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Intracellular Posttranslational Modification of Aspartyl Proteinase of *Candida albicans* and the Role of the Glycan Region of the Enzyme

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Using two drugs, tunicamycin and brefeldin A, which affect protein processing, we investigated the intracellular processing mechanism of secreted aspartyl proteinase 1 (SAP1) of *Candida albicans*. Three intracellular forms of SAP1 were detected by immunoblotting using monoclonal antibody (MAb) CAP1. Their molecular weights were approximately 40, 41 and 45 kDa, respectively. The 41 kDa protein is a glycoprotein and may be the same as the extracellular form judging by its molecular mass. The 40 kDa protein was the unglycosylated form and its molecular mass coincided with deglycosylated SAP1 and the 45 kDa protein was also the unglycosylated form. Neither the 40 and 45 kDa proteins were detected in the culture supernatant of *C. albicans*. These suggested that the 40 and 45 kDa proteins might be intracellular precursor forms of SAP1. These results show that SAP1 is translated as a 45 kDa precusor form in the endoplasmic reticulum and the 45 kDa precursor form undergoes proteolytic cleavage after translocation into the Golgi apparatus, generating the 40 kDa precursor form. This 40 kDa precursor is converted into a 41 kDa mature form through glycosylation in the Golgi apparatus. The mature form of the 41 kDa protein is sorted into secretory vesicles and finally released into the extracellular space through membrane fusion. When the glycan region of SAP1 was digested with N-glycosidase F, both stability and activity of the enzyme decreased. These results indicate that the glycan attached to the enzyme may, at least in part, be related to enzyme stability and activity.

Key words: *Candida albicans,* aspartyl proteinase, intracellular modification, glycan

As with many fungal species, *Candida albicans*, a medically important yeast and opportunistic pathogen capable of causing severe candidiasis in immunocompromised and debilitated patients (7, 10), produces several secreted aspartyl proteinases (SAPs) (9, 16, 17, 22, 23). Although their pathological roles are not completely clear, SAPs are believed to be virulence factors which play important roles during invasive hyphal growth of *C. albicans* by degrading the surface barrier prior to hyphal formation and deeper invasion into host tissues (1, 20, 26).

Lytic enzymes such as proteinases are normally translated as inactive, larger precursors which are subsequently processed during secretion and activated only at their final destination (6, 14). SAPs are also thought to be produced as precursor forms containing a signal peptide or a sorting signal peptide (25) and the amino acid sequence of SAPs deduced from its DNA sequence suggested that it be produced in this way (8).

To clarify the transport mechanisms underlying SAPs production, the studies on complexity and specificity of transport pathway and processing of precursors of SAPs must be performed. However, the complexity and specificity of these steps are not totally understood. The definitions of intracellular precursors, transport pathway and processing of precursors of SAPs have been hampered by several reasons. One of the reasons is that the pathway of protein secretion in the yeast appears to resemble the pathways in plants and animals, although less endoplasmic reticulum (ER), Golgi apparatus, and secretory vesicles exist. These observations are consistent with rapid transit time and low levels of precursors.

In the present study, we investigated the intracellular posttranslational processing of SAP1 precursors by detecting intracellular forms of the enzyme using a monoclonal antibody. Furthermore, we also investigated the putative roles of glycan attached to SAP1.

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Materials and Methods

Enzyme purification

C. albicans KIT 1113 was cultured under aerobic conditions in yeast nitrogen base (YNB; Difco Laboratories, Detroit, Mich.) broth supplemented with 1% bovine serum albumin (BSA) and 2% glucose at 30°C for 48 h. SAP1 was purified from the culture supernatant as described previously (17).

Assay of aspartyl proteinase activity

Aspartyl proteinase activity was determined spectrophotometrically following the digestion of BSA as the substrate as described by Crandall and Edwards (2). To 30 μl of enzyme solution, 270 μl of 1% BSA (w/v) in 50 mM KCl-HCl buffer (pH 2.5) was added, and the mixture was incubated at 37°C for 2 h. The reaction was then stopped by adding 700 μl of ice-cold 10% (w/v) trichloroacetic acid (TCA). Precipitated protein was removed by centrifugation at 10,000 rpm for 5 min. The amount of proteolysis was determined by measuring the A_{280} of the supernatant. One unit of enzyme activity was defined as the amount of enzyme needed to increase 0.1 unit of O.D. at A_{280}.

Monoclonal antibody (MAb)

MAb CAP1 (immunoglobulin G1 type) used in this study was described previously (18). The antibody is highly specific for SAP1. The epitope of SAP1 recognized by the antibody was the protein part of the enzyme and the putative epitope of the antibody was located in the Asp^{77} to Gly^{103} sequence.

Treatment of tunicamycin (TM) and brefeldin A (BFA)

TM was used to prevent protein glycosylation (4) and BFA was used to block the translocation of the proteins through the Golgi apparatus (3, 5, 13). *C. albicans* was precultured in YNB-BSA broth at 30°C overnight, pelleted by centrifugation at 10,000 rpm for 20 min, and resuspended in fresh YNB-BSA broth. To 50 ml of suspension, TM (Sigma Chemicals Co., St. Louis, Mo.; final concentration to 10 μg/ml) or BFA (Sigma; final concentration to 5 μg/ml) were added and cultured at 30°C for 2, 4, and 8 h. The control without any reagent was also included. At the indicated time, aliquots of cultures were taken out and used to measure cell growth and SAP1 activity in the supernatant. The cell pellets obtained by centrifugation of each culture were mechanically disrupted with a French Pressure cell (Aminco, Rochester, N.Y.) at a pressure of 18,000 lb/in^2. After centrifugation at 12,000 rpm for 20 min at 4°C, the cellular supernatant fractions were collected and used for further studies.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (11) using 10% (w/v) polyacrylamide gels. Proteins in the gel were stained with 0.1% Coomassie Brilliant Blue R-250 and destained. The protein concentration was measured by the method of Lowry *et al.* (12) using BSA as the standard.

Western blot analysis

Electrophoretic transfer of proteins from the polyacrylamide gel to the nitrocellulose membrane was performed in Tris-Glycine buffer (pH 8.3) as described previously (24). Nonspecific sites were blocked with 3% skim milk in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST, pH 7.2). The blocked sheets were immersed in MAb CAP1 diluted in 3% skim milk in PBST for 2 h with gentle shaking and washed with PBST three times (10 min each wash). The sheets were incubated with peroxidase-conjugated anti-mouse IgG (Sigma) diluted 1 : 1,000 in PBST for 2 h. The reaction was visualized by incubating the sheets with substrate solution of 0.05% 3,3'-diaminobenzidine (DAB) and 0.01% H_2O_2.

Treatment of N-glycosidase F

Treatment of N-glycosidase F was carried out essentially as described by Misumi *et al.* (15). In brief, N-glycosidase F (Boehringer Mannheim GmbH, Germany; finally 0.1 U/ml) was added to purified SAP1 (5 μg) or *C. albicans* whole cell lysates (30 μg) and incubated at 37°C for 12 h. Proteins were concentrated by precipitation in 25% TCA and prepared for further studies. Deglycosylation of SAP1 after treatment of N-glycosidase F was confirmed by Western blot using peroxidase-conjugated concanavalin A (Sigma).

Stability of enzyme

Purified SAP1 was incubated in 50 mM KCl-HCl buffer (pH 3.0), 50 mM Tris-HCl buffer (pH 7.0) and 50 mM Glycine-NaOH buffer (pH 10.0) at 37°C for various time intervals with or without pretreatment of N-glycosidase F. SDS-PAGE was performed followed by Western blotting using MAb CAP1 and an enzyme assay using BSA as the substrate was performed.

Results

Effects of TM and BFA on growth of C. albicans and secretion of SAP1

The effects of TM and BFA on growth of *C. albicans* and secretion of SAP1 were investigated. In the normal control, cell growth and SAP1 activity in the culture supernatant increased linearly in a time-dependent manner. When the cells were treated with TM, cell growth and SAP1 activity in the culture supernatant also increased linearly in the same manner as the normal control but were lower than those of the normal control. When the

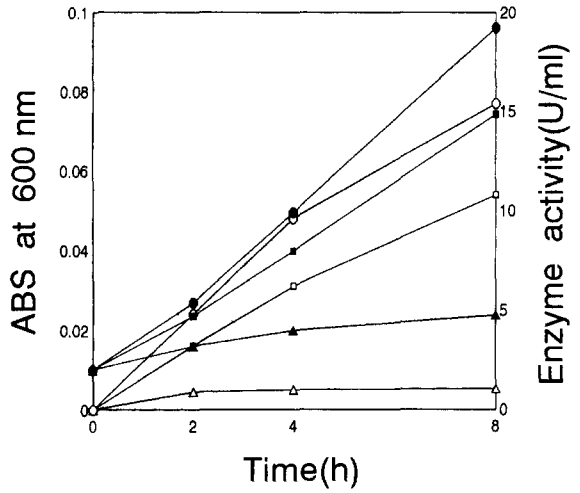

Fig. 1. Effects of TM and BFA on growth of *C. albicans* and secretion of SAP1. *C. albicans* (2×10^5 cells) were precultured in BSA-YNB broth at 30°C overnight, pelleted by centrifugation, and resuspended in fresh BSA-YNB broth. After adding TM and BFA at a final concentration of 10 μg/ml and 5 μg/ml, respectively, and cultured at 30°C. At the indicated times, aliquots of the culture were taken and used for measurement of the cell density at A_{600} and SAP1 activity in the culture supernatant. Normal growth (●), TM growth (■), BFA growth (▲), Normal activity (○), TM activity (□), and BFA activity (△).

cells were treated with BFA, cell growth and SAP1 activity in the culture supernatant did not increase significantly, even after 8 h (Fig. 1).

Secretion patterns of SAP1 under cultivation with drugs are shown in Fig. 2. In the normal control, the amount of SAP1 in the culture supernatant increased in a time-dependent manner. Treatment with N-glycosidase F resulted in a decrease in the molecular weight of SAP1 from 41 kDa to 40 kDa. However, TM-treated *C. albicans* secreted 40 kDa SAP1. The amount of secreted 40 kDa SAP1 also increased in a time-dependent manner and was not significantly different compared to the normal control in the immunoblot. This protein was not affected by the treatment of N-glycosidase F and showed the same mobility as the deglycosylated 41 kDa SAP1. In BFA-treated *C. albicans*, initial secretion of 41 kDa SAP1 was found in the culture supernatant, but the amount of secreted SAP1 did not increase thereafter. This result was consistent with the fact that the activity of SAP1 in the culture supernatant did not increase as shown in Fig. 1. The SAP1 secreted under BFA treatment was 41 kDa and the molecular weight was reduced to 40 kDa after treatment of N-glycosidase F.

Detection of intracellular precursors of SAP1

Three intracellular precursors of SAP1 were detected by immunoblot analysis of the whole cell lysate of *C. albicans*. Their approximate molecular weights were 40 kDa, 41 kDa, and 45 kDa (Fig. 3). The 41 kDa protein detected in normal *C. albicans* had the same mobility as SAP1 purified from the culture supernatant and its molecular weight decreased to 40 kDa after treatment with N-glycosidase F. When *C. albicans* cells were cultivated with TM, a drug known to prevent protein glycosylation, a 1 kDa smaller intracellular precursor of 40 kDa was accumulated. The 40 kDa SAP1 showed the same mobility as the deglycosylated mature SAP1 and was the unglycosylated form. On the other hand, when *C. albicans* was cultivated with BFA, a drug that blocks the translocation of the proteins through the Golgi apparatus, accumulation of the 45 kDa protein was detected. Treatment with N-glycosidase F did not influence the 45 kDa protein. It suggested that the 45 kDa protein was also unglycosylated protein.

Roles of the glycan region of SAP1

To investigate the putative roles of glycan attached to

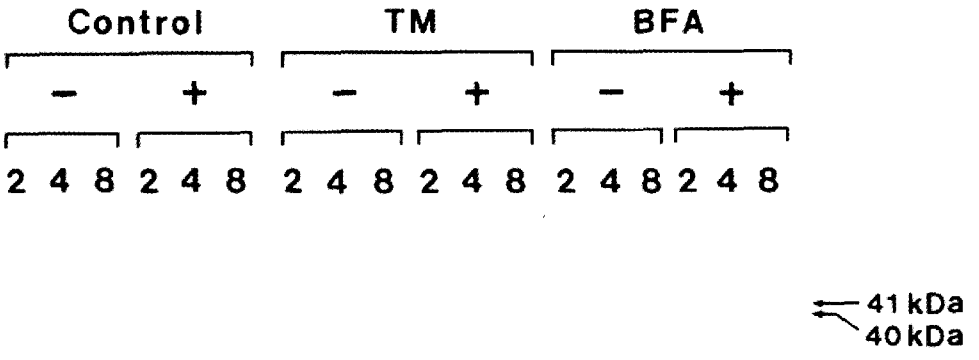

Fig. 2. Effects of TM and BFA on secretion and glycosylation of SAP1. *C. albicans* was cultured at 30°C for the indicated times in the absence or presence of TM and BFA. At the indicated times, the culture supernatants were prepared and used for immunoblot using MAb CAP1. The immunoblot before and after N-glycosidase F treatment were also performed. TM, tunicamycin-treated; BFA, brefeldin A-treated; –, before N-glycosidase F digestion; +, after N-glycosidase F digestion.

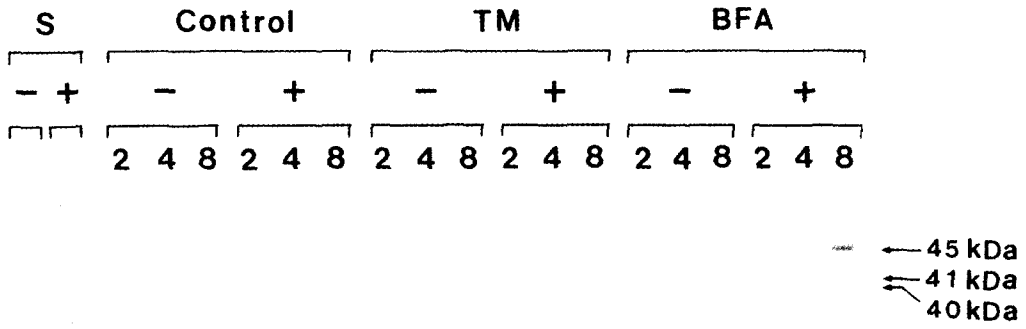

Fig. 3. Detection of intracellular precursors and mature form of SAP1. *C. albicans* was cultured at 30°C for the indicated times in the absence or presence of TM and BFA. At the indicated times, cells were collected and disrupted. The cell lysates were prepared and used for immunoblot using MAb CAP1. The immunoblot before and after N-glycosidase F treatment was also performed. S, supernatant, TM, tunicamycin-treated; BFA, brefeldin A-treated; –, before N-glycosidase F digestion; +, after N-glycosidase F digestion.

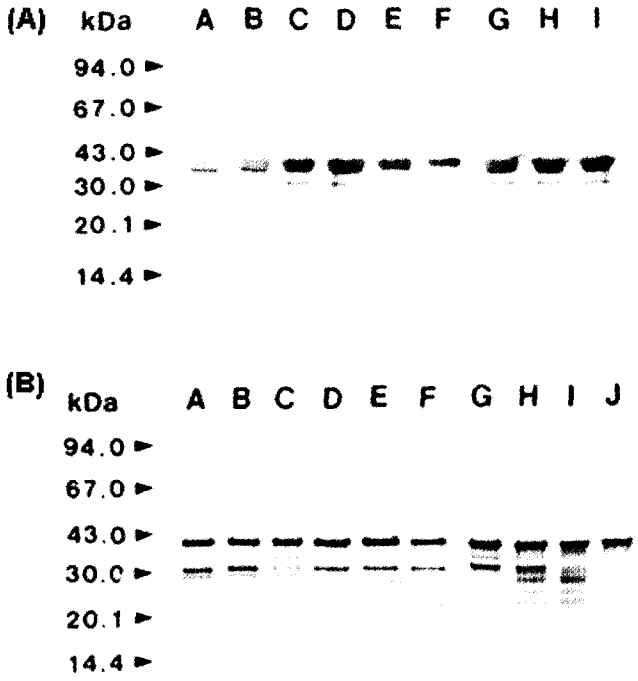

Fig. 4. Effect of deglycosylation on SAP1 stability. (A) SAP1 not treated with N-glycosidase F. (B) SAP1 treated with N-glycosidase F. Lanes A-C, SAP1 incubated in 50 mM KCl-HCl buffer (pH 3.0) at 37°C for 0.5, 1, and 2 h, respectively. Lanes D-F, SAP1 incubated in 50 mM Tris-HCl buffer (pH 7.0) at 37°C for 0.5, 1, and 2 h, respectively. Lanes G-I, SAP1 incubated in 50 mM Glycine-NaOH buffer (pH 10.0) at 37°C for 0.5, 1, and 2 h, respectively. Lane J, SAP1 control.

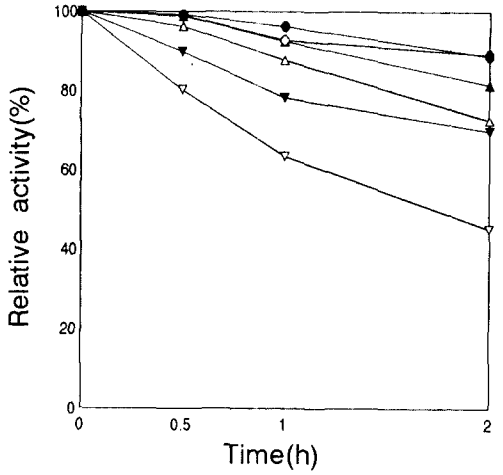

Fig. 5. Effect of deglycosylation on SAP1 activity. SAP1 and deglycosylated SAP1 were incubated in various pH buffers, 50 mM KCl-HCl buffer (pH 3.0), 50 mM Tris-HCl buffer (pH 7.0) and 50 mM Glycine-NaOH buffer (pH 10.0), at 37°C for 0.5, 1, and 2 h, respectively. The remaining activities of SAP1 were measured using BSA as the substrate. SAP1, pH 3.0 (●); Deglycosylated SAP1, pH 3.0 (○); SAP1, pH 7.0 (▲); Deglycosylated SAP1, pH 7.0 (△); SAP1, pH 10.0 (▼); Deglycosylated SAP1, pH 10.0 (▽).

SAP1, the stabilities of glycosylated (native) SAP1 and deglycosylated SAP1 were compared. Deglycosylated enzyme was less stable than the glycosylated enzyme and especially greatly unstable at alkaline pHs (Fig. 4). To further investigate the role of glycan, the activities of SAP1 with or without treatment of N-glycosidase F were determined. The deglycosylated enzyme showed lower activity than the glycosylated enzyme (Fig. 5). These suggest that the glycan attached to the enzyme may be related to stabilizing the enzyme from degradation and enhancing or maintaining the enzyme activity.

Discussion

In this study, we investigated the intracellular posttranslational processing pathway of SAP1 of *C. albicans* by analyzing the secretion patterns and detecting the intracellular precursors of the enzyme.

TM-treated *C. albicans* secreted 40 kDa SAP1, a 1 kDa smaller than 41 kDa SAP1 secreted by the normal control.

The 40 kDa SAP1 showed the same mobility as the deglycosylated 41 kDa SAP1 and was an unglycosylated protein. Since SAP1 is a single polypeptide chain, the change in molecular weight reflects the absence of glycan. Therefore, the 40 kDa SAP1 might be an unglycosylated form of SAP1 resulting from the action of TM which inhibits lipid carrier-dependent protein glycosylation by inhibiting UDP-GlcNAc:dolichyl phosphate-GlcNAc-1-phosphatetransferase that catalyzes the first step in lipid carrier-dependent glycosylation. Although the amount of secreted SAP1 under cultivation with TM was not significantly different with that of the normal control, *C. albicans* growth and SAP1 activity in the culture supernatant were lower than those of the normal control. Since SAP1 is known to be essential for growth of *C. albicans* (19, 21), lower growth of TM-treated *C. albicans* may be explained by lower enzyme activity of unglycosylated SAP1. On the other hand, BFA-treated *C. albicans* secreted 41 kDa SAP1 at initial growth; however, this secretion may not have any value because the secretion did not increase thereafter. Furthermore, there was no significant increase of SAP1 activity in the supernatant. Therefore, the initial secretion of SAP1 which occurred in the initial growth of BFA-treated *C. albicans* may be due to the secretion of fully processed SAP1 prior to the action of BFA. The growth of BFA-treated *C. albicans* was also not observed. The fact that the growth of *C. albicans* was inhibited by treatment with BFA, a drug that blocks the translocation of proteins through the Golgi apparatus, confirms that SAP1 is essential for the growth of *C. albicans*.

Three intracellular precursors of SAP1 were detected by immunoblot analysis of *C. albicans* cell lysates by using MAb CAP1 that is highly specific to SAP1. Their approximate molecular weights were 40 kDa, 41 kDa, and 45 kDa. The 41 kDa protein detected in normal *C. albicans* was the glycosylated form and had the same mobility as SAP1 purified from the culture supernatant. Therefore, it seemed likely that it corresponded to the mature form that was detected in the culture supernatant. However, the 40 kDa and 45 kDa proteins were not detected in culture supernatants. Therefore, these proteins were thought to be intracellular precursor forms of SAP1. *C. albicans* cells treated with TM accumulated a 1 kDa smaller precursor of 40 kDa that showed the same mobility as the deglycosylated 41 kDa mature enzyme. It was not affected by treatment with N-glycosidase F. Therefore, the 40 kDa SAP1 detected in TM-treated *C. albicans* was thought to be a precursor form without glycan that existed prior to processing to mature form. When cells were treated with BFA, accumulation of the 45 kDa protein was detected. Treatment with N-glycosylase F had no effect on the 45 kDa protein. On the basis of these data, we can postulate a secretion pathway of SAP1 (Fig. 6). After translocation into the Golgi apparatus, the 45 kDa precursor form undergoes proteolytic cleavage, generating the 40 kDa precursor form. This 40 kDa precursor is converted to the 41 kDa mature form through glycosylation in the Golgi apparatus. The mature form of the 41 kDa protein is sorted into secretory vesicles and finally released into the extracellular space.

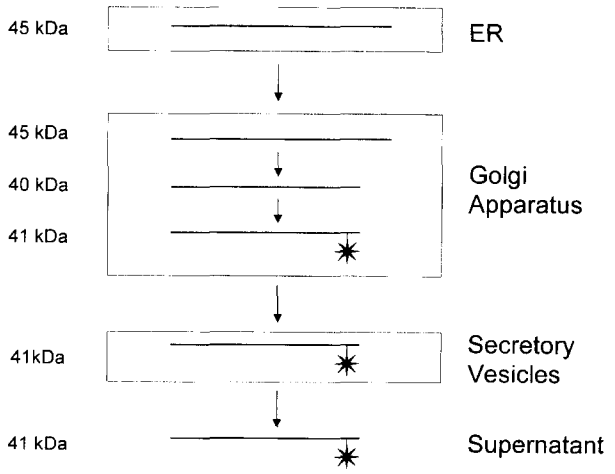

Fig. 6. Summary of SAP1 maturation pathway. The figure is explained in the discussion. The asterisk represents the carbohydrate chain.

Glycosylation of proteins is a common modification to all eukaryotes and represents one of the most important post-translational events and glycans attached to proteins are known to play various important biological roles. However, the roles of glycan attached to SAP1 are not totally understood. In this study, we partially investigated the putative roles of glycan attached to SAP1. Deglycosylated SAP1 after treatment of N-glycosidase F was less stable than glycosylated (native) SAP1. Especially, this instability is greatly exhibited at alkaline pHs. SAP1 is known to undergo alkaline denaturation as other aspartic proteinases (16, 17, 19, 22). The data obtained from the present study demonstrates that once denaturation has occurred, the enzyme was thought to be more susceptible to degradation. However, glycosylated SAP1 showed some resistance against degradation compared to the deglycosylated enzyme. These results suggest that the glycan attached to SAP1 might play a role in stabilizing the enzyme from degradation. The effect of glycan on activities of enzymes with or without treatment of N-glycosidase F was also investigated. Deglycosylated SAP1 showed lower activity than glycosylated SAP1. This coincided with the fact that the activity of SAP1 produced by TM-treated *C. albicans* cells was lower than that produced by normal *C. albicans*. These results further suggest that the glycans attached to the SAP1 might be, at least in part, related to enhancing or maintaining SAP1 activity.

References

1. Borg, M. and R. Rüchel. 1988. Expression of extracellular acid

proteinase by proteolytic *Candida* spp. during experimental infection of oral mucosa. *Infect. Immun.* 56, 628-631.
2. Crandall, M. and J.E. Edwards. 1987. Segregation of proteinase-negative mutants from heterozygous *Candida albicans*. *J. Gen. Microbiol.* 133, 2817-2824.
3. Doms, R.W., G. Russ, and J.W. Yewdell. 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* 109, 61-72.
4. Duksin, D., M. Seiberg, and W.C. Mahoney. 1982. Inhibition of protein glycosylation and selective cytotoxicity toward virally transformed fibroblasts caused by B_1-tunicamycin. *Eur. J. Biochem.* 129, 77-80.
5. Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ikehara. 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* 263, 18545-18552.
6. Hasilik, A. and W. Tanner. 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxy-peptidase Y. *Eur. J. Biochem.* 85, 559-608.
7. Horn, R., B. Wong, T.E. Kiehn, and D. Armstrong. 1985. Fungemia in a cancer hospital: changing frequency, earlier onset, and results of therapy. *Rev. Infect. Dis.* 7, 646-654.
8. Hube, B., C.L. Turver, F.C. Odds, H. Eiffert, G.J. Boulnois, H. Kochel, and R. Rüchel. 1991. Sequence of the *Candida albicans* gene encoding the secretory aspartate proteinase. *J. Med. Vet. Mycol.* 29, 129-132.
9. Kaminish, H., Y. Hagihara, S. Hayashi S, and T. Cho. 1986. Isolation and characteristics of collagenolytic enzyme produced by *Candida albicans*. *Infect. Immun.* 53, 312-316.
10. Kown-Chung K.J. and J.E. Bennett. 1992. Candidiasis, p. 280-286. *In* K.J. Kwon-Chung and J.E. Bennett (eds.), Medical Mycology. Lea & Febiger, Philadelphia.
11. Laemmli, D.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
12. Lowry, O.H., N.J. Rosebrugh, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
13. Magner, J.A. and E. Papagiannes. 1988. Blockade by brefeldin A of intracellular transport of secretory proteins in mouse pituitary cells: effects on the biosynthesis of thyrotropin and free alpha-subunits. *Endocrinology* 122, 912-920.
14. Mechler, B., M. Muller, H. Muller, and D.H. Wolf. 1982. *In vivo* biosynthesis of the vacuolar proteinase A and B in the yeast *Sacchromyces cerevisiae*. *J. Biol. Chem.* 257, 11203-11206.
15. Misumi, Y., Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara. 1986. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* 261, 11398-11403.
16. Morrison, C.J., S.F. Hurst, S.L. Bragg, R.J. Kuyhendall, H. Diaz, D.W. McLaughlin, and E. Reiss. 1993. Purification and characterization of the extracellular aspartyl proteinase of *Candida albicans*: removal of extraneous proteins and cell wall mannoprotein and evidence for lack of glycosylation. *J. Gen. Microbiol.* 139, 1177-1186.
17. Na B.K., S.I. Lee, S.O. Kim, Y.K. Park, G.H. Bai, S.J. Kim, and C.Y. Song. 1997. Purification and characterization of extracellular aspartic proteinase of *Candida albicans*. *J. Microbiol.* 35, 109-116.
18. Na B.K., G.T. Chung, and C.Y. Song CY. 1999. Production, characterization, and epitope mapping of a monoclonal antibody against aspartic proteinase of *Candida albicans*. *Clin. Diagn. Lab. Immunol.* 6, 429-433.
19. Ray, T.L. and C.D. Payne. 1990. Comparison production and rapid purification of *Candida* acid proteinase from protein-supplemented cultures. *Infect. Immun.* 58, 508-514.
20. Ray, T.L., C.D. Payne, and B.J. Morrow. 1991. *Candida albicans* acid proteinase: characterization and role in candidiasis. *Adv. Exp. Med. Sci.* 306, 173-183.
21. Ross, I.K., F. De Bernardis, G.W. Emerson, A. Cassone, and P.A. Sullivan. 1990. The secreted aspartate proteinase of *Candida albicans*; physiology of secretion and virulence of a proteinase-deficient mutant. *J. Gen. Microbiol.* 136, 687-694.
22. Rüchel R. 1981. Properties of purified proteinase from the yeast *Candida albicans*. *Biochim. Biophys. Acta.* 695, 99-113.
23. Rüchel R., K. Uhlemann, and B. Böning. 1983. Secretion of acid proteinases by different species of the genus *Candida*. *Zbl. Bakt. Hyg. I. Abt. Orig.* A225, 524-536.
24. Tsang, V.C.W., J.M. Peralta, and A. Bartlett. 1983. Enzyme-linked immuno-electrotransfer blot techniques for studying the specificities of antigens and antibodies separated by gel electrophoresis. *Method Enzymol.* 92, 377-391.
25. Verner, K. and G. Schatz. 1988. Protein translocation across membranes. *Science* 241, 1307-1313.
26. Wingard, J., J. Dick, W. Merz, G. Sandford, R. Saral, and W. Burns. 1982. Differences in virulence of clinical isolates of *Candida tropicalis* and *Candida albicans* in mice. *Infect. Immun.* 37, 833-836.