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The Determination of Chitin Synthases by Varying pH and Divalent Cations in *Candida albicans*

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Abstract The Chs1, Chs2, and Chs3 activities of a pathogenic fungus, *Candida albicans*, perform the same biochemical reactions, but exert different functions. Therefore, the determination of each enzyme activity is important. The three chitin synthases differ in their optimal pH and the effect of divalent cations as either stimulatory or inhibitory factors. The CAChs1, CAChs2, and CAChs3 activities are optimal at pH 7.5, 6.5, and 8.5, respectively. Co^{2+} stimulates CAChs1 and CAChs3, but inhibits CAChs2. Ni^{2+} inhibits CAChs1 and CAChs2 with little effect on CAChs3. Mg^{2+} stimulates CAChs2 and CAChs3, but hardly affects CAChs1. These characteristics are similar to those of the *Saccharomyces cerevisiae* enzymes except in degree. The sensitivity against Ni^{2+} of CAChs1 is higher than that of CAChs2, whereas the reverse is true in *S. cerevisiae*. Metal dependence of chitin synthases in *C. albicans* is less marked than that in *S. cerevisiae*, except for CAChs2. The activities of CAChs1 and CAChs3 from EDTA-treated membranes were increased 1.5 fold, while that of CAChs2 was stimulated 7 fold in the presence of divalent cations. These results could provide new criteria for screening systems of antifungal agents.

Key words: Chitin synthase, *Candida albicans*, pH, divalent cations

Candida albicans has three chitin synthase activities encoded by *CACHS1, CACHS2*, and *CACHS3* genes. All these genes were cloned and characterized [1, 4, 17]. Although these isozymes catalyze the same biochemical reaction using UDP-*N*-Acetyl Glucosamine (UDP-GlcNAc) as a substrate to synthesize insoluble chitin, their functions and enzymatic characteristics are quite different. *CACHS1*, an equivalent of *SCCHS2*, is involved in septum formation [10, 16]. It is likely that *CACHS1* is an indispensable gene, because the homozygous deletion of *CACHS1* has been unsuccessful (personal communication with Dr. M. Sudoh, Nippon Roche Research Center, Japan). *CACHS3* is involved in chitin synthesis in the cell wall [10, 17]. Because fungal chitin synthases require different divalent cations as stimulators, these cations are widely used to distinguish each isozyme from the others [15]. In *S. cerevisiae*, the mutants in chitin synthase 2 and chitin synthase 3 which contain only chitin synthase 3 and 2 activity respectively were isolated [16], and used for the characterization of each enzyme by their pH optimum and divalent cations stimulation [5]. Those characteristics led to the measurement of the activity of each individual enzyme even in a mixture containing all three chitin synthases [5]. In *C. albicans*, the measurement of individual chitin synthases has not been reported, even though total chitin synthase activities were measured [1, 10].

Unlike *S. cerevisiae, C. albicans* is an opportunistic dimorphic pathogen which can cause serious infections in immunocompromised persons such as AIDS patients [12]. Even though the hyphal form is generally believed to be the pathogenic form, the yeast form is also found to be involved in candidiasis in such host cells as corneocytes [14]. Since the host cell lacks a cell wall, inhibition of cell wall synthesis in *C. albicans* is a target for the development of antifungal agents. The three chitin synthases are expressed differentially depending on the morphology [2] and differentiation stage [11]. Accordingly, the three individual chitin synthases of *C. albicans* need to be characterized for an effective search for antifungal agents as well as for elucidation of their biochemical mechanism. Recently, homozygous chitin synthase-deleted null mutants have been isolated with the use of these mutants [10]. It has become possible to distinguish each enzyme according to the pH optimum and divalent cation effect.

As shown in this report, the properties of the *Candida* chitin synthases differ somewhat from those of their *S. cerevisiae* counterparts.

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MATERIALS AND METHODS

Strains and Media

Candida albicans, CAI-4 (*ura3*△::*imm34*/*ura3*△::*imm34*), and chitin synthase null mutants derived from CAI-4 [10] were kindly provided by Dr. Masayuki Sudoh (Nippon Roche Research Center, Japan). Chitin synthase-deleted mutants were used as sources of CAChs1, CAChs2, and CAChs3 activity. Homozygous *chs2*△ mutant (*chs2*△::*hisG*/*chs2*△::*hisG*) and homozygous *chs3*△ mutant (*chs3*△::*hisG*/*chs3*△::*hisG*) were used for the CAChs3 activity and the CAChs2 activity, respectively. Homozygous *chs2*△ *chs3*△ mutant (*chs2*△::*hisG*/*chs2*△::*hisG chs3*△::*hisG*/*chs3*△::*hisG*) was used for the CAChs1 activity. The strains were grown in Sabouraud-Dextrose medium (4% glucose, 1% peptone) in the yeast form.

Membrane Preparation and Enzymatic Assay

Membranes were isolated as described by Orlean [13], and suspended in 50 mM Tris-chloride pH 7.5, containing 33% glycerol, to a final volume of 1.6 ml/ g (wet weight) of yeast used. ethylenediaminetetraacetic acid (EDTA)-treated membranes were prepared by homogenizing the membranes with 2 ml of 50 mM Tris, pH 7.5, containing 1 mM EDTA. Homogenized membranes were collected by centrifugation at 40,000×g for 30 min. The pellet was suspended in 1.6 ml of 50 mM Tris, pH 7.5, containing 33% glycerol [5]. Chitin synthase activities were measured as follows. For the proteolytic step of CAChs1, reaction mixtures contained 32 mM Tris-chloride, pH 7.5, 2 mM cobalt acetate, 1.1 mM UDP-[U-^{14}C]GlcNAc (400,000 cpm/ μmol, Dupont), 2 μl of trypsin at the optimal concentrations for activation (0.25, 0.5, 0.75 mg/ml), and 20 μl of membrane suspension in a total volume of 46 μl. For CAChs2, the reaction mixture contained 32 mM 2-(*N*-morpholino)ethanesulfonate (Mes) pH 6.5, 8 mM magnesium acetate, 10 mM nickel acetate, 2 μl of trypsin at the optimal concentration for activation (0.25, 0.5, 0.75 mg/ml), and 10 μl of membrane suspension. For CAChs3, reaction mixture contained 32 mM Tris-chloride, pH 8.5, 10 mM magnesium acetate, 4 mM cobalt acetate, 10 mM nickel acetate, 1.1 mM UDP-[U-^{14}C]GlcNAc (400,000 cpm/μmol, Dupont), 2 μl of trypsin at the optimal concentration for activation (0.25, 0.5, 0.75 mg/ ml), and 20 μl of membrane suspension in a total volume of 46 μl. Mixtures were incubated for 15 min at 30°C. Proteolysis was stopped by adding 2 μl of a soybean trypsin inhibitor solution at a concentration 1.5 times that of the trypsin solution used, and tubes were placed on ice. GlcNAc was added to a final concentration of 32 mM, followed by incubation at 30°C for 90 min. In the case of CAChs2, 1.1 mM UDP-[U-^{14}C] GlcNAc (400,000 cpm/μmol, Dupont) was added after proteolytic activation. In all cases, the insoluble chitin formed was assayed by measurement of radioactivity after addition of 10% trichloroacetic acid and filtration through glass fiber filters. Specific activity is expressed as nanomoles of GlcNAc incorporated per hour per milligram protein. Protein was measured by the method of Lowry *et al.* [9].

RESULTS

Metal Dependency of Chitin Synthases

Chitin synthase activities were compared in the EDTA-treated membrane fractions with and without divalent cations (Fig. 1). By washing the membranes with EDTA, endogenous divalent cations were eliminated to determine the metal dependency of chitin synthases. For CAChs1 activity and CAChs2 activity, 2 mM Co^{2+} and 5 mM Mg^{2+} were added to the reaction, respectively. The activities of CAChs1 and CAChs3 were increased 1.7 fold and of CAChs2 7 fold in the presence of divalent cations.

The Chitin Synthase 1 Activity

A membrane fraction containing only CAChs1 activity was prepared from the homozygous *chs2*△*chs3*△ null mutant. Since CAChs1 activity was protease dependent (data not shown) as in *S. cerevisiae*, an appropriate concentration of trypsin was used. The optimum pH of CAChs1 was found at 7.5 (Fig. 2a). The metal specificity showed that 2 mM of Co^{2+} was stimulatory and Mg^{2+} was not effective at all. Ni^{2+} was inhibitory as in *S. cerevisiae* (Fig. 3a). However, the sensitivity to the inhibitor was different: 5 mM Ni^{2+} inhibited most of the activity in *S. cerevisiae* [5], while 10 mM Ni^{2+} inhibited 80% of activity in *C. albicans* (Fig. 3a).

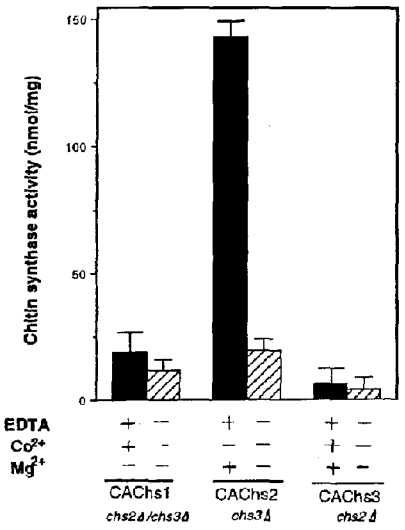

Fig. 1. Metal dependency of chitin synthases on EDTA-treated membranes from chitin synthase null mutants.

The activities from EDTA-treated membranes were lower than those from untreated membranes because the enzymes became unstable after EDTA treatment.

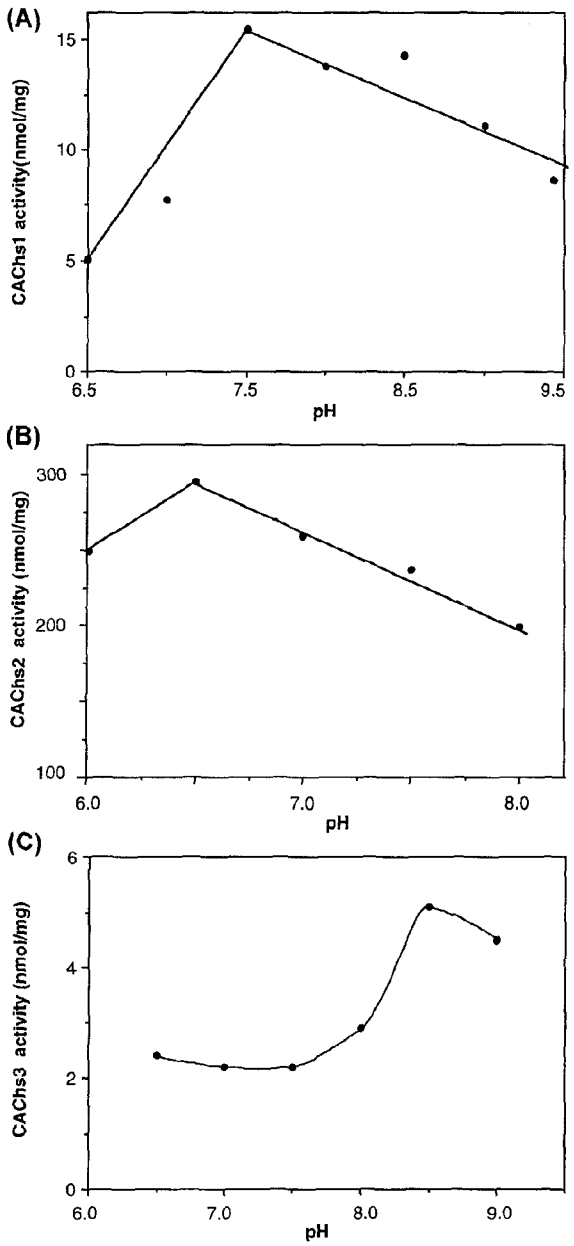

Fig. 2. The pH dependence of CAChs1 (a), CAChs2 (b), and CAChs3 (c).

Membranes were prepared from *chs2*△*chs3*△ null mutant and *chs3*△ mutant for CAChs1 and CAChs2, respectively. For the former, the activity was measured with 2 mM Co^{2+} and for the latter, the activity was measured with 8 mM Mg^{2+}. For CAChs3 activities, membrane fractions were prepared from *chs2*△ mutant and activity was measured with 10 mM Mg^{2+} and 6 mM Co^{2+}.

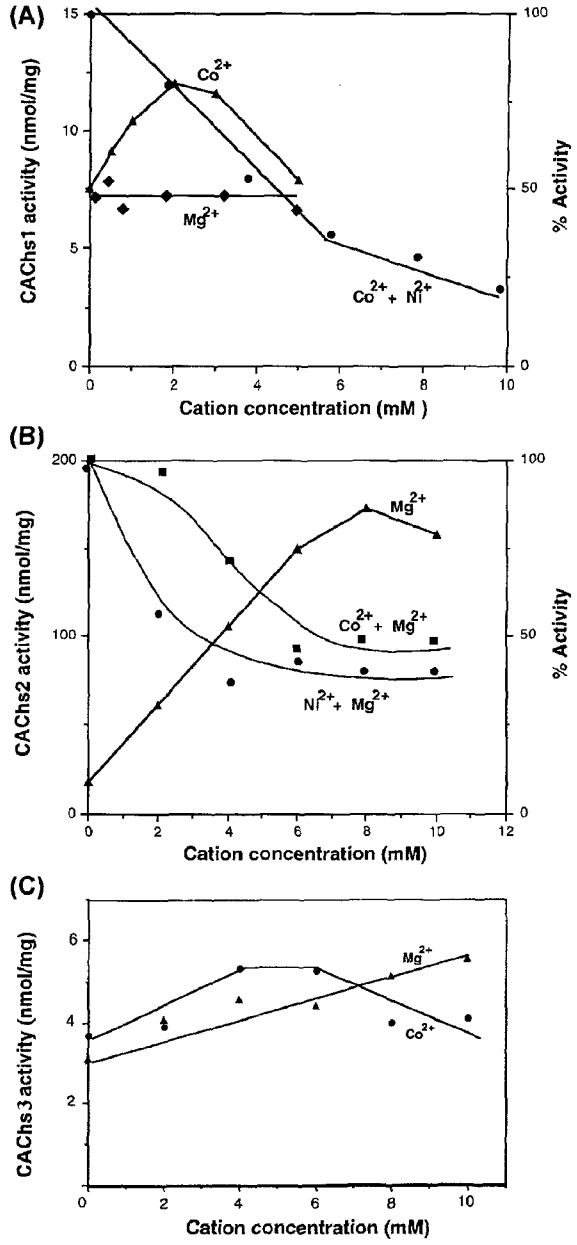

Fig. 3. The effect of different divalent cations on CAChs1 (a), CAChs2 (b), and CAChs3 (c) activities.

Left side indicates chitin synthase activity with stimulator: a, CAChs1 activity with Mg^{2+} (◆) or with Co^{2+} (▲); b, CAChs2 activity with Mg^{2+} (▲); c, CAChs3 activity with Mg^{2+} (▲) or with Co^{2+} (●). Right side indicates % activity with inhibitors: a, CAChs1 activity with varied concentrations of Ni^{2+} and 2 mM Co^{2+} (●); b, CAChs2 activity with varied concentrations of Ni^{2+} and 8 mM Mg^{2+} (●), or CAChs2 activity with varied concentrations of Co^{2+} and 8 mM Mg^{2+} (■).

The Chitin Synthase 2 Activity

A membrane fraction prepared from the homozygous *chs3*△ mutant, containing CAChs1 and CAChs2 activities, was used to measure the CAChs2 activity. Chitin synthase activity of the *chs3*△ mutant was measured at various pHs under the same condition used for SCChs1 activity (Fig. 2b). The chitin synthase activity of the *chs3*△ mutant (Fig. 2b, approximately 300 nmol/mg) at the optimum pH was 20 times higher than the CAChs1 activity of *chs2*△ *chs3*△ mutant (Fig. 2A, approximately 15 nmol/mg). Therefore, we concluded that most of *Candida* chitin synthase activity was derived from CAChs2, a homolog

of SCChs1 which also shows dominant chitin synthase activity in *S. cerevisiae*. Accordingly, the CAChs2 activity could be measured even in the presence of CAChs1 activity.

When assayed under the condition as described in Materials and Methods with the membrane fraction of the *chs3*△ mutant, the CAChs2 activity was highest at pH 6.5 (Fig. 2b). The metal specificity showed that Mg^{2+} was stimulatory and both Co^{2+} and Ni^{2+} were inhibitory (Fig. 3b). At 10 mM Ni^{2+}, 40% of CAChs2 activity remained, in contrast with SCChs1 activity, most of which was inhibited by 1 mM of Ni^{2+} [5].

I was interested in confirming that optimal reaction conditions established separately for CAChs1 and CAChs2 activities still could be used to distinguish one from the other in a strain containing both activities like the *chs3*△ mutant. As shown in Fig. 4, the activity measured under CAChs1 conditions was one-sixth of the CAChs2 activity (50 to 300 nmol/mg) rather than one-twentieth (see above). The CAChs1 activity was increased from 15 nmol/mg in *chs2*△*chs3*△ mutant containing only CAChs1 activity, to 50 nmol/mg in the *chs3*△ mutant. This discrepancy is thought to result from incomplete inhibition of the CAChs2 activity under the condition for the CAChs1 activity of the *chs3*△ mutant activity, in which 2 mM of Co^{2+} was used as a stimulator. Consequently, the CAChs1 activity assayed here represented combined activities of CAChs1 and CAChs2.

The Chitin Synthase 3 Activity

The membrane fraction prepared from homozygous *chs2*△ null mutant contains CAChs1 and CAChs3 activities. Since Ni^{2+} strongly inhibits SCChs2 activity with little effect on SCChs3 in *S. cerevisiae*, CAChs3 activity was also measured by using Ni^{2+} to inhibit the CAChs1 activity. Previously, the CAChs3 was reported as non-zymogen [1] in contrast to SCChs3 [7]. However, in this study, the CAChs3 activity turned out to possess zymogenic property. Preactivation with trypsin gave rise to enhancement of the enzymatic activity (Fig. 5, a and c).

When assayed for the CAChs3 activity, the optimum pH was 8.5 (Fig. 2c), and Co^{2+} and Mg^{2+} were stimulatory factors (Fig. 3c). Since a mutant strain containing only CAChs3 was not available, the sensitivity of CAChs3 against Ni^{2+} could not be measured. However, it seems that CAChs3 was not inhibited by Ni^{2+} as it is in *S. cerevisiae*, because optimum pH and metal specificity could not be determined if CAChs3 were inhibited by Ni^{2+}.

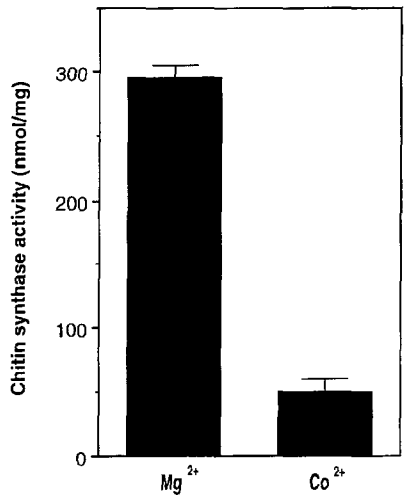

Fig. 4. Independent measurement of CAChs1 and CAChs2 from the *chs3*△ null mutant which contains both activities.

For CAChs1 and CAChs2 activities, reactions were incubated at pH 7.5 with 2 mM Co^{2+} or at pH 6.5 with 8 mM Mg^{2+}, respectively.

DISCUSSION

In this study, conditions were presented for the determination of three chitin synthases in *C. albicans*.

In *S. cerevisiae*, three chitin synthases were determined by taking advantage of the differences in pH optimum and in divalent cations requirements [15, 5]. Likewise, each chitin synthase of *C. albicans* was distinguished by pH optimum and the effect of divalent cations. In summary, CAChs1 activity is optimal at pH 7.5, is stimulated by Co^{2+}, but inhibited by Ni^{2+}, whereas Mg^{2+} shows no effect; CAChs2 activity is optimal at pH 6.5, is enhanced by Mg^{2+}, but inhibited by Co^{2+} and Ni^{2+}. The

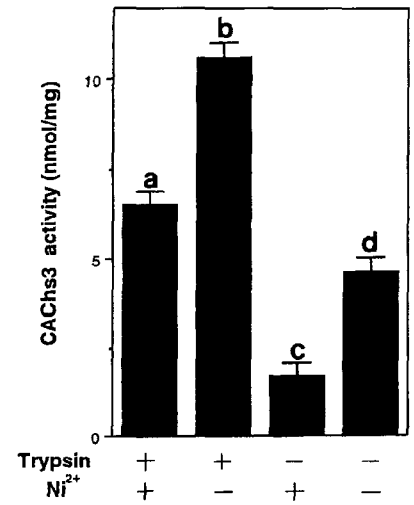

Fig. 5. Zymogenic property of CAChs3. Membranes were prepared from the *chs2*△ null mutant which contains CAChs1 and CAChs3 activities. CAChs3 activity was measured in the presence of Ni^{2+} to inhibit the CAChs1 activity (a and c).

These fractions contained CAChs1 activity partly because the inhibition of CAChs1 by Ni^{2+} was not complete. CAChs3 activity was increased three times by trypsin (a/c). CAChs1 activity (b-a) was lower than that from *chs2*△*chs3*△ null mutant which has CAChs1 activity only. d, Unstimulated activities of CAChs1 and CAChs3 without trypsin.

inhibitions of CAChs2 activity by Co^{2+} and Ni^{2+} were not complete. One possible explanation is that these divalent cations are poor activators. CAChs3 activity is optimal at pH 8.5, and stimulated by 6 mM Co^{2+} and 10 mM Mg^{2+}. These characteristics are not dramatically different from those of *S. cerevisiae*. It should be noted that the sensitivity to Ni^{2+} of CAChs1 was higher than that of CAChs2, in contrast to the case of *S. cerevisiae*, where the sensitivity of SCChs2, a homolog of CAChs1, was lower than that of SCChs1 [5]. The sensitivity of CAChs3 to Ni^{2+} cannot be detected until the mutant strain containing only CAChs3 activity is available.

S. cerevisiae chitin synthases showed metal dependency [16]; the activities of SCChs2 and SCChs1 were increased approximately three times in the presence of divalent cations. *C. albicans* chitin synthases also showed metal dependency but with different patterns (Fig. 1); the activities of CAChs2 and CAChs1 were increased about 7 times and 1.5 times, respectively, in the presence of divalent cations. While the chitin synthase activities from EDTA-treated membranes of *S. cerevisiae* showed no activity, the enzymes of *C. albicans* prepared under the same conditions had some activity. These results indicate that the chitin synthases in *C. albicans* are different from those in *S. cerevisiae*. Because *C. albicans* is a pathogen whereas *S. cerevisiae* is not, the former is in principle a better organism for the screening of antifungal agents. However, the use of *C. albicans* for screens of compounds that affect chitin synthesis and thereby cell wall formation has been limited by lack of knowledge about the properties of *Candida* chitin synthases. The information contained in the present report fills that void and can be used to determine which chitin synthase, and consequently which physiological process, is affected by a given antifungal agent.

That certain antifungal agents discriminate among chitin synthases was already known. In *S. cerevisiae*, nikkomycin Z inhibits Chs3 activity, but rarely affects Chs2, a CAChs1 equivalent [3, 6]. However, when the sensitivity to nikkomycin Z was tested in homozygous *chs2*△*chs3*△ mutants of *C. albicans*, CAChs1 activity was strongly inhibited by nikkomycin [8]. These results highlight the importance of using the pathogen in the screening and of being able to discriminate among the different enzymatic activities.

Acknowledgments

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