***Candida albicans* aspartic proteinase cleaves and inactivates human epidermal cysteine proteinase inhibitor, cystatin A**

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It is known that the cysteine proteinase inhibitor, cystatin, has a defence function against exogenous pathogens. Human epidermal cysteine proteinase inhibitor, cystatin A, which is a member of the cystatin family, is localized in the upper epidermal layer. In this study, the relationship between cystatin A and *Candida* aspartic proteinase (CAP), a putative *Candida* virulence factor, was studied. CAP activity was not affected by human epidermal cystatin A, while 90% of cystatin A activity was lost after incubation with CAP for 12 h at 37 °C. Human epidermal cystatin A was cleaved into small peptides by CAP, and the released peptides had no cystatin activity. These results suggest that CAP may induce an imbalance between cysteine proteinase and its inhibitor in cutaneous *Candida* infectious lesions through the degradation and inactivation of epidermal cystatin A.

Keywords: *Candida albicans*, aspartic proteinase, virulence factor, cystatin A

INTRODUCTION

Cysteine proteinase inhibitors have been found in many human tissues and fluids (Barrett *et al.*, 1986). The cystatin superfamily of cysteine proteinase inhibitors was defined on the basis of similarities in primary structure (Barrett *et al.*, 1986); it comprises three families, 1–3. The cystatins are known to have a regulatory role in intracellular protein catabolism (Katunuma, 1989). They also act as antiviral and/or antibacterial agents (Korant *et al.*, 1985; Björck *et al.*, 1989, 1990). The maintenance of homeostasis and defence against exogenous pathogens are assumed to be the main physiological functions of cystatins. Human epidermal cysteine proteinase inhibitor, cystatin A, is a member of cystatin family 1 (Barrett *et al.*, 1983). The human epidermis, especially the upper epidermal layer, contains an abundance of cystatin A (Hopsu-Havu *et al.*, 1983).

Candida albicans is a common pathogenic fungus in skin infections (Ahearn, 1978), and an aspartic proteinase excreted by this organism (*C. albicans* aspartic proteinase: CAP) is a putative virulence factor (Germaine *et al.*, 1978;

Rüchel, 1981; MacDonald & Odds, 1983; Odds, 1985; Rüchel *et al.*, 1985; Remold *et al.*, 1968; Kwon-Chung *et al.*, 1985; Ghannoum & Elteen, 1986; Borg & Rüchel, 1988). It has already been reported that CAP can degrade various extracellular proteins, such as keratin (Hattori *et al.*, 1981), collagen (Kaminishi *et al.*, 1986, 1988), immunoglobulins (Rüchel, 1986), and albumin, laminin and fibronectin (Ray & Payne, 1991).

It was unknown whether CAP can degrade and inactivate epidermal cystatin A. In this study, we showed that CAP can degrade and inactivate human epidermal cystatin A. We also determined the cleavage sites of cystatin A by CAP.

METHODS

Materials. Bovine serum albumin, bovine haemoglobin, papain, phenylmethylsulfonyl fluoride and α -N-benzoyl-DL-arginine β -naphthylamide (BANA) were purchased from Sigma. Pepstatin A was obtained from the Peptide Institute (Osaka, Japan). DEAE-Sephacel was from Pharmacia and DE-52 was from Whatman Biosystems. Carboxymethyl (CM)-papain Sepharose affinity chromatography was done according to the method of Anastasi *et al.* (1983). The reverse-phase HPLC column was a Bondasphere S-5 C18 (Nihon Waters). All other chemicals used were of analytical grade.

Organism. *C. albicans* IFO 1060 was maintained on Sabouraud glucose agar at room temperature.

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Abbreviations: CAP, *Candida* aspartic proteinase; BANA, α -N-benzoyl-DL-arginine β -naphthylamide; CM, carboxymethyl; TFA, trifluoroacetic acid.

Culture of *C. albicans*. The growth medium used contained 12.0 g yeast carbon base and 2.0 g bovine serum albumin in 1 litre of distilled water. The medium was sterilized by filtration and then *C. albicans* was inoculated into a 50 ml aliquot of medium. When the pH had reached near 3.0 after 7 d, culture was stopped. Four hundred millilitres of 7 d culture medium were centrifuged at 4000 *g* for 30 min to remove cells. This supernatant was used as the starting material for purification of CAP.

Purification of CAP. The collected culture medium was lyophilized before purification of the proteinase. The lyophilized material was solubilized with 10 mM sodium citrate buffer (pH 6.7), and then dialysed against the same buffer. The dialysed sample (20 ml) was applied to a DEAE-Sephacel column (2.5 × 4.5 cm), and the adsorbed proteins were eluted with a linear gradient of 10–400 mM sodium citrate buffer (pH 6.7) as described by Kaminishi *et al.* (1986). The active fractions were collected, dialysed against distilled water, and then lyophilized. The purity of the enzyme was confirmed by SDS-PAGE.

Purification of human epidermal cystatin A. The purification method described previously (Tsushima *et al.*, 1992) was used. Human epidermal cystatin A was obtained from human stratum corneum cells, collected by scraping the heels of healthy males. The skin scrapings (6.7 g) were homogenized with a glass homogenizer in 67 ml 0.01 M Tris/HCl buffer (pH 8.0) and extracted overnight at 4 °C. After centrifugation at 30000 *g* for 30 min, the supernatant thus obtained was used as the starting sample for cystatin A purification. The sample was run through a DE-52 ion-exchange column (1.5 × 5.7 cm) equilibrated with 0.01 M Tris/HCl buffer (pH 8.0). The active fractions were eluted with a linear gradient of 0–0.3 M NaCl/0.01 M Tris/HCl buffer (pH 8.0). They were collected and dialysed against 0.01 M Tris/HCl buffer (pH 8.0), then applied to a CM-papain affinity column (2.5 × 4.0 cm) that had been equilibrated with the same buffer. The adsorbed active fractions were eluted with 5 mM NaOH, collected, neutralized, and then dialysed against distilled water. The purity of the cystatin A thus obtained was confirmed by SDS-PAGE.

Assay of proteinase activity, proteinase inhibitory activity, and protein concentration. CAP activity was determined with 1.25% (w/v) bovine haemoglobin as the substrate by a modification of the method of Anson (1938). The reaction mixture contained 0.2 ml 0.1 M sodium acetate buffer (pH 3.8), 0.2 ml buffered haemoglobin solution, and 0.1 ml sample. After incubation at 37 °C for 60 min, 1.0 ml 5.0% trichloroacetic acid was added. The mixture was then centrifuged at 3000 r.p.m. for 10 min and filtered through Whatman no. 50 paper, after which the A_{280} was measured. Control assays were done with incubation mixtures to which CAP was added just before the addition of trichloroacetic acid.

Cysteine proteinase inhibitory activity was assayed by measuring the inhibition of papain activity, as reported previously (Järvinen, 1976; Tsushima & Hopsu-Havu, 1989). The sample (0.1 ml) and 0.1 ml of a 1.5 µg ml⁻¹ papain solution were mixed with 0.1 ml 0.2 M Tris/HCl buffer (pH 8.0) containing 8 mM EDTA and 4 mM dithiothreitol. After preincubation for 10 min at room temperature, 0.1 ml 5 mM BANA was added, followed after another 30 min by 0.4 ml *p*-dimethylamino-benzaldehyde. The A_{450} was read 30 min later. Protein concentrations were determined by the Lowry method, with bovine serum albumin as the standard.

Electrophoresis. SDS-PAGE was performed according to the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R 250.

Interaction of CAP with human epidermal cystatin A. Twenty-five microlitres of 0.13 µM CAP diluted with 0.1 M sodium acetate buffer (pH 4.0) was prewarmed with 25 µl of the same buffer at 37 °C for 5 min, and then mixed with 25 µl 1.3 µM human epidermal cystatin A and incubated at 37 °C for various times. The reaction was stopped by acidifying to pH 2.0 with 10% (w/v) trifluoroacetic acid (TFA; Pierce), then the incubated sample was applied to a reverse-phase HPLC column (2.1 × 150 mm) that had been equilibrated with 0.1% TFA. HPLC was performed using a linear gradient of 0–50% acetonitrile (Nacalai Tesque, Kyoto, Japan) in 0.1% TFA for 60 min at a flow rate of 0.3 ml min⁻¹. The peak fractions monitored by A_{214} were collected and used as sequential samples. Pepstatin (2 µM) treated samples were also applied to the HPLC column.

Sequence analysis. The NH₂-terminal amino acid sequence of the digestion products of human epidermal cystatin A was determined by automated sequence analysis with an Applied Biosystems 477 A protein sequencer.

RESULTS

Purification of CAP and human epidermal cystatin A

CAP was purified from *C. albicans* IFO 1060 culture medium containing bovine serum albumin. Its molecular mass was 42 kDa on SDS-PAGE (Fig. 1a). When 2 µM pepstatin was added to CAP at pH 4.0, its activity was completely inhibited (data not shown). Phenylmethylsulfonyl fluoride (4 mM) and EDTA (10 mM) had no inhibitory effect upon CAP. CAP activity was lost following dialysis against 10 mM sodium phosphate buffer (pH 7.5) at 4 °C overnight. These results confirmed that this purified enzyme is identical with the enzyme already characterized as CAP.

Human epidermal cystatin A was purified from skin scrapings. From 6.7 g scrapings, 1.54 mg purified inhibitor was obtained. It migrated as a single distinct band with a molecular mass of 12 kDa on SDS-PAGE (Fig. 1b). This was the same molecular mass as previously reported for cystatin A (Barrett *et al.*, 1986). The sequence of the first 30 N-terminal amino acid residues of purified epidermal cystatin A was identical with that previously reported for epidermal cystatin A (Takeda *et al.*, 1989).

Interaction of CAP with human epidermal cystatin A

CAP was unaffected by human epidermal cystatin A, even when incubation was continued for 12 h (data not shown). In contrast, the inhibitory activity of cystatin A decreased according to the duration of incubation with CAP (Fig. 2). After 12 h incubation, 90% of the original activity was lost. The possibility of inactivation by acid denaturation was ruled out, because cystatin A is a stable protein within the pH range 2–12 (Barrett *et al.*, 1986). The activity of human epidermal cystatin A was not changed when pepstatin was added to a mixture of cystatin A and CAP.

To further investigate the digestion of cystatin A, we used reverse-phase HPLC and sequenced each of the digested peptides. The main cleavage patterns found after 30 min and 12 h incubation with CAP are shown in Fig. 3(a, b).

Fig. 1. SDS-PAGE of purified CAP (a) and purified human epidermal cystatin A (b) under reducing conditions. (a) The arrow indicates the band of purified CAP with a molecular mass of 42 kDa. (b) Lane A, purified human epidermal cystatin A (arrow). Lane M, molecular mass standards. From the top: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α -lactalbumin (14.4 kDa).

Fig. 2. Changes of human epidermal cystatin A activity after incubation with CAP for up to 12 h. Twenty-five microlitres 0.13 μ M CAP was preincubated with 25 μ l 0.1 M sodium acetate buffer, pH 4.0, and then reacted with 25 μ l 1.3 μ M human epidermal cystatin A for 30 min, 75 min, 3 h and 12 h at 37 $^{\circ}$ C. After the reaction, 50 μ l 0.5 M Tris/HCl buffer (pH 8.0) was added, and then papain-inhibitory activity was assayed. The cystatin activity in the absence of CAP was taken as 100%.

None of the digested peptides had any papain-inhibitory activity. On adding 2 μ M pepstatin, cystatin A digestion was completely inhibited (data not shown). The cleavage sites are summarized in Fig. 4. The most common sites were QE, EA, DK, EE, KT, QY, QV, YY, KV, KY, DL, YQ and KN. After incubation for 12 h, additional cleavage sites appeared. Two residues at positions 19 and

Fig. 3. Reverse-phase HPLC profiles of cleavage peptides obtained from human epidermal cystatin A after incubation with CAP. (a) After 30 min incubation; (b) after 12 h incubation. One hundred microlitres of the reaction mixture was applied to the reverse-phase HPLC column. The peaks obtained after 30 min incubation are designated 1–16 and the new peaks appearing after 12 h incubation are designated 17–21. Their sequential positions are indicated in Fig. 4. —, A_{214} ; ----, CH_3CN gradient.

Fig. 4. Cleavage of human epidermal cystatin A by CAP. The double-headed arrows indicate the regions of the cleavage peptides. The top and middle lines show the cleavage patterns after 30 min incubation. The bottom line shows new cleavage patterns appearing after 12 h incubation.

20 were not detected in a search of the digested peptides (Fig. 4). It seems that they were in peptide peaks that were missed by HPLC, since we detected these residues in the sequence of the native inhibitor (data not shown). These

results show that the inhibitory activity of human epidermal cystatin A is lost due to cleavage by CAP.

DISCUSSION

Many researchers have suggested CAP as a possible virulence factor in *C. albicans* infection (Germaine *et al.*, 1978; Rùchel, 1981; MacDonald & Odds, 1983; Odds, 1985; Kwon-Chung *et al.*, 1985; Rùchel *et al.*, 1985; Ghannoum & Elteen, 1986; Borg & Rùchel, 1988). With regard to *C. albicans* infection of human skin, it seemed important to determine whether epidermal cystatin A, which may have a protective function against various pathogens (see Introduction), could be degraded and inactivated by CAP.

CAP is an aspartic proteinase, like pepsin, cathepsin D and cathepsin E. In this study, it was shown that CAP can degrade human epidermal cystatin A *in vitro*. CAP was shown to have endopeptidase activity, but its cleavage patterns were different from (and broader than) those reported for human cathepsin D (Lenarcic *et al.*, 1988).

Matsuda (1986), studying the growth of *C. albicans* in medium supplemented with human stratum corneum, suggested that *C. albicans* is able to adjust the environmental pH to around 4 (which is the optimum pH of CAP) for its growth by obtaining nutrients from human stratum corneum through digestion by the released CAP. Ray & Payne (1988) also speculated that actively growing *Candida* cells produce a circumjacent acidic milieu conducive to CAP activity. In cutaneous *C. albicans* infectious lesions, epidermal cystatin A might be cleaved and inactivated by CAP.

Conclusions

The results of this study suggest that CAP may function as a virulence factor by altering the proteolytic balance in the skin. We speculate that the degradation of epidermal cystatin A by CAP may induce an imbalance of cysteine proteinase and its inhibitor, and this imbalance may promote infection. However, the existence and physiological roles of *C. albicans* cysteine proteinase remain to be studied.

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