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PROTEINASE B IS, INDEED, NOT REQUIRED FOR CHITIN SYNTHETASE 1 FUNCTION IN *SACCHAROMYCES CEREVISIAE*

Sanford J. Silverman†, J. Andrew Shaw and Enrico Cabib*

Laboratory of Biochemistry and Metabolism
National Institute of Diabetes and Digestive and Kidney Diseases
National Institutes of Health
Bethesda, MD 20892

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Previous genetic evidence led to the conclusion that proteinase B of yeast was not involved in the function of chitin synthetase 1 (Chs1), based on the demonstration of normal septum formation, cell division and chitin deposition in mutants devoid of the proteinase (Zubenko, G.S., Mitchell, A.P., and Jones, E.W. (1979) Proc. Natl. Acad. Sci. USA 76, 2395-2399). Later, however, it was found that the essential enzyme for septum formation is chitin synthetase 2, whereas Chs1 acts as an auxiliary enzyme, whose absence results in daughter cell lysis under acidic conditions (Cabib, E., Sburlati, A., Bowers, B. and Silverman, S.J. (1989) J. Cell Biol. 108, 1665-1672). By using the lytic behavior as a criterion, we have now found that *prb1* strains are not defective in Chs1 function. Certain strains contain a recessive suppressor of lysis which could mask the Chs1 defect. However, appropriate crosses and transformation experiments showed that the *prb1* mutants do not harbor the suppressor. It may now be concluded with confidence that proteinase B is not required for chitin synthetase 1 function.

Chitin is an important structural component of the cell wall in many fungi. In *Saccharomyces cerevisiae* it is the main or sole constituent of the primary septum that grows to separate mother and daughter cell at division (1). Fungal chitin synthetases (E.C. 2.4.1.16) are membrane proteins, usually found in a latent or zymogenic state (2). They can be converted into active forms *in vitro* by the action of proteases (2). Activation of the zymogen is probably essential for the control of chitin deposition in vivo, therefore it is very important to identify the physiological activators, be they proteases or other systems. This has not yet been accomplished.

When the zymogenic nature of a yeast chitin synthetase (now called chitin synthetase 1 or Chs1) was first demonstrated, a proteinaceous factor was found in the same organism with the ability to convert zymogen into active enzyme (3). The factor was later identified as the vacuolar enzyme proteinase B (4) and was postulated to be the natural activator of the Chs1 zymogen (3, 4). This hypothesis was disputed by Zubenko *et al.* (5), based on the finding that mutants devoid of proteinase B showed no abnormalities in septum formation, cell division or chitin deposition. The conclusion seemed appropriate and was generally accepted (6).

It is correct that proteinase B is not required for the formation of the chitinous septum, but recent evidence indicates that the data of Zubenko *et al.* were insufficient to prove that the protease

†Present address: American Cyanamid, P.O. Box 400, Princeton, NJ 08543-0400.

*Corresponding author.

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is not the physiological activator of Chs1. We now know that Chs1 is not required for septum formation, cell division or maintenance of normal chitin levels, the parameters considered by Zubenko *et al.* (5). The essential enzyme for primary septum formation has been found to be chitin synthetase 2 or Chs2 (7). Cells deficient in Chs1 grow normally in well-buffered media, but show lysis of daughter cells in media that allow acidification during growth (8,9), an abnormality that would not have been detected in previous studies. The lysis appears to be caused by a chitinase that normally acts to facilitate cell separation and may cause excessive breakdown of chitin at low pH because of its very acidic pH optimum (9,10). The remedial effect of Chs1 is presumably attained by synthesizing additional chitin to make up for the excessive loss caused by the chitinase (9).

Two other considerations suggested the possible involvement of proteinase B in the activation of Chs1. First, as previously mentioned (11) and as will be shown below, the proteinase acts *in vitro* on Chs1 but not on Chs2. Second, the yeast chitinase was found to be localized partly in the periplasmic space and partly in the vacuole (12), the locale of proteinase B (13). In protoplasts, chitinase is secreted from the vacuole to the outside (12). In view of the balancing functions of chitinase and Chs1 during cell separation, it seemed possible that the hydrolytic enzyme and the synthetase-activating protease might be transported at the same time from the vacuole to the surface of the cell, to exert their opposing effects.

Because of all these reasons, it was deemed worthwhile to reinvestigate the possible link between Chs1 and protease B, using lysis in poorly buffered medium as a criterion of Chs1 function.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this work are enumerated in Table 1. Construction of strains and genetic analysis were carried out with standard methods (14). Growth was in minimal medium (0.7% Difco yeast nitrogen base, 2% glucose) plus required supplements where needed.

Introduction of a *CHS1* disruption in *prb1* strains by transformation. To obtain a disrupted *CHS1* gene, DNA was isolated by the method of Holm, *et al.* (15) from strain D3B, containing disruption 3 of Bulawa *et al.* (8). The DNA was used as template in a standard polymerase chain reaction (Perkin Elmer/Cetus) employing synthetic oligonucleotide primers (5'-ATCACTCAAACCGGAAG-3' and 5'-ACCCAGCGGATTTGCCA-3'). The primers were selected to generate a linear fragment with approximately 300 bp of *CHS1* sequences flanking the 1.2kb *URA3* insertion (8). The identity of the product was verified by restriction analysis. The fragment was phosphorylated with T4 polynucleotide kinase (New England Biolabs) and then used to transform strains ECY30-2A and ECY30-2D by the lithium acetate procedure (16) to uracil prototrophy.

Detection of lysed daughter cells. In *chs1* strains, daughter cells that appear refractile under phase contrast have lost their permeability barrier and present an orifice in their cell wall at the birth scar (9). The refractile (lysed) cells were counted as described (9). The results are given as percent of total cells, where a mother and daughter pair was counted as 2 cells.

Enzyme preparations and assays. The proteinase B preparation was a vacuole extract obtained from *S. cerevisiae* X2180, essentially as described previously (13) but omitting the Ficoll gradient.

For the experiment of Table 2, Chs1 was a digitonin-solubilized preparation (17) obtained from *S. cerevisiae* D3C. Chs2 was also a digitonin-solubilized preparation, obtained from membranes as previously described (11). In this case, the strain used was *S. cerevisiae* SSY563-9B which is devoid of Chs1 but harbors a high-copy plasmid with the *CHS2* gene (Table 1). This strain expresses Chs2 at a high level. In these experiments, the composition of the assay

Table 1. Strains

	GENOTYPE	LYSIS OF DAUGHTER CELLS	SOURCE
ECY4-10A	*MATa CAN^S chs1-23 ura3-52 his4 scs1*	-	This laboratory
ECY4-10B	*MATa his4 can^R chs1::URA3 ura3-52 SCS1*	+	"
ECY4-10C	*MATα can^R chs1-23 ura3-52 SCS1*	+	"
ECY4-10D	*MATα CAN^S chs1::URA3 ura3-52 scs1*	-	"
ECY22-12D	*MATa ura3-52 leu2 chs1::URA3 can^R*	+	"
SSY504-6D	*MATa can^R chs1::URA3 ura3-52 leu2-3,112 SCS1*	+	"
SSY563-9B	*MATa trp1-1 ura3-52 leu2-2 chs1-23 chs2::LEU2 SCS1* [YEp352-*CHS2*]	+	"
MSY121	*MATα can^R chs1-23 ura3-52 scs1*	-	"
D3B	*MATa his4 chs1::URA3 ura3-52 SCS1*	+	"
D3C	*MATα ura3-52*	-	"
BJ132	*MATa prb1-1122 his5*	-	E. Jones
BJ136	*MATα prb1-1122 trp1 SCS1*	-	E. Jones
2602	*MATα can^R ura3-52 his6 leu2-3,112 scs1*	-	R. Wickner
X2180	*MATa/MATα*	-	Yeast Genetic Stock Center

mixture was as described for Chs1 (17) and for Chs2 (Ref. 11, with Co^{2+} as the activating cation). For both enzymes, the cation, Mg^{2+} or Co^{2+}, was also present in the activation mixture. Incorporation of [^{14}C]GlcNAc from UDP[^{14}C]GlcNAc into chitin was measured as reported. During treatment of Chs2 with proteases, 1.2 mM unlabeled UDPGlcNAc was present to protect the enzyme against inactivation (E. Cabib, in preparation). Carrier-free UDP[^{14}C]GlcNAc was added before the assay in an amount sufficient to obtain a final specific activity of $4X10^5$ cpm/μmol. Other details are given under Table 2.

In the experiment of Table 4, cells were permeabilized with digitonin and Chs1 assayed as described (18).

RESULTS AND DISCUSSION

The cloning of the structural gene for Chs2 made available to us strains that express the enzyme at an elevated level because they harbor multicopy plasmids containing the *CHS2* gene. In these strains, a 20-fold increase in activity after trypsin treatment is easily accomplished, compared to 3- to 4-fold in strains with wild type levels of Chs2 (7); therefore a comparison of protease effects between the two synthetases becomes much easier. The results confirmed our preliminary findings (11), that trypsin activates the zymogens of both chitin synthetases, whereas proteinase B is only effective with the Chs1 zymogen (Table 2). Addition of antipain, an inhibitor of proteinase B, blocked the effect of the protease on Chs1 (Table 2).

The above results suggested that proteinase B might be the physiological activator of Chs1. If this were true, strains with a normal *CHS1* but a defective *PRB1* gene would show a defect in Chs1, manifested as the appearance of lysed (refractile in phase contrast) cells, when grown in a poorly buffered medium, such as minimal medium (9). Two strains containing the *prb1-1122* mutation, BJ132 and BJ136 (Table 1) were tested in that fashion. Both had a normal aspect with no apparent lysis, an indication that the *PRB1* gene is not required for Chs1 function. However, this evidence was not deemed to be definitive, because as previously mentioned (9) certain strains have been found to contain a suppressor of the lysis that results from deficiency in Chs1.

Table 2. Effect of trypsin and proteinase B on activity of chitin synthetase 1 and 2

Enzyme[a]	Activity after treatment (nmol GlcNAc incorporated/mg protein)			
	Untreated	Trypsin[b]	Proteinase B[c]	Proteinase B + antipain[d]
Chitin synthetase 1	0	355	120	6
Chitin synthetase 2	6	136	4	2

[a] The amount of protein used in the assay of the Chs1 preparation was 24 μg; for Chs2, 72 μg. For protease treatment, the incubation time was 15 min., followed by addition of excess trypsin inhibitor or of antipain (final concentration 8 μg/ml), for trypsin or proteinase B, respectively. In the subsequent assay, the incubation time was 30 min. For assay conditions see Methods.

[b] The amount of trypsin added that yielded maximal activation was 1 μg per assay for Chs1 and 2 μg per assay for Chs2.

[c] The amount of the proteinase B preparation added was 15 μg protein per assay (optimum amount) for Chs1 and 60 μg protein per assay for Chs2.

[d] Antipain added before, rather than after incubation with proteinase B.

The suppressor was first detected in strain MSY121, which has a mutation in *CHS1* but did not show lysis when grown in minimal medium. To determine if this trait was a characteristic of the specific *chs1* allele of MSY121 or was due to another gene, MSY121 was crossed (ECYX4) to a strain that did have the refractile character (D3B) as well as a *CHS1* disruption. Sporulation and dissection resulted in a 2:2 segregation of refractile to non-refractile cells. The refractile character was not linked to *CHS1* as demonstrated by the finding that the two different *chs1* alleles segregated independently of the refractile character (Table 1). The 2:2 segregation of this trait was reproduced when spores from ECYX4 were back crossed (e.g. ECY4-10B (refractile) x ECY4-10D (nonrefractile) or out-crossed (e.g. SSY504-6D (refractile) x ECY4-10D). The genetic locus responsible for the suppression was designated *SCS1* (suppressor of chitin synthetase 1) and cells that contained a *CHS1* mutation but were not refractile were considered to have an *scs1* mutation. Another strain that contains a suppressor of lysis is 2602. A cross with ECY4-10A (*scs1*) failed to segregate any refractile cells, suggesting that the suppressor in 2602 is, as well, *scs1*. An additional check of the independent segregation of *scs1* was performed by crossing strain SSY504-6D (*SCS1*) with strain 2602. The resulting 3:1 and 4:0 segregation of nonrefractile:refractile cells confirmed that *scs1* segregated independently of *CHS1*.

To ascertain whether the *scs1* mutation was dominant or recessive, three diploids were constructed that were *SCS1/scs1* (ECY4-10B X ECY4-10C; SSY504-6D X ECY4-10D; ECY4-10C X ECY4-10A). All of these showed the refractile character, so we conclude that the suppressor of chs1 lysis (*scs1*) is recessive. The results indicate that suppression of lysis results from loss of function. It may be caused by absence or diminished level of a protein required in addition to chitinase for cell wall digestion and cell lysis, such as a glucanase (9). Levels of chitinase and Chs1 in cells containing the suppressor did not differ significantly from those of wild type (results not shown).

Table 3. Segregation of lytic behavior in a cross between a *prb1* mutant and a strain harboring a disrupted *CHS1* gene

Tetrad	Colony	Lysis	Ura+[a]	can^R[b]
1	A	-	-	+
	B	+	+	+
	C	-	+	-
	D	+	+	-
2	A	-	-	-
	B	+	+	+
	C	+	+	+
	D	-	-	-
3	A	-	+	+
	B	-	-	+
	C	+	+	-
	D	+	+	-
4	A	-	+	+
	B	+	+	-
	C	+	+	-
	D	-	+	+
5	A	+	+	-
	B	+	+	+
	C	-	+	+
	D	-	+	-
6	A	-	-	+
	B	-	+	-
	C	+	+	-
	D	+	+	+
7	A	+	+	-
	B	-	+	+
	C	-	-	-
	D	+	+	+
8	A	+	+	+
	B	-	+	+
	C	+	+	-
	D	-	-	-

The strains crossed were BJ136 (*prb1-1122 trp1-1 CAN*^{S}) and ECY22-12D (*ura3-52 leu2 chs1*::*URA3 can*^{R}).

[a] Ura$^-$ strains are necessarily *CHS1*.

[b] *CAN*^{S} strains are presumed to be *prb1*, because of the tight linkage between the two genes (19).

To determine whether *prb1* strains contained *scs1*, one of them (BJ136) was crossed to a strain containing a disruption of the *CHS1* gene and exhibiting the refractile character. After sporulation and dissection, the progeny was examined for lysis in minimal medium and for *PRB1* allele (by detection of the closely linked (19) *can*^{R} character). All of the 8 tetrads analyzed in this fashion yielded a 2:2 segregation for lysis (Table 3). If the *prb1* strain had contained a suppressor, only one tetrad out of six should have segregated 2:2 (14). Furthermore, the 4 strains that could be positively identified as *CHS1 prb1* (*ura3 CAN*^{S}) i.e. 2A, 2D, 7C and 8D, did not show lysis.

Table 4. Effect of introducing a *CHS1* gene disruption in *prb1* cells by transformation

Strain	Chs1 activity[a]	Percent refractile cells[b]	
		Logarithmic phase	Early stationary phase
ECY30-2A	17	0	n.d.[c]
ECY30-2A (transformed)	<0.1	22	25
ECY30-2D	17.1	1	n.d.
ECY30-2D (transformed)	<0.1	19	33

[a] nmol GlcNAc incorporated into chitin/h/mg cells wet wt. The activity is given per mg cells rather than mg protein because intact permeabilized cells were used in the assay (18).

[b] For measurement of refractile (lysed) cells, see Methods.

[c] n.d., not determined.

Another, somewhat remote, possibility had not been eliminated, i.e. that *prb1* strains contained a suppressor of lysis closely linked to the *CHS1* gene. In such a case, both genes would segregate together and the suppressor would escape detection. To investigate this possibility, strains ECY30-2A and ECY30-2D (from tetrad #2 in Table 3) were transformed with a DNA fragment containing the *CHS1* gene disrupted by a *URA3* gene insertion (disruption 3 of Ref. 8). The transformants lacked Chs1 activity and showed lysis in daughter cells (Table 4). These results confirm that no suppressor of lysis was present in the *prb1* cells.

In conclusion, strains harboring the *prb1* mutation did not show a defect in Chs1 function, as manifested by lysis of daughter cells in a poorly buffered medium. The possibility of the presence of a lysis suppressor in those strains has been effectively eliminated.

Therefore, we can now state with assurance that proteinase B is not required for Chs1 function. Although the nature of the activator and of the activation mechanism of Chs1 and Chs2 remain unresolved, the specificity shown by proteinase B suggests that other proteases or different enzymes may distinguish between the two synthetases and act on their regulation.

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