0.88	16
2.0	5	+	0.58	11
2.0	50	—	ND	ND
0.2	0.025	—	4.76	89
0.02	0.025	—	2.3	43
0.002	0.025	—	0	0

* +, Cultures were supplemented with a mixture of 13 amino acids.

† ND, Proteinase activity below the limit of detection ($< 0.0014 \mu\text{g ml}^{-1}$; $< 0.03\%$ of control).

Similar to repression of expression of yeast peptide permeases (Island *et al.*, 1987; Payne *et al.*, 1991), amino acids seemed to repress proteinase production less than

Table 2. Induction of *C. albicans* aspartyl proteinase by polymers

Cells were grown in NL-YNB/glucose and amino acid supplements in the presence of polymer (0.4 mg ml^{-1}). Proteinase activity was monitored in culture supernatants during growth, and peak activities were detected using the fluorogenic peptide substrate.

Polymer	M_r	Proteinase production ($\mu\text{g ml}^{-1}$)*
Polyglycine	5000	ND
Poly-L-glutamate	8400	1.64
Poly-D-glutamate	12000	1.17
Poly-L-lysine	4900	0.36
Poly-D-lysine†	11000	ND
Heparin sulphate	14000	ND
Dextran sulphate	500000	ND
Dextran	500000	ND

* ND, Proteinase activity below the limit of detection ($< 0.0014 \mu\text{g ml}^{-1}$; $< 0.03\%$ of control).

† Poly-D-lysine was toxic and inhibited fungal growth.

$(\text{NH}_4)_2\text{SO}_4$. This seems a reasonable cellular response, since the presence of amino acids would be a likely indicator that proteins were also present, and cells would thus benefit from the partial induction of proteinase synthesis.

Survey of proteinase induction by proteins and large polymers

The amino-acid-supplemented NL-YNB/glucose medium was used to monitor induction of proteinase by various compounds, some of which might not be good nitrogen sources. The charge and stereospecificity requirements of the sensory apparatus involved in induction of proteinase production were examined using D and L isomer forms of peptide homopolymers (Table 2). Both the D and L isomers of polyglutamate efficiently induced proteinase production. Poly-L-lysine also induced proteinase, but poly-D-lysine caused cell lysis. These results suggest that the sensor for proteinase production is not highly stereoselective and does not show a charge preference. Interestingly, polyglycine did not induce proteinase production, suggesting that the sensor may require interaction with side-chains of amino acid residues in proteins. Neither charged (heparin and dextran sulphate) nor neutral (dextran) non-protein polymers induced proteinase production. These results indicate that the sensory apparatus involved in induction of proteinase production must interact with both the main- and side-chains of protein molecules; however, the stereochemistry about the α -carbon atom is not critically important. This contrasts with the requirement for L stereochemistry in the transport of peptides into *Candida*

Table 3. *Induction of secreted proteinase by peptides of defined length and sequence*

Cells were grown in NL-YNB/glucose and amino acid supplements in the presence of peptide (0.2 mg ml⁻¹). Proteinase activity was monitored in culture supernatants during growth, and peak activities were detected using the fluorogenic peptide substrate.

Peptide	No. of amino acid residues	Proteinase production (µg ml ⁻¹)*	Peptide sequence
Lys-Val	2	ND	KV
Arg-Gly	2	ND	RG
Laminin fragment	5	ND	YIGSR
[Met ⁵]Enkephalin	5	ND	YGGFM
Cholecystokinin fragment	6	ND	MGWMDF
β-Lipotropin fragment	7	ND	KKDSGPY
Cholecystokinin fragment	7	ND	YMGWMDF
Angiotensin III	7	0.42	RVYIHPF
Bradykinin fragment	7	ND	RPPGFSP
[Met ⁵]Enkephalin-RGL	8	ND	YGGFMRGL
Angiotensin II	8	0.91	DRVYIHPF
Neurotensin fragment	8	0.043	ELYENKPR
Bradykinin	9	ND	RPPGFSPFR
Angiotensin I	10	1.50	DRVYIHPFHL
Neurotensin fragment	11	0.32	ELYENKPRRPY
Neurotensin	13	0.76	ELYENKPRRPYIL

* ND, Proteinase activity below the limit of detection (< 0.0014 µg ml⁻¹; < 0.03 % of control).

albicans (Milewski *et al.*, 1988). Furthermore, the induction of proteinase by poly-D-glutamate is strong evidence that neither small peptides, nor transport of small peptides, is required for proteinase induction, because poly-D-glutamate should not be a substrate for proteinase, nor would poly-D-glutamate oligopeptides be substrates for peptide transport.

Survey of proteinase induction by peptides

The minimal size required for induction of proteinase production was examined using a panel of 18 peptides ranging in length from 2 to 13 residues. Generally peptides 8 or more residues in length induced proteinase production while peptides of 7 or fewer residues did not (Table 3). Exceptions to this general rule include [Met⁵]enkephalin-Lys-Gly-Leu and bradykinin, 7- and 9-mer peptides which did not induce, as well as angiotensin III, a 7-mer which did induce proteinase.

While this panel of peptides is not exhaustive, a few trends are evident from the composition and sequences of inducing and non-inducing peptides. All peptides examined which induced proteinase production contained the dipeptide sequence (His/Lys)-Pro and lacked Gly residues. Two preferred peptide cleavage sites for *Candida* proteinase are His-Thr (Capobianco *et al.*, 1992), and Lys-Thr (S. Kadam, personal communication), indicating the preference for a basic residue in the P1 site. At present the relationship between these

two pieces of data is unknown. All non-inducing peptides, larger than dipeptides, contained at least one Gly residue and lacked the dipeptide (His/Lys)-Pro motif. Since homopolymers of glutamate and lysine induced proteinase production the (His/Lys)-Pro motif is not strictly required for induction of proteinase production. However, the observation that the poly-glycine homopolymer and all glycine-containing peptides did not induce proteinase production suggests that the sensory mechanism may detect side-chains at each residue along a specific length of the inducing peptide. Exceptions to these trends may be expected for peptides involved in pathogenesis or host defence. Such peptides, although larger than generally needed to elicit proteinase production, may not be recognized by the signalling system involved in induction of proteinase production. For example, the bradykinin 9-mer peptide, which was unable to elicit proteinase production, is the same peptide as released during activation of the kallikrein-kinin system, thus causing increased vascular permeability (Kaminishi *et al.*, 1990; Cutler, 1991). Cutler (1991) suggested that bradykinin-mediated activation of the kallikrein-kinin system may lead to the inflammatory reactions associated with the presence of *C. albicans*. Interestingly, Kaminishi *et al.* (1990) reported that *Candida* proteinase can activate the kallikrein-kinin system. Thus it might be beneficial to the host if a peptide associated with activation of the inflammatory response were unable to induce production of proteinase.

Fig. 3. Inhibition of growth of *C. albicans* strain ATCC 10261 on BSA by proteinase inhibitor A-70450. Nitrogen-starved cells were inoculated into YNB/glucose containing 0.025 mg $(\text{NH}_4)_2\text{SO}_4$ ml⁻¹, 2.0 mg BSA ml⁻¹, and various concentrations of proteinase inhibitor. Growth was monitored at 420 nm after 18 h.

Proteinase production in presence of potent inhibitor of proteinase activity

Previous reports have clearly established that the presence of exogenous protein leads to induction of proteinase. We have shown that peptides of sufficient size can also induce. However, from these data it is not clear whether fully intact protein can act as an efficient inducer of proteinase production. It is possible that only peptide fragments generated by a low basal level of constitutively produced proteinase act as inducer while full-length protein does not. We assessed the production of proteinase in medium containing inducer (BSA) plus the fast-tight binding proteinase inhibitor, A-70450 ($\text{IC}_{50} = 1.3$ nM, apparent $K_i = 0.17$ nM; Capobianco *et al.*, 1992), in order to answer this question. Since formation of proteolytic fragments would be blocked under these conditions, production of proteinase should not occur if peptide fragments are required for induction. Addition of inhibitor effectively blocked proteolysis in culture, since it completely inhibited cell growth on BSA as a sole nitrogen source (Fig. 3). Consistent with the fact that A-70450 is a tight-binding inhibitor (Capobianco *et al.*, 1992) and should thus bind near-stoichiometrically with proteinase, the minimal growth inhibitory concentration of the inhibitor (0.1–0.2 μM) was very close to

the maximal molar concentration of proteinase produced (0.14 μM, based on an M_r of about 40000). Since proteinase activity could not be assayed in cultures grown in the presence of A-70450, the amount of proteinase antigen production was measured by immunoblotting. Western blots of protein present in the culture supernatants were probed with anti-proteinase antiserum (Fig. 4). Addition of BSA without inhibitor resulted in production of proteinase activity as well as a large amount of proteinase antigen. Addition of inhibitor alone, in the absence of BSA, did not result in proteinase antigen production. The presence of proteinase inhibitor also did not inhibit growth when ammonium sulphate or amino acids were the low-molecular-mass nitrogen sources. Lower, but clearly detectable levels of proteinase antigen were detected in culture supernatants containing BSA plus the proteinase inhibitor, indicating that fully intact protein can induce proteinase production. However, peptides do seem to contribute to high-level induction of proteinase production, inasmuch as maximal production of proteinase antigen was observed in the absence of A-70450. Perhaps the degradation of BSA yields peptide sequences not exposed on intact BSA, sequences that are more potent and effective inducers of proteinase.

The fact that intact protein can induce proteinase may be relevant to the infectious process. Attachment of *Candida* to host cells and tissues should put a putative receptor in contact with intact host protein. Subsequent induction of proteinase could thus assist in the attachment and invasion process. Proteinase is produced *in vivo* (MacDonald & Odds, 1980; Ruchel *et al.*, 1983; De Bernardis *et al.*, 1990), and *in vitro* when *Candida* comes into contact with host cells or tissue (Ruchel *et al.*, 1986; Borg & Ruchel, 1988, 1990).

General conclusions

We have developed a chemically-defined synthetic growth medium in which induction of the *C. albicans* secreted aspartyl proteinase can be determined (i) under conditions where previously nitrogen-starved cells deplete $(\text{NH}_4)_2\text{SO}_4$ while they are adapting to a protein nitrogen source, and (ii) with compounds that are not necessarily adequate nitrogen sources for cell growth. Studies with peptide homopolymers suggest that proteinase induction occurs via a sensory mechanism which requires both side- and main-chain interactions with the inducing polypeptide. Evaluation of a panel of peptides of defined size and sequence indicates that proteinase production is generally induced by peptides of 8 or more residues in length. Preliminary evidence suggests some sequence specificity involving a preference for a (His/Lys)-Pro motif in inducing peptides while peptides

Fig. 4. Effect of proteinase inhibitor A-70450 on BSA induction of proteinase production. Nitrogen-starved *C. albicans* strain ATCC 10261 cells were inoculated into YNB/glucose containing 0.025 mg $(\text{NH}_4)_2\text{SO}_4$ ml⁻¹, amino acid supplements, 2.0 mg BSA ml⁻¹ and proteinase inhibitor, as indicated. Proteinase inhibitor was added to a final concentration of 0.5 μM and samples were harvested for analysis after 9 h growth, which is near the peak for maximum proteinase production (see Fig. 2). Concentrated supernatant proteins were electrophoresed and transferred to nitrocellulose. Western blots were probed with antisera to the secreted aspartyl proteinase, CAP, *C. albicans* proteinase.

containing glycine residues apparently are unable to elicit proteinase production.

The existence of a mechanism which detects extracellular protein and signals proteinase induction is supported by the fact that (i) although *Candida* possesses multiple peptide transport systems which are repressed by ammonium ion, and induced by peptides (Payne *et al.*, 1991), the size of peptides which induce proteinase production is greater than the size of peptide known to be transported by *Candida* peptide permeases (Milewski *et al.*, 1988); (ii) poly-D-glutamate is an inducer, but not a substrate for proteinase, nor are D-peptides substrates for peptide transport (Milewski *et al.*, 1988; Shallow *et al.*, 1991), and (iii) significant induction of proteinase by BSA was observed in the presence of the tight-binding inhibitor A-70450. Furthermore, the yeast *Saccharomyces cerevisiae* contains G-protein-linked receptors for the peptide pheromone α -factor, a peptide of 13 amino acids (Raths *et al.*, 1988), demonstrating that peptides can initiate signal transduction in fungal cells. *C. albicans* also contains a homologous G-protein α -subunit that functions in *Saccharomyces* (Sadhu *et al.*, 1992), indicating that *Candida* may also possess the components of a functional signal transduction system. The possibility of proteinase induction following fluid-phase or receptor-mediated endocytosis can be excluded for peptides, since peptide permease mutants no longer accumulate, nor grow on, peptides (McCarthy *et al.*, 1985; Payne & Shallow, 1985; Payne, 1986; Milewski *et al.*, 1988). Fluid-phase or receptor-mediated endocytosis of protein, followed by transmission of the induction signal, can not, however, be excluded.

Recently the genes for secreted aspartyl proteinase

from various *Candida* strains and species have been cloned and sequenced (Hube *et al.*, 1991; Togni *et al.*, 1991; Ganesan *et al.*, 1991; Morrow *et al.*, 1992; Wright *et al.*, 1992). It now appears that a family of related aspartyl proteinase genes may exist in *Candida*, which raises the question as to how the various genes are regulated *in vitro* and *in vivo*, and what each of their roles may be in the infectious process. It may be that an ancestral gene used primarily for nutritional purposes duplicated and evolved specific functions related to pathogenesis. The expression, regulation, and function of this gene family both *in vivo* and *in vitro* is amenable to study, now that specific gene probes are available.

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