FEBS Letters 351 (1994) 263–266

FEBS 14486

A 70-kDa heat shock cognate protein suppresses the defects caused by a proteasome mutation in *Saccharomyces cerevisiae*

Masayuki Ohba*

Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo, Japan

Received 24 June 1994; revised version received 30 July 1994

Abstract An allele of mutation in the proteasome subunit gene *Y7*, *y7-1*, caused a temperature-sensitive growth in *S. cerevisiae*. One of the multi-copy suppressor genes for this growth defect was identical to *SSB1*, which encodes a 70-kDa heat shock cognate protein of the yeast. Introduction of the multi-copy *SSB1* gene into the *y7-1* mutant cells suppressed defects in the degradation of X-β-galactosidase (X = Arg or Pro) observed in the mutant cells. Thus, the *SSB1* protein, one of the chaperons of the yeast, facilitated intracellular protein degradation.

Key words: Proteasome; Heat-shock protein; N-End rule; Yeast

1. Introduction

In living cells, renewal of cellular proteins is guaranteed by the coordinate balance of two processes which mainly occur in the cytoplasm, namely protein synthesis and degradation. Changes in the efficiency of either one of the processes will cause severe effects on cell growth. While the process of protein synthesis has been extensively studied both biochemically and genetically, the understanding of the degradation process has started to expand recently. In the cytoplasm, the major process of protein degradation is catalyzed by a proteasome complex(s), whose catalytic core is the 20S proteasome complex composed of more than twenty subunits with a molecular mass of 20–31 kDa [1]. Both ubiquitin-conjugated and ubiquitin-free proteins were degraded through this pathway and their intracellular half-lives are governed by the N-end rule [2]. Several genes were isolated for the 20S proteasome subunits from rat, human and yeast. Genetic manipulation to delete the subunit genes from the yeast *S. cerevisiae* showed that they are indispensable for cell viability, except for the subunit *Y13* gene [3]. This indicates the physiological significance of the proteasome complex and the protein degradation process catalyzed by it. However, the components which initially recognize and lead abnormal proteins into the proteasome-dependent degradation pathway are largely unknown, except for a gene (*UBR1*) of the N-end recognizing protein of the yeast [4]. Chaperon proteins are one of the candidates for such components [5], since they preferentially bind to misfolded or loosely-folded proteins, which are often the targets of proteolytic degradation.

The yeast mutant of the 20S proteasome subunit gene *Y7* has been isolated. The mutation is associated with a temperature-sensitive (ts) growth defect. Taking advantage of the conditional lethality of this mutant, the multi-copy suppressors for the defect were isolated. One of the suppressor gene was *SSB1* [6], a gene for a cytosolic chaperon in yeast.

2. Experimental procedures

2.1. Plasmids

A plasmid pmc8 encodes a *COXIV* derivative, MGIPPKKKRKGI-SISLRQSIRFFKPATRTLCSSRSRYLL sequence fused to the mature part of *COXIV*, which was constructed from pmc4 [7]. A single-copy plasmid pRSY7 has a 1.3 kb *Sac*I *Hin*dIII fragment of the proteasome subunit *Y7* gene [3] inserted into pRS315 [8]. A multi-copy plasmid of the *SSB1* gene, pYEX1, was constructed by subcloning a 3.5 kb *Sph*I–*Sac*I fragment derived from CX clone into YEP351 [9]. A single-copy plasmid of SSB1 gene, pRSX1, carried a 2.6 kb *Xba*I–*Nhe*I fragment of the CX clone on pRS315.

2.2. Yeast strains

The strain BYL18 was the transformant of BYL1 (*a, leu2, his4, ade2, ura3, coxIV::LEU2*) [10] with pmc8. This strain barely grew on glycerol as the sole carbon source (*Gly*$^-$) because the *COXIV* molecule coded by pmc8 has a short half-life in the cells (data not shown). The BYL18 cells were mutagenized with ethyl methane sulfonate and screened for the ability to grow rapidly on glycerol (*Gly*$^+$). One of the *Gly*$^+$ mutants, a strain G116, bore a new allele of mutation in a proteasome subunit gene *Y7* (named *y7-1*) which is associated with ts growth phenotype on YPD medium. The *y7-1* mutation probably caused the *Gly*$^+$ phenotype by impairing the rapid degradation of the *COXIV* molecule (coded by pmc8) in the cytoplasm. The details of the screening, along with the analysis of the other *Gly*$^+$ mutant strains, will be described elsewhere. Strain CG13 (*a, leu2 ade2 ura3 y7-1*) was a progeny of the diploid between G116 (*a leu2 his4 ade2 ura3 coxIV::LEU2 y7-1*) and DBY746 (*α his3 leu2 ura3 trp1*), and was used for the isolation of multi-copy suppressors and for the analysis of the mutant phenotypes described below .

2.3. Isolation of multi-copy suppressor

The CG13 cells were transformed with a yeast genomic library constructed in YEP13 [11] and the transformants were screened for the growth on YPD at 37°C, a non-permissive temperature for the growth of the CG13 cells. Plasmids were recovered from the transformants which grew at 37°C and classified according to the restriction map of their inserts. After removing the *Y7*-containing clones, five different clones were obtained as multi-copy suppressor for the ts growth.

2.4. Assay of β-galactosidase activity

The plasmids pUB23-M, pUB23-P and pUB23-R [12], which encode ubiquitin-X-β-galactosidase (Ub-X-βgal; X = Met,Pro or Arg), were generous gifts from Dr. Varshavsky. The CG13 cells were transformed with pRSY7 (*Y7*), pYEX1 (*SSB1*) or YEP351. The transformants were further transformed with pUB23-M, pUB23-R or pUB23-P to give the strains carrying nine combinations of the plasmids (see Fig. 3). These cells were grown overnight in minimal medium (SD) supplemented with growth requirements (leucine, adenine and uracil), and then inoculated into a galactose-medium (2% Galactose, 2% Raffinose, 2% Glycerol, 2% Lactate, 0.05% Glucose and 0.67% Nitrogen base) supplemented with the growth requirements. After overnight culture at 30°C to 1.5–2.0 OD_{600} the cells were harvested and their intracellular β-galactosidase

*Corresponding author. Fax: (81) (427) 29 1252.

SSDI 0014-5793(94)00873-6

activities were assayed at 25°C according to the method [13] using *o*-nitrophenol as the substrate.

2.5. Miscellaneous methods

Published procedures were used for the transformation yeast cells [14], for recombinant DNA methods [15], for yeast genetic methods [16] and for the dideoxy chain-termination methods [17]. Except when scoring temperature sensitivity or selecting for temperature resistant clones, the routine growth temperature for all other manipulations was 24°C and standard yeast media [16] were used in this study.

3. Results and discussion

The strain CG13 bore a mutation in the proteasome subunit gene *Y7* (*y7-1*) and showed a ts growth phenotype at 37°C. To get insight into the defects caused by the *y7-1* mutation, multicopy suppressors were isolated for the ts defect. By screening a yeast genomic library on YEP13, the clones which restored the growth of the CG13 cells at 37°C to varying degrees were isolated. Of the total five suppressors obtained by the screening, the plasmid clone named CX was further analyzed and described here. As shown in Fig. 1A, the suppression by CX was nearly at the same level as that by the authentic *Y7* gene (compare the suppression by clone C39, a weak suppressor obtained by the screening). Fig. 2 shows the restriction map of the insert and the localization of the suppression activity in the CX clone. The full suppression was obtained by subcloning a 4.1 kb *Xba*I–*Nhe*I fragment into the multi-copy vector YEP351. The partial sequence from the *Hin*dIII sites of the fragment indicated that it contained the entire coding region of *SSB1* [6] and the 3′-flanking region of *MER1* [18]; the activity was ascribed to the *SSB1* gene. The SSB1 locus was adjacent to *MER1* (a gene for meiotic recombination), which is genetically mapped on the left arm of chromosomes XIV [19]. (The analysis of the C39 clone identified *PED1* [20] as the other suppressor gene.)

The *SSB1* gene encodes a heat shock cognate protein having a molecular mass of 70-kDa (Ssb1p), which belongs to the stress-seventy (70) protein family of yeast [21]. Mainly based on their sequence homology, eight proteins are reported as members of this family. They are further classified into four subgroups named SSA through SSD [22]. Although the degree of sequence homology and the subcellular distribution differ from each other, they are supposed to share chaperon activities which facilitate folding/unfolding of the cellular proteins. Localized in the cytoplasm, Ssb1p is thought to be a chaperon for cytosolic proteins. The suppression by the *SSB1* gene was dosage-dependent, which requires the presence of more than two copies of the *SSB1* in the cell; a single-copy plasmid of *SSB1* (pRSX1) could not suppress the growth defect of the mutant, as shown in Fig. 1 (B). This is consistent with the idea that the Ssb1p plays a structural role rather than a catalytical role in the suppression. Since the synthesis of Ssb1p decreases at high growth temperatures [21], the supplementing of the Ssb1p from the multi-copy plasmid will be necessary to accommodate the large amount of undegraded proteins which will accumulate in the cytoplasm of the mutant cells.

To know whether the Ssb1p directly plays a role in protein degradation, I measured the stability of X-βgal (X = Met, Pro or Arg) molecules in the mutant cells in the presence of the multi-copy *SSB1* gene. The respective Ub-X-βgal was expressed in the mutant cells which carried pRS315 (vector), pRSY7 (single-copy *Y7*) or pYEX1 (multi-copy *SSB1*), and their intracellular β-galactosidase activities were measured to

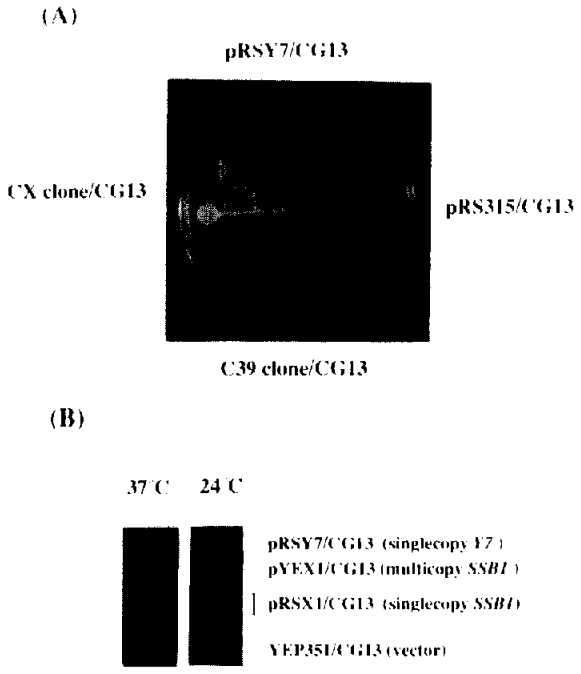

Fig. 1. (A) Suppression of ts growth of CG13 by CX or C39 clones. The growth of the CG13 cells carrying pRSY7 (*Y7*), YEP351, CX (*SSB1*) or C39 (*PED1*) was assayed on a YPD plate with incubation at 37°C for 2 days. (B) The growth of CG13 cells carrying *SSB1* gene on multi-copy or single-copy vector. The cell suspensions were dropped on a YPD plate and incubated at 24°C or 37°C for 2 days. The transformant with pSRY7 (*Y7*) and YPE351 (vector) were also spotted as a control.

monitor the stabilities of the X-βgal molecules in these cells. The Ub-X-βgal is hydrolyzed to X-βgal, which is then degraded according to the N-end rule through a proteasome-dependent proteolytic pathway; Met-βgal is metabolically stable, while Pro- (or Ub-Pro-, see [24]) and Arg-β gal molecules are less stable and rapidly degraded in the cells. As shown in Fig. 3, the activities from Pro-X-βgal and Arg-X-βgal exhibited 21% and 2.1% of that from Met-X-βgal, respectively, in pRSY7/CG13 cells, where the *Y7* gene on the plasmid suppressed the effect of the chromosomal y7-1 mutation (Fig. 3B). On the other hand, the corresponding values increased up to 87% and 27%, respectively, in the mutant cells with the control vector, pRS315/CG13 (Fig. 3A). Thus, the *y7-1* mutation impaired the proteolytic activity of the proteasome and thereby stabilized the fusion proteins which were otherwise short-lived in the cells. This is consistent with the results obtained from other proteasome-deficient mutants [24,25]. Under these conditions, the multi-copy *SSB1* gene suppressed the effects of the y7-1 mutation; the values for Pro- and Arg-βgal in pYEX1/CG13 cells were 32% and 12%, respectively, which were significantly lower than those observed in the pRS315/CG13 cells (Fig. 3C). Thus, the multi-copy *SSB1* gene suppresses not only the growth defect of the mutant but also facilitates the process of protein degradation impaired by the *y7-1* mutation.

What is the mechanism of the suppression by multi-copy *SSB1*? Nelson et al. [26] reported that nearly half of Ssb1/2p is associated with the nascent polypeptides on translating ribosomes, and suggested that the Ssb1/2p functions as a chaperon to prevent the misfolding of nascent polypeptides during their

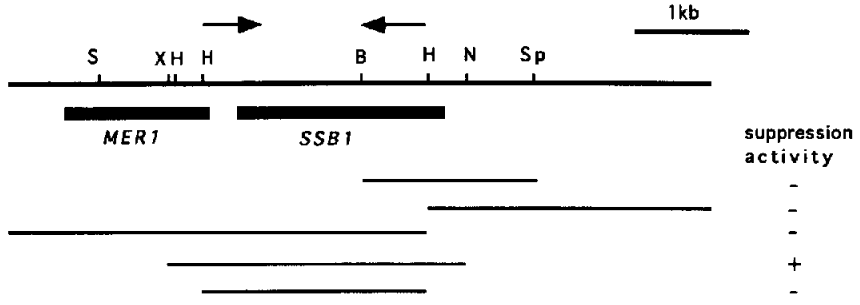

Fig. 2. Restriction map of the insert of CX clone and suppression of ts growth by its subclones. The restriction enzyme abbreviations were B, *Bgl*II; H, *Hin*dIII; N, *Nhe*I; S, *Sac*I; Sp, *Sph*I and X, *Xba*I. The coding sequence (thick black line) and the direction of sequencing (→) are indicated. The subclones were obtained by deleting or subcloning (on YEP351) the respective restriction fragments of the inserts and introduced into the CG13 cells to measure the suppression activity (+, growth; −, no growth).

synthesis (*SSB2* is the other member of the SSB subgroup and codes a functional homologue of Ssb1p). Analogous to this, Ssb1/2p will function as a chaperon to facilitate the protein degradation by directly interacting with the target proteins. If the nascent polypeptides fail to fold correctly by the end of translation, they will be released into the cytoplasm in a complex with Ssb1/2p. Ssb1/2p will also bind to the cellular proteins whose conformations were damaged by environmental stress such as heat shock and aging. In either a co- or post-translational manner, the binding of Ssb1/Ssb2p to misfolded/unfolded proteins will help to maintain the loose conformations of the bound proteins and thereby prepare them for their proteolytic degradation. In *E. coli*, mutations in the heat shock genes including a *dnaK* mutation, result in defective proteolysis of puromycin-released polypeptides [27]. Since the *DnaK* product is a 70-kDa heat shock protein and can bind to short-lived proteins in vitro [28], this bacterial heat shock protein also functions as a chaperon in the degradation of the cellular proteins.

Another possibility for the mode of suppression by the *SSB1* is that the Ssb1/2p facilitates the transfer of misfolded proteins

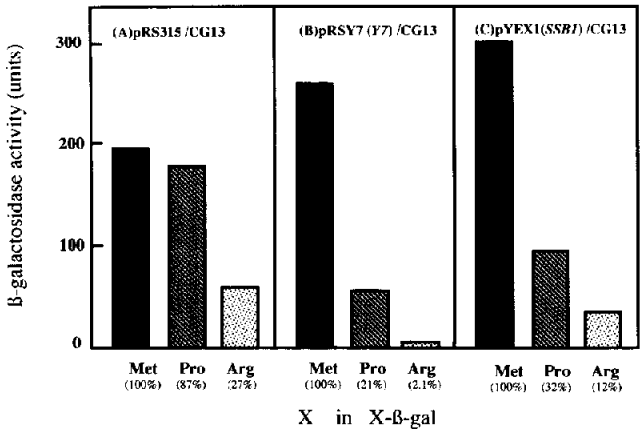

Fig. 3. Effect of multi-copy SSB1 on metabolic instability of X-β-gal protein. The CG13 strain carrying *Y7* gene (pRSY7), multi-copy *SSB1* gene (pYEX1) or a vector (YEP351) was transformed with the plasmid encoding the respective Ub-X-β-gal. The transformant cells were grown near a late log phase at 30°C in the galactose-medium to induce the expression of Ub-X-β-gal, and assayed for the intracellular β-galactosidase activity as described in section 2. The values of β-galactosidase activity were the means of two experiments and given in Miller units [14]. Their relative values to those of the respective Met-β-gal (in%) were described in the parentheses.

to lysosomes, thereby bypassing the proteasome-dependent degradation pathway. Although the significance of the lysosomal pathway in the intracellular protein degradation is not clear, the RNaseA molecules microinjected into culture cells are rapidly degraded in a lysosome-dependent manner [29]. Chiang et al. [30] further suggested that Hsc70 protein (a heat shock 70-kDa cognate protein in mammalian cells) is a cytosolic factor to promote this process. In the yeast, the shift from glyconeogenic to glycolytic growth induces the rapid degradation of cytosolic fructose-1,6-bisphosphate in the vacuole, and this process is defective in *pep4* vacuolar hydrase-deficient mutants [31]. It will be interesting to determine whether the *pep4* mutation also affects the suppression by the *SSB1*.

The analysis of the CX suppressor gene provided information on the protein which will modulate the defects in protein degradation (*SSB1*). The molecular analysis of the other suppressor genes will help toward further understanding of these points.

Acknowledgements: I thank Dr. A. Varshavsky for the plasmids encoding ubiquitin-X-β-gal, and Drs. M.Tokunaga, A. Sakai and F. Hishinuma for their helpful discussions during this study.

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