Son1p is a component of the 26S proteasome of the yeast *Saccharomyces cerevisiae*

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Abstract A *son1* mutant was isolated as a mutant showing synthetic lethality with *nin1-1* which is defective in the p31 component of the regulatory subunit of the yeast 26S proteasome. *son1*Δ showed a synthetic effect with *sen3*Δ and *sun1*Δ, both components of the 26S proteasome, and with *cdc28-1N*. The 26S proteasome was partially purified from the wild type yeast. The FPLC fractions were analyzed by Western blotting using anti-Son1p antibody and antibodies against some authentic subunits of the 26S proteasome, and we found that Son1p co-migrated with components of the 26S proteasome. The 26S proteasome containing fraction was immunoprecipitated with anti-Son1p antibody. The resultant precipitate contained Nin1p, Sun1p, TBP1, and the 20S proteasome. Combining genetic and biochemical results together, we concluded that Son1p is a component of the yeast 26S proteasome.

Key words: Proteasome; *SON1*; Synthetic-lethal; *Saccharomyces cerevisiae*

1. Introduction

Accumulating evidence indicates that proteolysis by the ubiquitin-proteasome system plays pivotal roles in many cellular processes. Key factors of cell cycle regulation, such as cyclins for example, are degraded by the ubiquitin-proteasome pathway [1]. The machinery for the degradation in this system is the 26S proteasome, an ATP-dependent, multicatalytic protease of 2000 kDa [2–4]. The 26S proteasome is composed of two subcomplexes, the 20S proteasome and regulatory components. The 20S proteasome is a catalytic core and is consisted of seven structurally related polypeptides designated α subunits and seven structurally related β subunits, which are assembled into a cylinder-like structure [5]. The regulatory component contains six ATPases and an unknown number of non-ATPase subunits [5,6]. Our major concern is how the activity of the 26S proteasome is regulated. To this goal it is necessary to know the subunit composition of the regulatory component. Attempts to elucidate the composition of subunits of the regulatory component have been conducted and the results indicated, fortunately, that many of the subunits are highly conserved among eukaryotes [7]: Sen3p/p112 [8,9], Nas1p/p97 [10], etc.

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Abbreviations: DTT, dithiothreitol; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; Suc-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide

Genes encoding subunits of the yeast 20S proteasome have been completely elucidated: seven genes for the α subunits and seven genes for the β subunits, all of which are essential except *y13*/*PRE9* for an α subunit [11]. On the other hand, subunits of the regulatory component are not yet completely known. *cim3*/*sug1*/*p45* and *cim5* were identified as mutants that showed synthetic lethality with *cdc28-1N* and were shown to be ATPases contained in the regulatory component [12]. *sug2*, like *sug1*, was first isolated as a suppressor of *gal4* and later identified as the yeast 26S proteasome subunit [13]. Other ATPase subunits were identified as homologues of the mammalian counterparts [14]. In a previous study, we obtained a temperature sensitive mutant *nin1-1* which has a defect in a subunit of the regulatory component, a counterpart of p31 of the human proteasome [15–17]. We also reported two multicopy suppressors of *nin1-1*, both of which encode a component of the regulatory subunit, *SUN1* and *SUN2* [18,19]. Sun1p is a homologue of S5a multiubiquitin binding protein and Sun2p is a homologue of p58 of the human proteasome subunit. In this communication, we describe the isolation of a *son1* mutant by exploiting the adenine color assay method [20] for screening of a synthetically lethal mutation with *nin1-1* and show that Son1p is a component of the yeast 26S proteasome. A *son1* mutation was first identified as a suppressor of the *sec63-101* mutation [21]. Another *son1* allele was found in the *ufd* (ubiquitin fusion degradation) mutations which had been screened for mutants defective in degradation of ubiquitin-β-galactosidase fusion protein [22]. The *son1* and *sen3* mutants were isolated as suppressors of the mating defect shown by the $gpa1^{val50}$ mutant [23].

2. Materials and methods

2.1. Strains, media, and genetic analysis

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Mutants isolated in this study were also used as described in the text. *Escherichia coli* strain DH1 (*end*A1 *gyr*A96 *hsd*R17 *rec*A1 *rel*A1 *sup*E44 *thi*-1 F^-) was used for propagation and construction of plasmids. YPD, YPGal, SD, omission media and sporulation medium were for yeast and prepared as described previously [24]. A permissive temperature for yeast was 25°C and a non-permissive temperature was 35–37°C depending on the mutant strains. *E. coli* was grown at 37°C in LB [25] supplemented with 50 μg/ml ampicillin as appropriate. Methods for yeast genetics were described previously [24].

2.2. Construction of pDL120 (URA3-ade3-2p-CEN4-ARS1)

First, a leaky *ade3* mutant gene, *ade3-2p* described by Koshland et al. [20], was integrated into a *CEN* plasmid, YCp50 [26]. The 4.5 kbp *Sal*I-*Sma*I fragment containing the *ade3-2p* gene was blunt ended and then ligated into the *Sma*I site of YCp50. The resulting plasmid was designated pKOM3. The 2 kbp *Sal*I fragment containing the *NIN1* gene was excised form pHN4 [15] and inserted into the *Sal*I site of pKOM3, resulting in pDL120.

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2.3. Biochemical analysis

The yeast 26S proteasome was partially purified according to the procedure described by Rubin et al. [27]. Yeast extract was prepared from W303D cells grown in 400 ml YPD at 25°C for 36 h with shaking. Cells were harvested by centrifugation (5000×*g*) for 5 min, resuspended in 6 ml buffer A (100 mM Tris-HCl, pH 7.6, 2 mM ATP, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 2% glycerol), and then disrupted by vortexing with glass beads (0.5 mm in diameter) at the maximum speed for 20 min at 4°C with occasional cooling. Debris was removed by centrifugation at 15 000×*g* for 15 min at 4°C and the resultant supernatant was subjected to high speed centrifugation at 100 000×*g* for 20 min at 4°C and then at 300 000×*g* for 3 h at 4°C. Precipitate was dissolved in 500 μl buffer B (100 mM NaH_2PO_4/NaOH, pH 6.5, 1 mM ATP, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) and loaded on a phosphocellulose column (1.5 cm in diameter×4 cm; bed volume, 8 ml) equilibrated with buffer B. Elution was conducted with buffer B at a flow speed of 0.6 ml/min and 0.6 ml was collected in each fraction. The flow through fractions (1.2 ml) containing the 26S proteasome were pooled and filtered through a membrane filter (pore size, 0.45 μm), applied to a Superose 6 column equilibrated with buffer C (20 mM Tris-HCl, pH 7.8, 1 mM ATP, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 M NaCl, 10% glycerol) and FPLC was performed. Each fraction (0.5 ml) was eluted with buffer C at a flow speed of 0.25 ml/min. Protease activity was assayed by using Suc-LLVY-MCA as substrate with or without ATP and 0.1% SDS to discriminate protease activity of the 26S proteasome from that of the 20S proteasome. Western blot analysis was carried out as described previously [17]. Protein concentration was determined by the method described by Bradford [28]. SDS-PAGE was performed by the Laemmli system [29].

2.4. Antibodies

Anti-Son1p peptide antibody was prepared as described previously. In brief, a peptide consisted of 18 amino acid residues (494–511 in the amino acid sequence of Son1p) was chemically synthesized and a cysteine residue was attached at the C terminus. The peptide was conjugated to keyhole limpet hemocyanin and the resulting conjugates emulsified with complete Freund adjuvant were injected to rabbits to raise antibodies. Anti-TBP1 antibodies [17], anti-Sun1p antibodies [19], anti-p45 antibodies [10] and anti-20S proteasome antibodies [30] were described previously.

2.5. Immunoprecipitation

Polyclonal antibodies against Son1p and non-immune rabbit IgG (60 μg each in 40 μl of buffer D which is buffer A lacking DTT) were mixed with protein A-Sepharose beads (Pharmacia) and mixtures were rotated at 4°C for 2 h. The beads were then treated with 1% skimmed milk in buffer D, washed three times with buffer D, and added to 20 μl of the 26S proteasome-containing fraction obtained from the Superose 6 column. After the mixtures were rotated at 4°C for 2 h, supernatant (40 μl) was recovered by centrifugation and mixed with sample buffer (20 μl) for SDS-PAGE, while the resulting beads were washed three times with buffer D and suspended in the sample buffer (60 μl). 20 μl each of supernatant and beads suspension were subjected to SDS-PAGE in a slab gel containing 12.5% polyacrylamide followed by Western blot analysis.

2.6. Determination of nucleotide sequence

Nucleotide sequences were determined by the dideoxy chain termination method described by Sanger et al. [31], using a DNA sequencer Model 373A (ABI, Palo Alto, CA, USA).

3. Results

3.1. Isolation of mutants

Mutants whose growth was dependent on the presence of plasmid carrying the wild type *NIN1* gene were screened by the colony color assay developed by Koshland et al. [20]. In brief, strain TM1-4B [pDL120] was mutagenized with ethylmethane sulfonate as described by Lindegren et al. [32] and an appropriate dilution of the mutagenized cells was spread on YPD plates at 25°C. Since the growth of the parental strain is not dependent on pDL120 at 25°C, colonies developing from the parental strain contain red and white sectors on YPD at 25°C. In contrast, a colony developing from a cell containing a mutation synthetically lethal with *nin1-1* displays uniformly red on YPD at 25°C since a strain containing such a mutation is dependent on the presence of plasmid pDL120. Among approximately 50 000 colonies tested, 14 uniformly red colonies were obtained and subjected to further analysis. Among them, five mutants (#14, #47, #77, #114, and #205) showed a reproducible phenotype. Each isolate was crossed to the *nin1-1* strain (YK110). The synthetic lethality was no longer shown in the resultant diploids, indicating that the additional mutations are recessive. The trait of synthetic lethality was segregated 2+:2− in every heterozygous diploid except a diploid containing strain #14 [9]. Complementation tests between these five mutant strains defined five complementation groups, each of which is represented by a single mutant.

To facilitate genetic analyses, pDL120 plasmid (URA3-NIN1-ADE3) was replaced with pHN19, whose *NIN1* is expressed by the *GAL1* promoter [15]. Each of *SUN1* and *SUN2* on a multicopy plasmid (YEp24) was introduced into each of the mutants and we examined whether *SUN1* or *SUN2* could be a suppressor of the lethality. Synthetic lethality of #47 and #77 was weakly suppressed by *SUN2* on a multicopy vector whereas that of #114 was partially suppressed by multicopy *SUN1*. The fact that neither *SUN1* nor *SUN2* fully suppressed the synthetic lethality indicates that they are not the genes responsible for the synthetic lethality. So far we have succeeded in isolating three genes by exploiting #14, #47, and #114 as hosts. We identified *SEN3* in #14 [9] and *CIM5* in #47 (to be published elsewhere). In this communication, our primary concern is with the gene identified in the #114 mutant.

A YCUp4 based genomic library was introduced into the #114 [pHN19] strain grown in YPgal and the transformation mixture was spread on a YPGal plate to estimate the number of total transformants and on YPD plates to recover transformants. From about 7000 colonies tested, 15 positive clones were obtained. Plasmid was recovered from each of the isolates. Only one plasmid was found to contain a gene other than *NIN1* and the rest contained *NIN1*. The plasmid that contained a non-*NIN1* sequence was partially sequenced at its cloning junctions. Homology search indicated that the

Table 1
Yeast strains

Strain	Genotype	Source or reference
W303D	*MAT*a/*MAT*α *leu2*/- *his3*/- *trp1*/- *ura3*/- *ade2*/- *can1*/-	[36]
W303-1B	*MAT*α *leu2 his3 trp1 ura3 ade2 can1*	A segregant of W303D
YK110	*MAT*α *nin1-1 leu2 his3 trp1 ura3 ade1*	[17]
TM1-4B	*MAT*a *nin1-1 leu2 trp1 ura3 ade2 ade3*	[9]

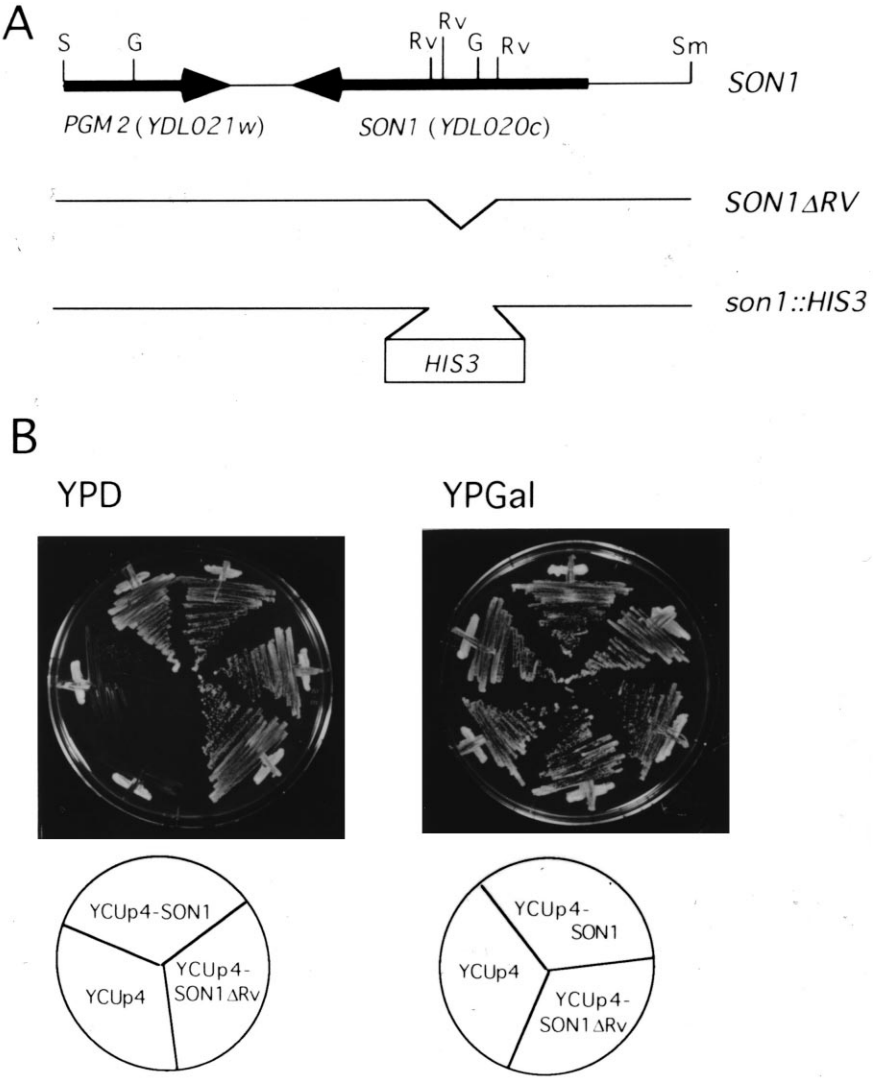

Fig. 1. Genetic analysis of the *SON1* gene. A: The map of the *SON1* locus. Arrows indicate the ORFs and their direction. Internal *Eco*Rv fragments were deleted from the *SON1*, resulting in *SON1*ΔRv. This deletion resulted in removing internal 97 amino acid residues (219–315) and the N-terminal portion was fused to the C-terminal portion in frame. YCUp4-SON1 was constructed by inserting the 3 kbp *Sal*I-*Sma*I fragment possessing the *SON1* gene into the *Sal*I-*Sma*I gap of YCUp4, a CEN-ARS plasmid containing *URA3* as a selective marker. The *SON1*ΔRv gene was inserted into YCUp4, resulting in YCUp4-SON1ΔRv. The *SON1* gene was disrupted by replacing the *Eco*RV fragments within the *SON1* ORF with the blunt ended fragment containing *HIS3* excised from pJJ217 [37]. B: Plate test for complementation. Each YCUp4, YCUp4-SON1, and YCUp4-SON1ΔRv was introduced into #114 [pHN19] and two representative transformants from each transformation experiment was streaked on YPD plate or YPGal plate which were incubated at 25°C for 4 days. Symbols: S, *Sal*I; Sm, *Sma*I; B, *Bam*HI; G, *Bgl*II; Rv, *Eco*RV.

DNA segment cloned was derived from chromosome 4 containing *SON1(YDL020c)* and the 5′ truncated *PGM2(YDL021w)* (Fig. 1A). The 3 kbp *Sma*I-*Sal*I fragment contains *SON1* as a sole complete ORF and a low copy plasmid (YCUp4-SON1) containing this DNA segment complemented the synthetic lethality of the #114 [pHN19] strain (Fig. 1B), suggesting that *SON1* is the gene responsible for this synthetic lethality. To our surprise, the derivative of YCUp4-SON1ΔRv constructed by deleting two *Eco*RV fragments which reside within the *SON1* ORF still retained the complementation activity (Fig. 1B). To further examine whether *son1* is the gene responsible for synthetic lethality in #114, plasmid DNA containing *SON1* and *URA3* was targeted to the *son1* locus of #114 [pHN19] and the resulting Ura^+ transformants were subjected to linkage analysis. The Ura^+ transformants were able to grow on YPD. One of these transformants was crossed to YK110 and the diploids were sporulated and dissected. No segregant showing synthetic lethality was obtained among 16 asci tested, indicating that the mutation concerned occurred in the *SON1* gene.

3.2. Synthetic lethality of son1 with other genes

The *SON1* gene of W303-1B was disrupted by the one step replacement method [33] using the *son1::HIS3* gene shown in Fig. 1A as donor. The correct disruption was confirmed by Southern hybridization (data not shown) and the resultant disruptant was viable as described previously [21]. The *son1::HIS3* strain was crossed with each of the *sen3::URA3*, *sun1::LEU2*, and *cdc28-1N*. In the crosses between *son1* and *sen3* and between *son1* and *cdc28-1N*, every double mu-

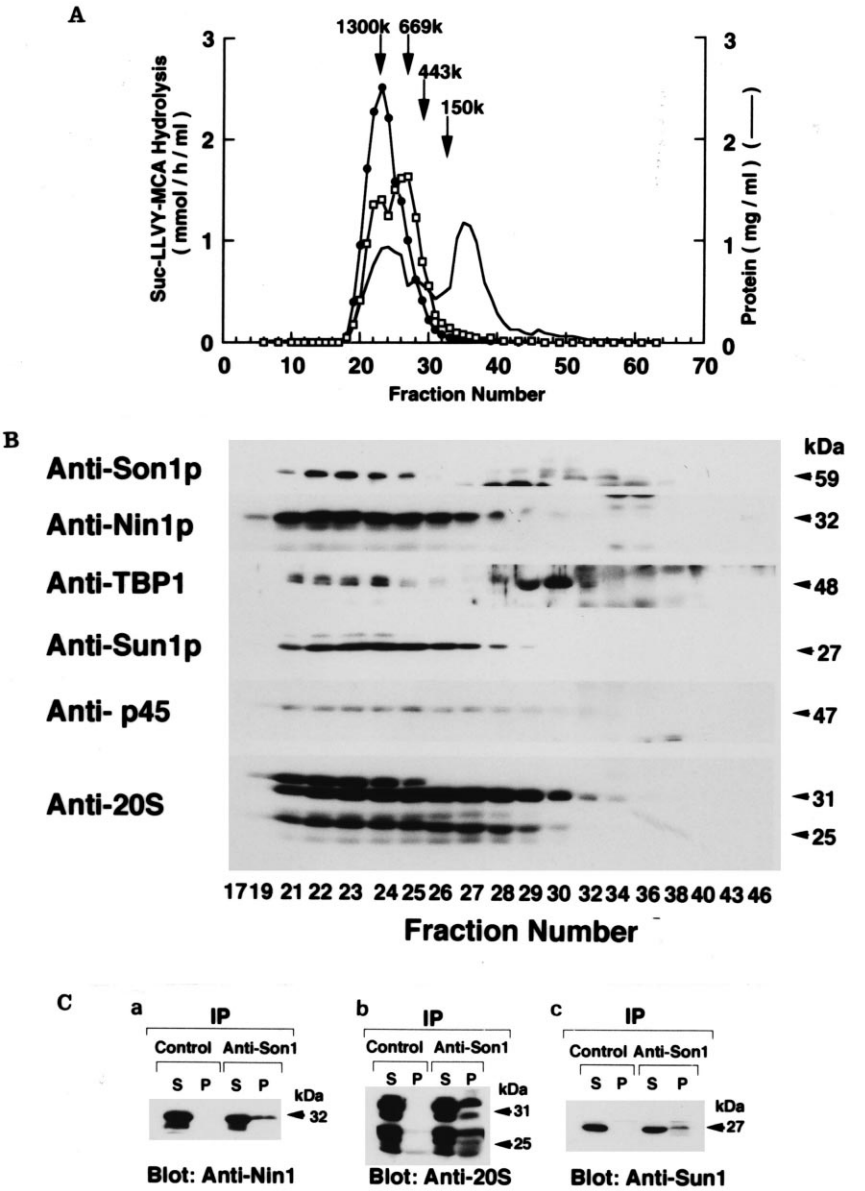

Fig. 2. Son1p is associated with the 26S proteasome. A: Elution pattern of Superose 6 FPLC chromatography. For the protease assay, Suc-LLVY-MCA was used as substrate. 5 μl of the indicated fraction was added to 100 μl reaction mixture containing 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 2 mM ATP, 10 mM $MgCl_2$, 0.1 mM Suc-LLVY-MCA (+ATP, −SDS, solid circles) or 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 5 mM EDTA, 0.02% SDS, 0.1 mM SucLLVY-MCA (−ATP, +SDS, open squares). Incubation was carried out at 37°C for 12 min. The reaction was stopped by adding 1 ml of 10% SDS. Molecular weight markers used were thyroglobulin (669 kDa), apoferritin (443 kDa), and alcohol dehydrogenase (150 kDa). B: Western blotting. The indicated fraction (20 μl) was subjected to 12.5% SDS-PAGE. The proteins separated were electrotransferred to nitrocellulose membrane filter. Menbrane was probed with antibodies indicated. C: Immunoprecipitation. 20 μl of the peak fraction 23 of FPLC (see A) was immunoprecipitated with 20 μl of anti-Son1p antibody conjugated to protein A-Sepharose beads and also with non-immune rabbit IgG conjugated to protein A-Sepharose beads as control. The resulting supernatant (S) and precipitate (P) were subjected to SDS-PAGE followed by Western blot analysis with anti-Nin1p antibody (a), anti-20S proteasome antibody (b), and anti-Sun1p antibody (c). IP, immunoprecipitation; Blot, Western blotting.

tant spore was inviable or very sick, whereas, in the cross between *son1* and *sun1*, double mutant spores were obtained but showed temperature sensitive growth (data not shown). We also confirmed the synthetic lethality between *nin1-1* and *son1::HIS3* (data not shown). Since Sen3p, Nin1p, and Sun1p are the regulatory subunits of the yeast 26S proteasome and since some genes showing synthetic lethality with *cdc28-1N* also encode the components of the 26S proteasome, we examined the possibility that Son1p can be a component of the yeast 26S proteasome.

3.3. Son1p as a component of the 26S proteasome

The 26S proteasome was partially purified from extract prepared from W303D cells grown in YPD for 36 h at 25°C as described in Section 2. Fig. 2A shows the elution pattern of FPLC on a Superose 6 column. Protease activity was assayed for each fraction in the presence of ATP and absence of SDS or in the presence of SDS and absence of ATP. The 26S proteasome activity was detected in the former condition and the 20S proteasome activity was enhanced in the latter condition. The 26S proteasome was eluted at a position peak-

ing at fraction 23 with a molecular weight of 1300 kDa, whereas the 20S proteasome eluted at position peaking at fractions 26–27 with a molecular weight of 700 kDa (Fig. 2A). Proteins in each fraction was separated by SDS-PAGE and analyzed by immunoblotting using anti-Son1p, anti-TBP1, anti-Sun1p, anti-Nin1p, anti-p45, and anti-20S proteasome antibodies. As shown in Fig. 2B, Son1p was distributed at the 26S proteasome area overlapping with TBP1, Sun1p, and p45. Fraction 23 was treated with anti-Son1p antibodies and the resulting precipitate was analyzed by Western blotting using anti-Nin1p antibody, anti-Sun1p antibody or anti-20S proteasome antibody. As shown in Fig. 2C, Son1p was precipitated with authentic 26S proteasome components, such as Nin1p, Sun1p and 20S proteasomes.

4. Discussion

Genetic characterization of *SON1* which was identified as a mutation showing synthetic lethality with *nin1-1*, which tends to show a synthetic effect with various mutations, occurred in components of the 26S proteasome. Furthermore, we found that Son1p is co-eluted with the yeast 26S proteasome in Superose 6 FPLC and that Son1p was co-precipitated with Nin1p, Sun1p, and the 20S proteasome. These genetic and biochemical lines of evidence indicate that Son1p is a component of the yeast 26S proteasome. The *SON1* gene can encode 531 amino acid residues. Substitution of a DNA segment encoding 97 amino acid residues from amino acid 219 to amino acid 315 by the *HIS3* marker destroyed the function of the *SON1* whereas a *SON1* derivative with an internal deletion of the 97 amino acid residues was found to be functional as *SON1* (Fig. 1). This result suggests that two acidic regions covered by the deletion exert no essential role and that the C-terminal region is important for the Son1p function.

SON1 was first identified [21] as a suppressor of temperature sensitive growth of *nlp1-1 (sec63-101)*. The *sec63-101* mutant showed temperature sensitive growth and mislocalized nuclear proteins. *son1* suppressed the temperature sensitivity of *sec63-101* but not the defect in nuclear transport. Suppression by *son1* was allele specific and the *son1* mutation alone resulted in a defect in nuclear transport. In this context, it should be noted that the *SEN3* encoding the largest subunit of the 26S proteasome, a deletion of which was synthetically lethal with *son1*, has a function in protein transport to the nucleus [9]. Genetic interaction between *son1* and *sen3*, along with the fact that both Son1p [21] and Sen3p [34] localize in nucleus, is consistent with the idea that both genes work in nuclear transport. Recently, Xu and Kurjan [23] isolated *son1* and *sen3* mutants as suppressors of the mating defect displayed by the $gpa1^{val50}$ mutant. They showed that *son1* and *sen3* are synthetically lethal. They explained their genetic results by assuming that some positive factor(s) might be involved in a mating pathway and that these factor(s) might be degraded by the ubiquitin-proteasome pathway.

Involvement of Son1p in protein degradation was directly indicated by Johnson et al. [22]; they identified *son1* as a mutant (*ufd5*) defective in degradation of ubiquitin-β-galactosidase fusion protein which is degraded by the 26S proteasome after polyubiquitination. The *son1/ufd5* mutant did polyubiquitinate ubiquitin-β-galactosidase but did not degrade it. Thus, it is likely that *SON1* protein is needed for the protease activity, consistent with our present result that *SON1* is a component of the 26S proteasome. How then can the suppression of *sec63-101* by *son1* be explained? A simple explanation is that Sec63-101p became unstable by the mutation and that its instability was cancelled by a *son1* mutation. However, this explanation is unlikely because Nelson et al. [21] reported that Sec63-101p showed the same stability in the presence or absence of Son1p. Another explanation is that the *sec63-101* mutation may destabilize protein(s) interacting with Sec63p, instead of itself. In this hypothesis, we assume that Sec63p interacts with protein(s) which are stabilized by making a complex with Sec63p. This interaction may be lost in the *sec63-101* mutant, rendering the interacting protein accessible to the 26S proteasome. The stability of the protein(s) might be improved by the *son1* mutation which reduces protease activity of the 26S proteasome. Actually, Brodsky and Schekman [35] demonstrated that Sec63p forms a complex with Bip in the endoplasmic reticulum lumen and with Sec66p and Sec67p in the cytosol. These factors or other(s) interacting with Sec63p could be a target for degradation by the 26S proteasome.

A counterpart of *SON1* in other organisms is not known. Since not all the subunits of the mammalian 26S proteasome are known at present, there is a possibility that a *SON1* counterpart may be found in future. Since Son1p is not essential, the 26S proteasome without Son1p is able to execute the 26S proteasome functions at an ordinary temperature but it is needed for degradation of a set of proteins, such as ubiquitin-β-galactosidase and a putative protein interacting with Sec63p or involved in the mating pathway. This result suggests that isoforms of the 26S proteasome exist and that each of them exerts a respective degradative function in various facets of cellular processes.

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