Modulation of intracellular protein degradation by SSB1–SIS1 chaperon system in yeast *S. cerevisiae*

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Abstract In prokaryotes, DnaK–DnaJ chaperon is involved in the protein degradation catalyzed by proteases La and ClpA/B complex as shown in *E. coli*. To extend this into eukaryotic cells, we examined the effects of hsp70 genes, *SSA1* and *SSB1*, and DnaJ genes, *SIS1* and *YDJ1*, on the growth of proteasome subunit mutants of the yeast *S. cerevisiae*. The results identified *SSB1* and *SIS1* as a pair of chaperon genes specifically involved in efficient protein turnover in the yeast, whose overexpression suppressed the growth defects caused by the proteasome mutations. Moreover, a single amino acid substitution in the putative peptide-binding site of SSB1 protein profoundly enhanced the suppression activity, indicating that the activity is mediated by the peptide-binding activity of this chaperon. Thus *SSB1*, with its partner DnaJ, *SIS1*, modulates the efficiency of protein turnover through its chaperon activity.

Key words: Protein degradation; Molecular chaperon; *SSB1*; *SIS1*; In vitro mutagenesis

1. Introduction

Protein turnover in eukaryotic cells depends on proteolytic degradation machinery localized in subcellular compartments [1]. Molecular chaperons in each compartment are speculated to facilitate this degradation process either by targeting misfolded proteins for the degradation and/or by unfolding protein conformation feasible to proteolytic attack. In *E. coli*, DnaK (Hsp70 in prokaryotes) and DnaJ proteins are involved in the efficient turnover of abnormal and short-lived proteins, probably acting as molecular chaperons in the protein degradation process [2,3]. The system of protein breakdown and chaperons in eukaryotic cells is more complex than that in prokaryotic cells. The eukaryotic chaperon system in the cytosol consists of members of Hsp70 and DnaJ heat shock protein families [4]. There, the degradation of cytosolic proteins proceeds through multiple steps, which include the modification of target proteins with ubiquitin and the final digestion by a large protease composed of more than 30 subunits [5], named the 26S proteasome complex. This system poses two questions as to the function of chaperons in the eukaryotic protein degradation: first, which of these multiple chaperons are specifically involved in protein degradation and, second, what is the role of these chaperons in the process. These question were addressed by examining the effects of chaperon genes on yeast proteasome mutant strains having defective protein degradation activity.

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2. Materials and methods

Construction of plasmids was carried out according to methods described [6–8]. Most of the experiments were done with the proteasome *y7* mutant previously described [9]. A proteasome *y13* mutant, which was isolated through the same screening as the *y7* mutant isolation, was also used. The details of their isolation procedure will be described elsewhere.

ssb1-A mutant gene was isolated as follows; YCp*SSB1* plasmid, which carries the *Xba*1–*Nhe*1 fragment containing *SSB1* on YCp50 vector, was treated with hydroxylamine (200 μg/mg YCp*SSB1* DNA in 0.5 M hydroxylamine-HCl, 0.5 M potassium phosphate, pH 6.0) at 70°C for 1 h, inducing mutational changes to a degree which reduce the number of Amp-resistant colonies to 1% of the untreated level. The mutagenized plasmids were introduced into the *y7* cells with the lithium-acetate method [10]. The transformants (140 000 clones) were screened for those which made colonies on YPD plate after incubation at 37°C for 2 days. *ssb1-A* plasmid was recovered from one of these rapidly growing colonies in the screening (total seven candidate clones were obtained).

3. Results and discussion

In the yeast *S. cerevisiae*, hsp70 proteins of SSA and SSB families (SSA1–4 and SSB1–2) and two DnaJ-related proteins (SIS1 and YDJ1) are known to localize in the cytosol [11]. To identify chaperons participating in cytosolic protein degradation in the cytosol, multicopy suppresser activity was examined for their representative genes. Fig. 1A shows the growth of *y7* mutant cells transformed with a multicopy of *SSA1*, *SSB1*, *SIS1* and *YDJ 1* genes. Multicopy of *SSB1* gene, a member of SSB family, suppresses the temperature-sensitive growth caused by the proteasome subunit Y7 mutation (YEp*SSB1*). As reported previously, it also suppressed a low efficiency of protein turnover observed in the mutant cells [9]. A gene for other hsp70 protein, SSA1, exhibited no suppression activity (YEp*SSA1*). *SSA1* codes for a hsp70 which is more abundantly present in the cytosol than SSB1 protein and whose expression significantly increases at high temperatures in vivo, and the amino acid sequence of SSA1 and SSB1 proteins share about 60% amino acid identity [12,13]. In spite of these, *SSA1* could not substitute for *SSB1* in the suppression of the proteasome defects. Among the genes introduced in the mutant cells, only *SIS1* exhibited a similar degree of suppression of the growth defect at 37°C to *SSB1*, when placed on a high copy-number plasmid (YEp*SIS1*). Another DnaJ homologue, *YDJ1*, exhibited no activity (YEp*YDJ1*). Thus the suppression activity is characteristic of *SSB1* and *SIS1* genes. The same pattern of suppression of *SSB1* and *SIS1* was observed in another proteasome subunit mutant, a Y13 subunit mutant with a temperature-sensitive growth phenotype. As shown in Fig. 1B, the growth defects of the mutant cells was suppressed by the introduction of multicopy of *SSB1* or *SIS1*, but not by multicopy of *SSA1*

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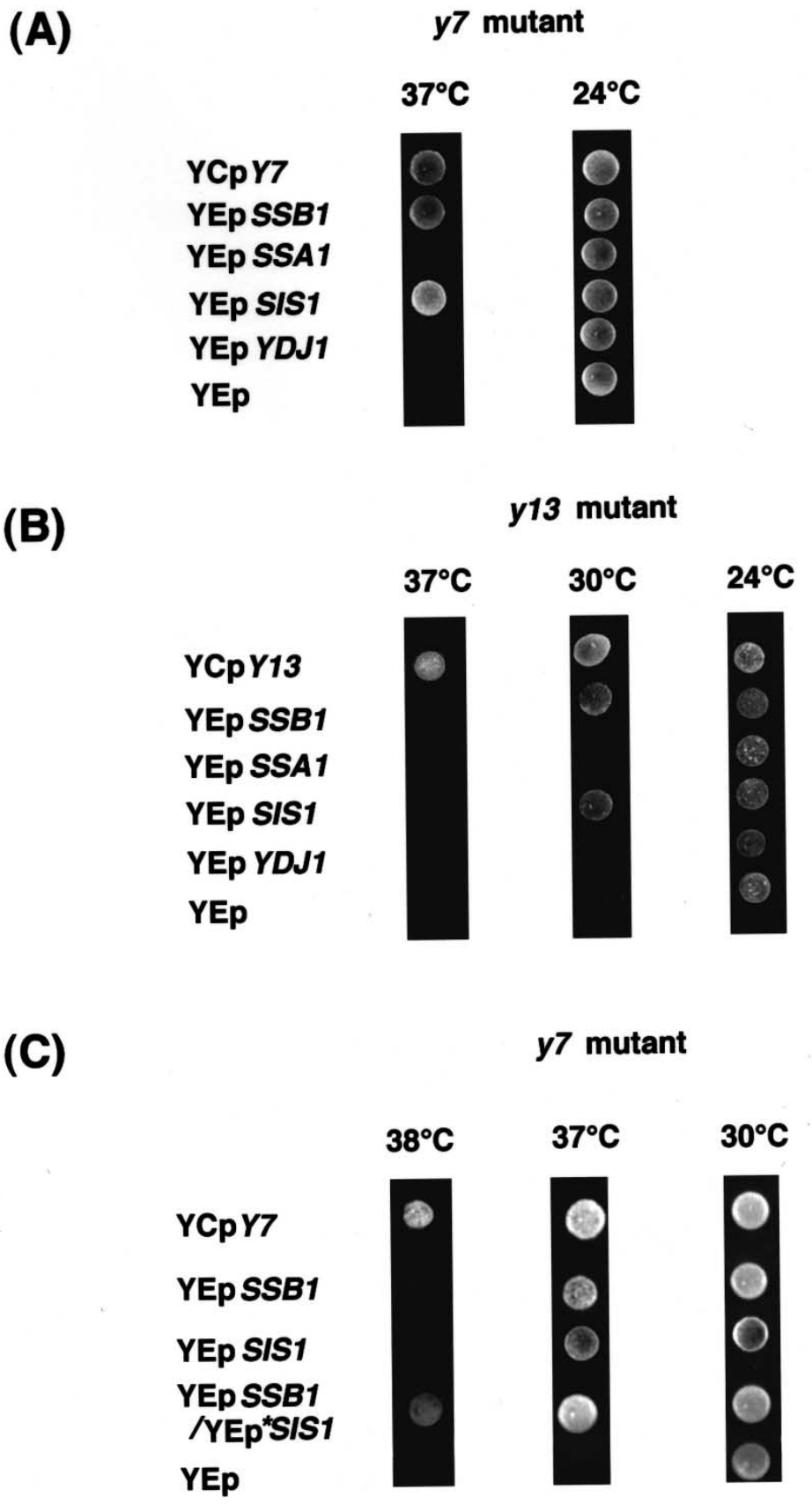

or *YDJ1*. The suppression was partial at 30°C and not observed at 37°C in the case of *y13* mutant, but the specificity is the same as in the *y7* mutants. This suggests that the activity of *SSB1* and *SIS1* is not related to the specific character of

Fig. 1. Suppression of the proteasome mutants by hsp70 and DnaJ genes. A: The temperature-sensitive growth of the proteasome Y7 subunit mutant was suppressed by multicopy of *SSB1* or *SIS1*. The culture of *y7* mutant cells carrying *SSB1*(YEp*SSB1*), *SSA1* (YEp*SSA1*) or SIS1 (YEp*SIS1*) gene on a multicopy vector (YEP351) was spotted on a YPD plate to examine growth at 37°C or 24°C. Transformants with wild-type *Y7* (YCp*Y7*) on a single-copy vector (pRS314) and the multicopy vector alone (YEp) were also spotted to represent the growth of the wild- and mutant-type cells, respectively. B: The temperature-sensitive growth of a proteasome Y13 subunit mutant was suppressed by multicopy of *SSB1* or *SIS1*. The *y13* mutant cells were transformed with the same set of plasmids as tested in (A). The cells were spotted on YPD and incubated at 24°C, 30°C, or 37°C. The specific suppressions were observed at 30°C in the cells with YEp*SSB1* and YEp*SSA1*. C: Coexpression of *SSB1* and *SIS1* in the Y7 subunit mutant further improved the cell growth. The growth of the *y7* mutant cells carrying the multiple copy of both *SSB1* (YEp*SSB1*) and SIS1 (YEp*SIS1**, *SIS1* on a multicopy vector pCF35) was compared with that of the mutant cells with either *SSB1* or *SIS1* at 30°C, 37°C or 38°C.
←

the proteasome subunit mutations but rather to the impaired activity of proteolytic protein degradation in the mutant cells. The presence of multicopy of *SSB1* or *SIS1* significantly improved the turnover of short-lived proteins in the *y7* mutant cells, as monitored by the stability of N-end rule substrates [14] in vivo (data not shown). Thus, the subset of Hsp70–DnaJ pairs, *SSB1* and *SIS1*, seems to be specialized for progression of the protein degradation catalyzed by the proteasome complex.

In prokaryotes, DnaJ protein is known to stimulate DnaK

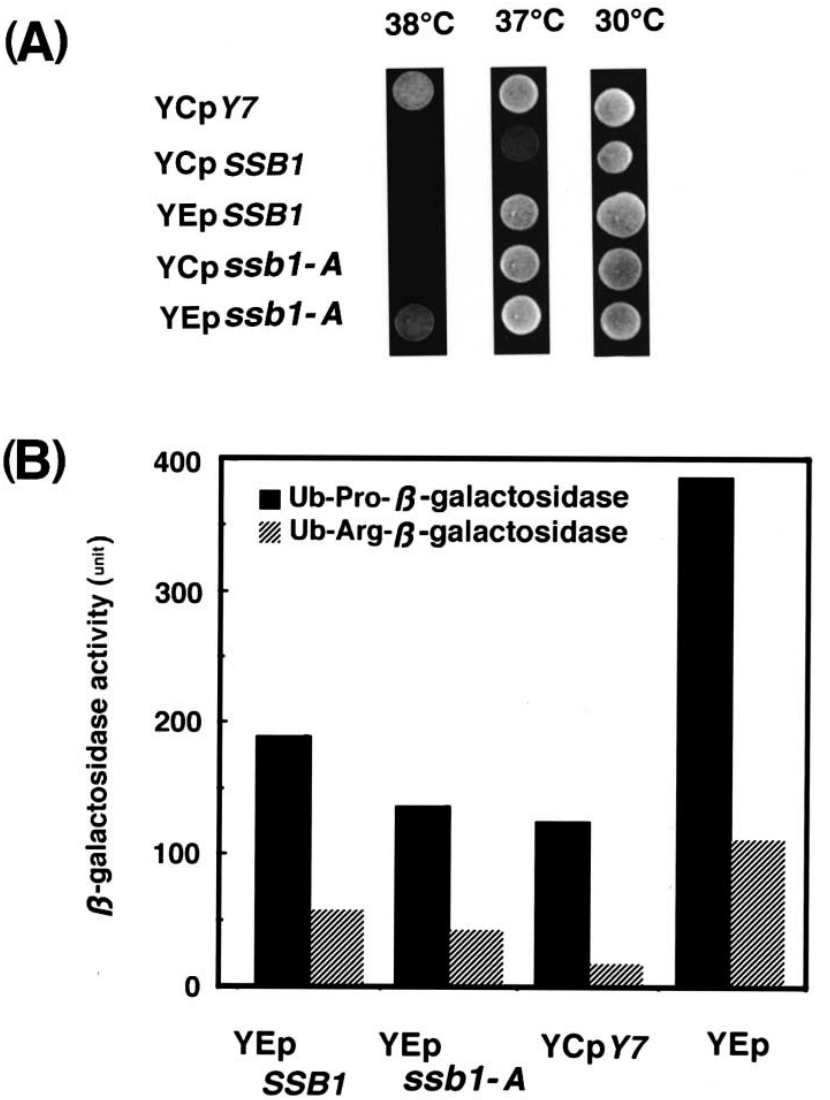

Fig. 2. Effects of *ssb1-A* mutation on the activity of *SSB1* gene. A: *ssb1-A* mutation elevated the suppression of *y7* mutant growth defect. The *y7* mutant cells transformed with *SSB1* or *ssb1-A* plasmids were spotted on YPD plates to monitor growth at 30°C, 37°C or 38°C for 3 days (the cells with YCp*Y7* corresponds to the wild-type cells). YCp*Y7*, YCp*SSB1* and YCp*ssb1-A* are single-copy plasmids, while YEp*SSB1* and YEp*ssb1-A* are multicopy plasmids. B: ssb1-A mutation elevated the turnover of Ub-Pro/Arg-β-galactosidase molecules in *y7* mutant cells. *y7* mutant cells carrying YCp*Y7*, YEp*SSB1*, YEp*ssb1-A* or YEp were further transformed with the pUb23 series plasmids to express Ub-Pro/Arg-β-galactosidase [17] as a marker for protein turnover in the cells. The cells were cultured at 30°C in a galactose-medium to induce the expression of the ubiquitinated β-galactosidase proteins, and the levels of β-galactosidase activity in the late-log phase cells were measured using a *o*-nitrophenyl-β-D-galactosidase assay. Units of β-galactosidase specific activity were calculated according to the Miller unit [18].

chaperon activity by accelerating the cycle of peptide-binding and -release coupled to ATP hydrolysis by this chaperon [15,16]. Similarly, the cooperation of *SIS1* and *SSB1* was expected to stimulate their suppresser activity in the yeast cells. In fact, the simultaneous presence of multicopy *SSB1* and *SIS1* in the cells expanded the temperature range of the growth-defect suppression. As shown in Fig. 1C, the mutant cells co-transformed with multicopy *SSB1* and *SIS1* (YEp*SSB1*/YEp*SIS1**) grow at 38°C at a rate comparable to that of wild-type cells, while those with only one of the plasmids ceased to grow at this temperature. The synergistic effect was specific for the pair of *SIS1* and *SSB1*; the double transformants with an alternative pair of genes, *SSB1–YDJ1* or *SSA1–SIS1* did not grow at 38°C. Thus *SIS1*, which itself has the suppresser activity, seems to serve as a SSB1-specific DnaJ homologue which stimulates the activity of *SSB1* in the degradation process.

Next, the molecular character of SSB1 protein which relates this chaperon-to-protein degradation was examined. To this end, a mutation on SSB1 which elevates suppression activity was created, and its mutational change was mapped on a structural model of SSB1 protein. A single-copy plasmid bearing *SSB1* (YCp*SSB1*) was mutagenized with hydroxylamine in vitro and introduced into the *y7* mutant cells. Since the efficiency of the suppression is dependent on the copy number of *SSB1* in the cell, the original single-copy plasmid exhibited only a weak suppression at 37°C. Colonies which showed rapid grow at 37°C, however, appeared in the transformants with the mutagenized plasmids. Analysis of the plasmids carried by these colonies identified a mutation on *SSB1*, *ssb1-A*, which enhances its suppression activity. As shown in Fig. 2A, the single-copy plasmid carrying *ssb1-A* (YCp*ssb1-A*) suppressed the growth defects of the *y7* mutant cells as efficiently as multicopy *SSB1* (YEp*SSB1*). The mutant cells bearing multicopy *ssb1-A* grew at a temperature up to 38°C, the temperature at which only the wild-type cells (YCp*Y7*) could grow. Thus *ssb1-A* is a gain-of-function mutation having hypersuppression activity. The mutation also affected the efficiency of protein turnover in the yeast cells. Fig. 2B compares the intracellular stabilities of ubiquitin(Ub)-Pro/Arg-β-galactosidase molecules [17,18] in the *y7* mutant cells with multicopy of *SSB1* or the mutant *ssb1-A* gene. Since Ub-Pro/Arg-β-galactosidase proteins are degraded through a proteolytic pathway dependent on the proteolytic activity of the proteasome complex (compare YEp and YCp*Y7* in Fig. 2B), the lower stability of these reporter proteins means the higher level of protein degradation activity in the cells. Here, the stabilities were more profoundly reduced by the presence of multicopy *ssb1-A* than multicopy *SSB1* in the cells. This indicates that the *ssb1-A* mutation enhanced the stimulatory activity of *SSB1* on the proteasome-dependent breakdown of short-lived proteins represented by Ub-Pro/Arg-β-galactosidase.

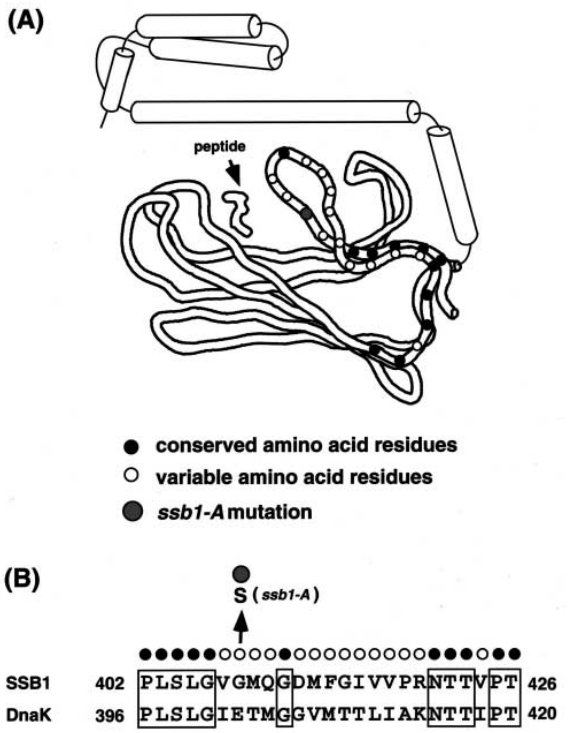

Fig. 3. The site of *ssb1-A* mutational change superimposed on the structural model of DnaK. A: *ssb1-A* mutational change superimposed on the structure of the peptide-binding domain of DnaK. Based on the amino acid sequence similarity of SSB1 to DnaK, the sequence around the ssb1-A mutation was mapped onto the structure of the peptide binding domain of DnaK (residues 384–638) (the model was based on [17]). The locations of the amino acid residues of the SSB1 sequence in (B) are indicated by circles on the loop structure in the model. The *ssb1-A* mutation site (which corresponded to Asp^{402} in DnaK) is shown by a shaded circle. Filled (●) and open (○) circles indicate the position of conserved and variable amino acids between DnaK and SSB1, respectively. B: Comparison of the amino acid sequence around *ssb1-A* mutation with the DnaK sequence. The amino acid sequence near the amino-terminal proximity of the peptide binding domain *E. coli* DnaK (residues 405–429) and its corresponding sequence of SSB1 (residues 399–423) are aligned. In closed boxes are the conserved amino acids (●). The *ssb1-A* change, gly^{409} of SSB1 to serine (shaded circle) occurred in the region with variable amino acids (○). The same symbols are used in the structure model shown in (A).

The analysis of the activity of chimera genes of *SSB1* and *ssb1-A* showed that the hypersuppression activity comes from the *Hin*dIII–*Bgl*II fragment of the *ssb1-A* gene, which covers the posterior third of the SSB1 coding region. Nucleotide sequence analysis of this region of *ssb1-A* revealed a single G-to-A transition which replaces glycine residue at 408 to serine in the sequence of SSB1 protein. The substitution has occurred in the carboxyl-terminal peptide-binding domain of this hsp70; hsp70 family proteins have two domains — an amino-terminal ATP-binding domain and a carboxyl-terminal peptide-binding domain. The structure of the peptide-binding domain of a prokaryotic Hsp70, DnaK protein of *E. coli*, was recently solved to 2.3 Å resolution in a complex form with a model peptide NRLLLTG [19]. As illustrated in Fig. 3A, the structure is composed of a peptide-binding platform formed by several loops of the polypeptide chain, accompanied by five successive and overhanging α-helix structures. When the amino acid sequences of SSB1 and the DnaK peptide-binding domains were aligned pairwise, blocks of amino acid identity (with ≈60% identity over its entity) were found, which reflect the evolutional conservation of hsp70 family protein sequences. These conserved amino acids allowed us to superimpose the SSB1 sequence on the DnaK structure, using them as landmarks. In the sequences around the mutation site (shown in Fig. 3B), the mutation site of gly^{408} is located in a less-conserved region of SSB1 sequence which is flanked by two blocks of conserved amino acids. This sequence was mapped on a loop structure in the peptide-binding platform in Fig.

3A. The mutation site is located in the middle of this loop, which is in the vicinity of the peptide trapped in the pocket structure. The amino acids shared by SSB1 and DnaK sequences are clustered near the two ends of this loop, which suggests that the structure of this region of SSB1 protein does not largely deviates from the DnaK model. Thus, the *ssb1-A* mutation occurred in the residue which could be directly involved in the specificity and/or efficiency of the peptide-binding activity of SSB1 protein. It is likely that the glycine-to-serine substitution at this position creates a new hydrogen bond to increase the interaction with substrate peptides. Or the substitution induces perturbation in the whole structure of this putative binding pocket, resulting in changes in the properties of the peptide-binding pocket. In either case, the *ssb1-A* mutational change should result in the modification of the peptide-binding site of SSB1 so as to enhance its effects on the proteasome-dependent process.

The results here suggested that the SSB1–SIS1 system is preferentially used in efficient intracellular protein degradation in the yeast. This, however, does not exclude the involvement of other chaperon proteins in the process; multicopy suppression approach only identified, at least theoretically, the genes which can affect rate-limiting steps for the cell growth. Since the protein degradation process consists of multiple steps, it is likely that several chaperon proteins participate in recognition of substrate protein structure in each step. In fact, *ydj1* mutation is reported to impair the degradation of both short-lived normal proteins and abnormal proteins, and to retard the degradation and phosphorylation of cyclin B [20]. Since ubiquitin-conjugated proteins failed to accumulate after heat treatment in this mutant cells, it is suggested that YDJ1 chaperon plays a role in substrate recognition or modification by ubiquitination enzymes [21]. Thus, the SSA1–YDJ1 system, whose functions in the protein translocation into mitochondria and the endoplasmic reticulum have been established, also play a role in the protein turnover, although their genes did not exhibit suppression of the proteasome mutant defect described here. It is possible that the involvement of SSB1–SIS1 is highlighted under proteasome-defective conditions. However, the turnover of ubiquitin-modified β-galactosidase molecules was promoted by the presence of multicopy *SSB1* in the wild-type cells as well as the proteasome mutant cells, and it was impaired under a conditions with the depletion of SSB chaperons (in *SSB1l2* disruptant cells, unpublished data). These results support that the intracellular contents of SSB1 chaperon is a major determinant of the efficiency of the protein degradation process under physiological conditions as well.

As reported [22,23], SSB1 and SIS1 chaperon are known to be required for efficient protein synthesis and their major intracellular location is in polysomes. The result above, therefore, rises the question whether the SSB1 and SIS1 proteins participating in protein synthesis also promote protein degradation at the same time. If this is the case, these chaperons would regulate the balance of these processes rather exclusive each other. Such a coupling of synthesis and breakdown of cellular components is observed in biosynthesis of other macromolecules which requires proof-reading for its quality control such as DNA synthesis and aminoacylation of tRNA. There, incorrectly synthesized molecules are degraded at the early stage of their synthesis in order to ensure the accurate synthesis of the macromolecule. Similarly, the proofreading of the folding-state of nascent polypeptides during protein synthesis, if any, would proceed both with the degradation of incorrectly folded polypeptides and with the promotion of proper folding of polypeptides. SSB1 chaperon may participate in both processes, controlling the quality of protein folding during polypeptide elongation. It is worth determining whether such a proofreading, possibly catalyzed by SSB1 protein or/and other hsp70 family proteins, takes place during the synthesis of proteins on polysomes.

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