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Regulatory Role of C-Terminal Residues of SulA in Its Degradation by Lon Protease in *Escherichia coli*[1]

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The SulA protein is a cell division inhibitor in *Escherichia coli*, and is specifically degraded by Lon protease. To study the recognition site of SulA for Lon, we prepared a mutant SulA protein lacking the C-terminal 8 amino acid residues (SA8). This deletion protein was accumulated and stabilized more than native SulA in *lon*$^+$ cells *in vivo*. Moreover, the deletion SulA fused to maltose binding protein was not degraded by Lon protease, and did not stimulate the ATPase or peptidase activity of Lon *in vitro*, probably due to the much reduced interaction with Lon. A BIAcore study showed that SA8 directly interacts with Lon. These results suggest that SA8 of SulA was recognized by Lon protease. The SA8 peptide, KIHSNLYH, specifically inhibited the degradation of native SulA by Lon protease *in vitro*, but not that of casein. A mutant SA8, KAHSNLYH, KIASNLYH, or KIHSNAYH, also inhibited the degradation of SulA, while such peptides as KIHSNLYA did not. These results show that SulA has the specified rows of C-terminal 8 residues recognized by Lon, leading to facilitated binding and subsequent cleavage by Lon protease both *in vivo* and *in vitro*.

Key words: ATP-dependent protease, cell division, Lon, protein recognition, SulA.

When *Escherichia coli* is exposed to such environmental stresses as heat or cold shock, a pH-shift, oxidative radicals or UV-irradiation, a series of protective responses occurs to maintain cell viability. For example, exposure of *E. coli* to agents or conditions that damage DNA results in the induction of more than 20 genes, which include that of the DNA-repairing enzyme (*1*). One of these genes, *sulA*, is a cell-division inhibitor, which prevents premature segregation of damaged DNA into daughter cells during DNA repair processes (*2*, *3*).

The *sulA* gene product, SulA, is an 18 kDa protein comprising 169 amino residues which interacts with an essential cell division protein, FtsZ (*3–6*). The polymerization of FtsZ is regulated by GTP hydrolysis and leads to the formation of a cytokinetic ring at the time of cell division (*7*, *8*). SulA forms a complex with FtsZ in a GTP-dependent manner, and thus inhibits the polymerization of FtsZ and GTP hydrolysis (*9*, *10*). After DNA repair is completed the production of SulA ceases, followed by rapid degradation by Lon protease (*11*, *12*).

The Lon protease in *E. coli* is an oligomeric enzyme composed of 87 kDa subunits, containing both ATP-binding and proteolytic sites (*13*). Protein substrates bind to two sites of the Lon protease; one is a catalytic site and the other an allosteric site (*14*). The binding of a protein substrate to the allosteric site stimulates ADP release from Lon protease and ATP binding, leading to proteolysis at the catalytic site. The degradation of the protein substrate in turn stimulates ATP hydrolysis, which is needed for decomposition of the secondary structure of the substrate (*15–18*).

Lon protease has two types of substrates; abnormal proteins, and physiological proteins such as the λN, RcsA, and CcdA proteins (*18–21*), which have been studied in detail. However, there is little information on the degradation of SulA, one of the most specific and physiological substrates for the Lon protease, especially in an *in vitro* system, because of the difficulty in preparing the SulA protein due to its instability. We have succeeded in preparing SulA fused to maltose binding protein (MBP-SulA) and have shown the rapid degradation of MBP-SulA by Lon protease in an ATP-dependent manner *in vitro* (*22*). Moreover, we showed that the SulA protein has a specific region at its C-terminus, which is only necessary for the interaction with Lon, but is different from the cleavage sites for Lon (*23*). The amino acid sequence of SulA is conserved in enterobacteria species (*26*). In particular, the C-terminal 8 amino acid residues (SA8) show high homology, suggesting that SA8 is indispensable for the interaction with Lon protease. Here, we focused on SA8 and found that SA8 is responsible for

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Abbreviations: DMSO, dimethylsulfoxide; DTT, dithiothreitol; IPTG, isopropyl-1-thio-β-D-galactopyranoside, LB, Luria Bertani; MBP, maltose binding protein; NHS, *N*-hydroxysuccinimide; MNA, methoxynaphthylamine, PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; TCA, trichloroacetic acid; SPR, surface plasmon resonance; Suc-, succinyl.

the specific recognition by Lon protease both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials—Chemically synthesized oligonucleotides were obtained from Funakoshi (Tokyo) and Sawady Technology (Tokyo). Restriction endonucleases, kinase, T4 polynucleotide kinase, T4 ligase, and Taq polymerase were obtained from Takara (Kyoto). Chemically synthesized oligopeptides, NH_2-KIHSNLYH-COOH (C-terminal 8 amino acid residues of SulA; SA8), NH_2-KAHSNLYH-COOH (SA8, except that isoleucine was changed to alanine; SA8I163A), NH_2-KIASNLYH-COOH (SA8, except that the second histidine from the N-terminus was changed to alanine; SA8H164A), NH_2-KIHSNAYH-COOH (SA8, except that leucine was changed to alanine; SA8L167A), NH_2-KIHSNLYA-COOH (SA8, except that the histidine at the C-end was changed to alanine; SA8H169A), and NH_2-ASSHATRQLSGLKIHSNLYH-COOH (C-terminal 20 residues of SulA; SA20), were prepared by and obtained from Sawady Technology. These peptides were stored at –20°C as 1 mM stock solutions in distilled water. Resolfin-labeled casein was obtained from Boehringer Mannheim (Germany). Succinyl-Phe-Leu-Phe-methoxynaphthylamine (Suc-Phe-Leu-Phe-MNA) was obtained from BACEM Feinchemilalien AG (Bubendorf, Switzerland) and stored at –20°C as a 10 mM stock solution in 100% DMSO.

Bacterial Strains—*E. coli* strains JM109 (for the purification of proteins), AB1157 (wild type), and JK405 (carrying *lon*::Tn10) were used and characterized as described previously (*22*).

Plasmid Construction—The construction of pB10a, pSulA5 (native *sulA*), pB10aSulAC161 (deletion of SA8 from *sulA*), pMAL-p2, and pMAL-p2-SulA was performed as described previously (*23*). For preparation of the maltose binding protein (MBP)–fusion protein with deletion of SA8 from SulA (MBP-SulAC161), pMAL-p2-SulAC161 was constructed by PCR using oligonucleotide primers, 5′-**ATG** TAC ACT TCA GGC TAT GCA C (as the upstream primer; boldface, the first codon of SulA) and 5′-GTA AGC **TTA** TAG CCC GGA AAG TTG TCT (as the downstream primer; underlining, *Hin*dIII site, and boldface, the stop codon), and pUA94 plasmid DNA (*22*) as the template for the *sulA* gene. The PCR product was phosphorylated with T4 polynucleotide kinase, cleaved with *Hin*dIII, and then subcloned between the *Xmn*I and *Hin*dIII sites of pMAL-p2. For preparation of the LonS679A protein, in which the serine of the proteolytic active site of Lon protease at residue 679 was changed to alanine, the DNA fragment corresponding to the Lon 3′ *Pst*I fragment was recovered from pBR322-lon3′ (*24*) by *Pst*I digestion. This fragment was cloned into M13mp18 digested with *Pst*I. The amino acid replacement of Ser679 to Ala was carried out by site-directed mutagenesis according to Amerik *et al.* (*27*), followed by subcloning into pMAL-Lon5′-LacZα (*24*) to construct pMAL-LonS679A.

Growth Media—All cultures were carried out in LB medium (Difco yeast extract 5 g/liter, Difco tryptone 10 g/liter, and NaCl 5 g/liter). This medium was solidified with 15 g/liter Difco agar for the preparation of LB-agar plates.

Assaying of the Cell Division–Inhibiting Effect of SulA—An overnight culture of AB1157 or JK405 harboring pB10a, pSulA5, or pB10aSulAC161, respectively, was streaked onto a LB agar plate using a cotton ear pick, rectangularly across a line of 0.05 M isopropyl-1-thio-β-D-galactopyranoside (IPTG) (*23*).

Western Blotting Analysis of SulA In Vivo—A overnight culture of AB1157 or JK405 harboring pB10a, pSulA5, or pB10aSulAC161, respectively, was diluted 100-fold with 10 ml of LB containing 50 μg/ml ampicillin and incubated at 37°C until the mid-log phase, and then 1 mM IPTG was added. After incubation for 30 min, 200 μg/ml chloramphenicol was added, and then 1 ml aliquots were collected at intervals and precipitated with 10% trichloroacetic acid (TCA). The acetone-washed pellets were suspended in 60 μl of a urea solution composed of 8 M urea, 1 mM dithiothreitol (DTT), and 1 mM EDTA in 50 mM Tris-HCl, pH 8.0. Samples containing the same amount of protein were mixed with 10 μl of SDS-sample buffer composed of 10% SDS, 38% glycerol, 9.3% DTT, and 0.012% bromphenol blue in 0.35 M Tris-HCl, pH 6.8, and then subjected to electrophoresis on 5–20% SDS-slab gels (PAGEL™; ATTO, Tokyo) and transferred to polyvinylidene difluoride (PVDF) membranes (ImmobilonP™; Millipore). Transferred SulA on the membranes was detected with a polyclonal anti-SulA antibody and a Phototope-HRP Western blot detection kit (New England BioLabs), and the reaction products were quantified with the NIH image program. To prepare the anti-SulA antibodies, the MBP-SulA fusion protein was digested with Factor Xa (*22*), and then the aggregated SulA was used to immunize rabbits. The antiserum was treated with saturated ammonium sulfate and the precipitates were resuspended in PBS (–), followed by pre-adosorption on an MBP-conjugated agarose column to remove a small amount of anti-MBP IgG. The pass-through fractions were applied to a SulA-conjugated agarose column to purify the anti-SulA IgG. The eluate with 0.1 M glycine-HCl (pH 2.5) was neutralized with 1 M Tris and stored at –80°C. The specificity of the anti-SulA IgG was examined by Western blotting using purified SulA and by immunoprecipitation using a UV-irradiated cell extract containing induced SulA.

Protein Purification—The Lon protease and LonS679A protein were purified as described previously (*24*). For purification of MBP-fusion proteins such as MBP-SulA, MBP-SulAC161, and MBP-LacZα, JM109 carrying pMAL-p2-SulA, pMAL-p2-SulAC161, and pMAL-p2, respectively, was grown to the mid-log phase in 1 liter LB medium containing 50 μg/ml of ampicillin at 37°C, and then cultured with 0.3 mM IPTG for 3 h, centrifuged, and resuspended in 40 ml of buffer A, which was composed of 20% sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.0025% lysozyme in 20 mM Tris-HCl, pH 8.0. After freezing and thawing, the cells were diluted 2-fold with buffer B, which was composed of 0.4 mM EDTA, 80 mM NaCl, and 8% glycerol in 20 mM Tris-HCl, pH 8.0, and then centrifuged at 13,000 rpm. The upper solution was applied to an amylose column equilibrated with buffer C, which was composed of 0.2 mM EDTA, 40 mM NaCl, and 4% glycerol in 20 mM Tris-HCl, pH 8.0. After washing the column with buffer C, MBP-fusion proteins were eluted with 10 mM maltose in buffer C, followed by immediate storage at –80°C.

Degradation of MBP-Fusion Proteins by Lon Protease In Vitro—MBP fusion proteins such as MBP-SulA, MBP-SulAC161, and MBP-LacZα were incubated at 0.25 μM in 50 μl of buffer D, which was composed of 7.5 mM $MgCl_2$ in

50 mM Tris-HCl, pH 8.0, containing 0.25 μM Lon protease and 4 mM ATP. After incubation for 30 min at 37°C, the reaction was stopped by mixing the samples with 10 μl of SDS sample buffer. The samples were then boiled at 95°C for 10 min and subjected to SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R250, destained and dried as transparent films.

ATPase Activity of Lon—Various concentrations of MBP-SulA or MBP-SulAC161 were incubated with 0.1 μM purified Lon protease and 1 mM [γ-^{32}P]ATP (specific activity, 5 μCi/μmol) in 50 μl of buffer D at 37°C for 1 h. The reaction was stopped by adding 250 μl of an ice-cold PBS (–) solution containing 6% Norit SX-3 (Wako, Japan) and 0.3% Dextran T500 (Pharmacia), and then the mixture was stood on ice for 30 min, followed by centrifugation at 13,000 rpm for 5 min at 4°C. The release of [^{32}Pi]orthophosphate was measured by counting 100 μl of the resultant supernatant in ACS-II (Amersham) using an Aloka liquid scintillation counter, LSC-3100II (Aloka, Tokyo).

Peptidase Activity of Lon Protease—Various concentrations of MBP-SulA or MBP-SulAC161 were incubated with 0.02 μM purified Lon protease, 5 μM Suc-Phe-Leu-Phe-MNA and 1 mM ATP in 100 μl of buffer D at 37°C for 120 min. After the reaction, the fluorescence of MNA was measured with a Fluoroskan II version 6.3 (Labsystems) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

Complex Formation between the MBP-Fusion Protein and Lon Protease—About 0.1 μM MBP-SulA, MBP-SulAC-161, or MBP-LacZα was incubated with 0.1 μM purified Lon protease in 625 μl of buffer D. After incubation at 37°C for 5 min, the reaction mixture was applied directly to an amylose column, with a 300 μl bed volume, pre-equilibrated with 5 ml of the reaction buffer. After washing with 3 ml of buffer D, the bound proteins were eluted with 10 mM maltose in buffer D and precipitated with TCA. The acetone-washed pellet was suspended in SDS sample buffer and boiled for 10 min, and then subjected to SDS-PAGE as described above.

Kinetic Analysis of the Lon–Substrate Interaction—Real time detection of Lon–substrate interactions and kinetic analyses were performed using a BIA core™ 1000 system (Amersham Pharmacia Biotech). The principle and application of the system employing the method of surface plasmon resonance (SPR) detection were according to Karlsson and Falt (*28*). A continuous flow of HBS buffer, which was composed of 3.4 mM EDTA, 150 mM NaCl, and 0.05% Tween20 in 10 mM HEPES, pH 7.4, over the sensor surface was maintained. The carboxylated dextran matrix of the sensor chips was activated by the injection of 50 μl of a solution containing 200 mM *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide and 50 mM *N*-hydroxysuccinimide (NHS). Then, substrates were injected under immobilization conditions as summarized in Table I. After binding of the substrates to the sensor surface, 35 μl of 1 M ethanolamine-HCl (pH 8.5) was injected to block the remaining activated NHS-ester groups. After washing with 50 μl of 10 mM NaOH and then with excess HBS buffer, the sensor chips were used. The SPR measurements of the binding of LonS679A protein to the immobilized substrates were performed under a flow of the HBS buffer at 5 μl/min. LonS-679A was used after exchange of the buffer with HBS using a Smart System™ equipped with a Superlose 12 column (Amersham Pharmacia Biotech). The concentration of Lon was varied from 2.6 to 160 μM on a monomer basis. Regeneration of the sensor chips after each analysis cycle was performed by injecting 15 μl of 10 mM NaOH. Kinetic parameters were determined using the BIAevaluation 3.0 software (Amersham Pharmacia Biotech).

TABLE I. **Conditions for immobilization of various substrates for LonS679A.**

Substrate	Concentration (mg/ml)	Concentration and pH of sodium acetate buffer[a]	Flow rate (μl/min)	Substrate volume (μl)	Immobilized (RU[b])
α-Casein	0 1	5 mM (pH 4.0)	5	120	978
MBP-SulA	0 05	5 mM (pH 5 5)	5	16	920
SA20	1	10 mM (pH 5 5)	5	40	1150
SA8	2	10 mM (pH 5 5)	2	100	1343
BSA	0 05	10 mM (pH 5 0)	5	20	2227

[a]Each substrate was diluted with the indicated buffer. [b]Resonance Units.

Degradation of MBP-Fusion Proteins by Lon Protease in the Presence of SA8 or SA8 Mutant Peptides In Vitro—Various concentrations of SA8 or an SA8 mutant peptide were preincubated with 0.25 μM Lon protease in 50 μl of buffer D at 37°C for 10 min, and then 0.25 μM MBP-SulA or MBP-SulAC161 was added to the reaction mixture in the presence or absence of 4 mM ATP. After incubation at 37°C for 30 min, the reaction was stopped by adding the SDS sample buffer. The samples were boiled, subjected to SDS-PAGE and then stained as described above. The degradation of a MBP-fusion protein was quantitated by analysis of the stained band remaining at the position of the MBP-fusion protein using the NIH image program.

Degradation of Resolfin-Labeled Casein by Lon Protease in the Presence of SA8—Various concentrations of SA8 were preincubated with 0.25 μM Lon protease in 400 μl of buffer D at 37°C for 10 min, and then 0.25 μM resolfin-labeled casein was added to the reaction mixture in the presence of 4 mM ATP. After incubation at 37°C for 30 min, the degradation of resolfin-labeled casein was stopped by adding 20 μl of 100% TCA. The samples were then centrifuged at 14,000 rpm for 5 min and 400 μl of each of the resultant supernatants was mixed with 600 μl of 0.5 M Tris-HCl, pH 8.8, and then the absorbance of the mixture was measured with a UV-visible Recording Spectrophotometer UV 1604 (Shimadzu) at the wavelength of 574 nm.

RESULTS

Deletion of SA8 from SulA Increases the Stability of SulA In Vivo—In the previous study, we showed that deletion of the C-terminal 8 amino acids (SA8) from SulA made more toxic to the wild type *E. coli* than the untreated control. Figure 1 shows streaked cells containing native *sulA* (SulA) or the SA8 deletion of *sulA* (SulAC161) gene plasmid vector under the control of the *lacUV5* promoter on an IPTG-streaked LB agar plate. SulAC161 in wild type cells made it more toxic to the cells than native SulA. However, native SulA and SulAC161 in *lon*$^-$ cells showed the same toxicity, suggesting that SA8 is necessary for SulA to be destabilized by Lon protease. To confirm this possibility, we measured the accumulation and stability of SulA or SulAC161 in both wild and *lon*$^-$ cells by Western blot analysis (Fig. 2). The accumulation of native SulA in wild type cells was very

low (Fig. 2A). However, the accumulation of SulAC161 in wild type cells was very high, similar to that of native SulA or SulAC161 in *lon*⁻ cells (Fig. 2A). Native SulA in wild type cells had disappeared by 2 min after the addition of chloramphenicol (Fig. 2, B and C). However, SulAC161 in wild type cells was stable, similar to native SulA and SulAC161 in *lon*⁻ cells (Fig. 2, B and C). These results indicate that SA8 is necessary for SulA to be destabilized by Lon protease.

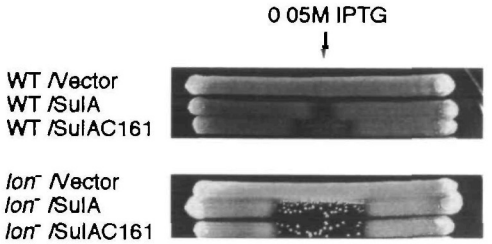

Fig 1. **Cell division-inhibiting effects of native SulA and a C-terminal deletion mutant of SulA.** *E coli* cells harboring plasmids carrying native *sulA* (SulA) or a C-terminal 8 amino acid residue deletion mutant of *sulA* (SulAC161) were cross-streaked against a 0.05 M IPTG solution on LB agar plates containing 50 μg/ml ampicillin, and then incubated at 37°C overnight. Two host strains, AB1157 (*lon*⁺) and JK405 (*lon*⁻), were used, which are indicated as WT and *lon*⁻, respectively

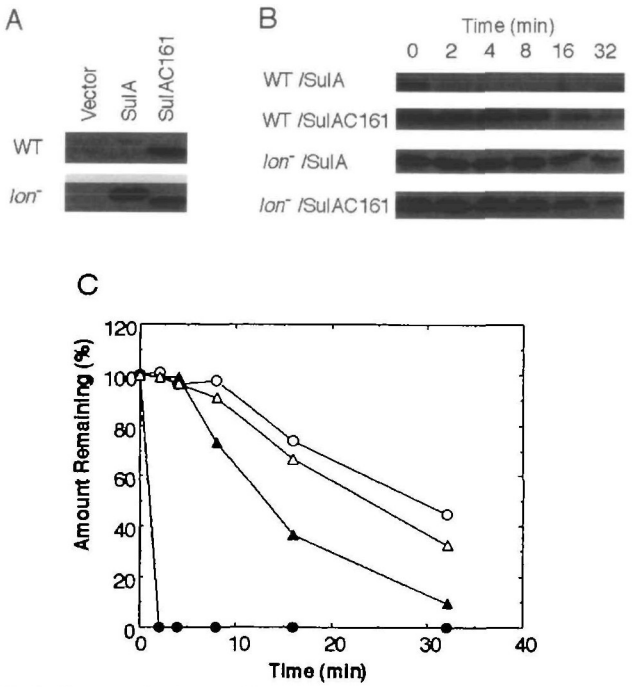

Fig. 2. **Accumulation and stability of native SulA and the SA8 deletion mutant of SulA *in vivo*.** Wild type cells (WT) and *lon*⁻ cells (*lon*⁻) carrying plasmids containing *sulA* (SulA) and *sulAC161* (SulAC161), respectively, were grown at 37°C to the mid-log phase in LB containing ampicillin, and then 1 mM IPTG was added. After incubation for 30 min, samples were taken and precipitated with TCA (panel A), or 200 μg/ml chloramphenicol was added to the cultures and aliquots were taken at 0, 2, 4, 8, 16, and 32 min, and precipitated with TCA (panel B). SulA and SulAC161 in the precipitates were analyzed by Western blotting as described under "MATERIALS AND METHODS." SulA (closed circles) and SulAC161 (closed triangles) in wild type cells, and SulA (open circles) and SulAC161 (open triangles) in *lon*⁻ cells in panel B were quantitated with the NIH program (panel C).

Deletion of SA8 Protects SulA from Degradation by Lon Protease—*In vivo* experiments have suggested that SA8 is necessary for SulA to be degraded by Lon protease. To confirm this possibility *in vitro*, we prepared MBP-SulA lacking SA8 (MBP-SulAC161) and examined whether or not this fusion protein was degraded by Lon (Fig. 3). The native SulA fused to MBP (MBP-SulA) was degraded by Lon protease in an ATP-dependent manner. However, MBP-SulAC161 was not degraded, similar to the control of LacZα fused to MBP (MBP-LacZα). These results show that SA8 of SulA also plays an important role in the susceptibility of SulA to digestion by Lon protease *in vitro*, as has been suggested *in vivo*.

Effects of SA8 on the ATPase and Peptidase Activities of Lon—Because protein substrates had been shown to enhance the ATPase activity of Lon protease (*15*), we examined whether or not MBP-SulAC161 stimulates the ATPase activity of Lon. MBP-SulA itself had no ATPase activity, but the addition of MBP-SulA increased the hydrolysis of ATP by Lon protease, about 2-fold stimulation being reached with a 10 molar excess to Lon. However, MBP-SulAC161 showed lower stimulatory activity toward the Lon ATPase than MBP-SulA (Fig. 4A). These results show that deletion of SA8 from SulA causes a decrease in the stimulatory activity of the latter toward Lon ATPase.

Lon protease was reported to hydrolyze some specific peptides (*25*) depending on ATP or non-hydrolyzable ATP analogs (*16*), and this ATP-stimulated peptidase activity was much more increased in the presence of a protein substrate (*14*). We examined whether or not MBP-SulAC161 enhances the ATP-dependent peptidase activity of Lon protease. The hydrolysis of Suc-Phe-Leu-Phe-MNA, a specific substrate peptide for Lon, increased more than 4-fold in the presence of MBP-SulA (Fig. 4B). On the other hand, MBP-SulAC161 increased it about 3-fold. These results show that deletion of SA8 from SulA decreased the stimulatory activity of SulA toward the ATP-dependent peptidase activity of Lon protease. Taken together, the results in Fig. 4 suggest the presence of some recognition site for Lon protease on SulA in the C-terminal region including SA8,

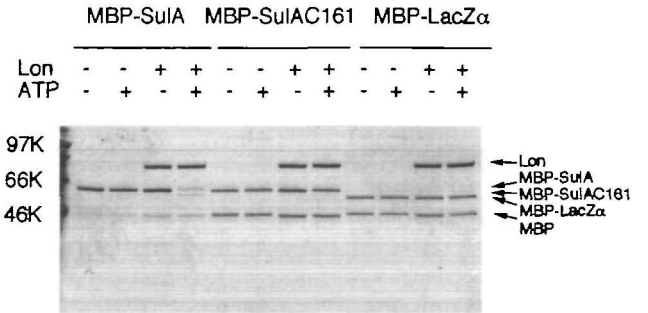

Fig. 3 **Degradation of native SulA and the SA8 deletion mutant of SulA fused to MBP by Lon protease *in vitro*.** Degradation of MBP-LacZα (as a negative control), MBP-SulA or MBP-SulAC161 was examined in the presence or absence of Lon protease and/or ATP, followed by SDS-PAGE analysis and Coomassie Brilliant Blue staining. Protein sizes are shown on the left, and the position of each protein on the right

which is associated with stimulation of the ATPase activity of Lon protease.

Effect of Deletion of SA8 from SulA on the Formation of a Complex of SulA with Lon Protease—To confirm the effect of deletion of SA8 from SulA on the interaction with Lon protease, we next examined its effect on the formation of a complex of SulA with Lon by means of amylose-MBP affinity column chromatography. MBP-SulA, MBP-SulAC161, or MBP-LacZα was incubated with Lon protease without ATP, and then the mixture was applied to the amylose column. Lon protease was adsorbed to the column and then co-eluted with MBP-SulA with 10 mM maltose. However, it was not co-eluted with MBP-SulAC161, as was the case with MBP-LacZα (Fig. 5). These results also provide evidence that deletion of SA8 from SulA results in the loss of formation of a complex SulA with Lon protease.

Interactions of SA8 with Lon Protease—Casein, MBP-SulA, SA20, or SA8 was immobilized directly on a carboxylate dextran matrix chip, and then LonS679A lacking proteolytic activity was injected over these proteins or peptides, and the interaction was monitored using the BIAcore system. The kinetic parameters analyzed in the sensorframes are summarized in Table II. k_{ass}/k_{diss} of SA8 for LonS679A was 7.29×10^5 M^{-1}. On the other hand, k_{ass}/k_{diss} of casein, MBP-SulA and SA20 (C-terminal 20 amino acid residues of the SulA peptide) were 1.93×10^7, 7.16×10^7, and 3.46×10^6 M^{-1}, respectively. BSA, which is not a substrate for Lon, did not interact with LonS679A. These results suggest that SA8 directly interacts with Lon protease, but its affinity is lower than that of casein, MBP-SulA, or SA20.

Inhibition of SulA Degradation by SA8 In Vitro—We next examined whether or not SA8 exerts an inhibitory or stimulatory effect on the degradation of SulA by Lon protease. Various amounts of SA8 were mixed with Lon protease, followed by preincubation for 10 min at 37°C. Then MBP-SulA and ATP were added to start the hydrolysis of SulA at 37°C for 30 min. SA8 inhibited the degradation of MBP-SulA depending on the dose to about 30% with 200

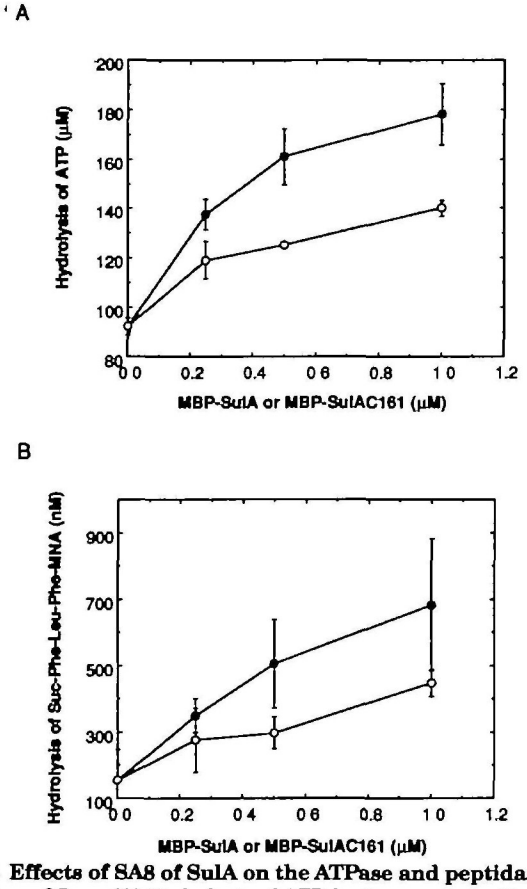

Fig 4 **Effects of SA8 of SulA on the ATPase and peptidase activities of Lon.** (A) Hydrolysis of ATP by Lon protease. Reaction mixtures containing 0.1 μM purified Lon protease (as the monomer), 1 mM [γ-^{32}P]ATP and various concentrations of fusion proteins [MBP-SulA (closed circles) or MBP-SulAC161 (open circles)] were incubated at 37°C for 1 h, and then the radioactivity of [^{32}P]orthophosphate released from [γ-^{32}P]ATP was measured as described under "MATERIALS AND METHODS." The means ± SD for three independent experiments are shown (B) Hydrolysis of Suc-Phe-Leu-Phe-MNA by Lon protease. Reaction mixtures containing 0.02 μM purified Lon protease (as the monomer), 5 μM Suc-Phe-Leu-Phe-MNA, 1 mM ATP, and various concentrations of fusion proteins [MBP-SulA (closed circles) or MBP-SulAC161 (open circles)] were incubated at 37°C for 2 h. The fluorescence of the free MNA liberated from Suc-Phe-Leu-Phe-MNA was measured with a spectrofluorometer at an excitation wavelength of 355 nm and an emission wavelength of 460 nm The means ± SD for three independent experiments are shown.

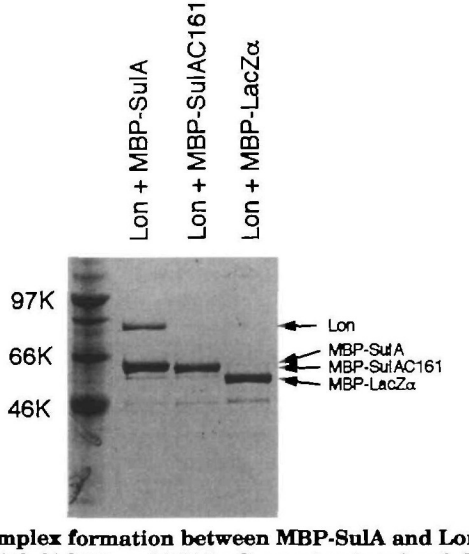

Fig. 5. **Complex formation between MBP-SulA and Lon.** MBP-SulA, MBP-SulAC161, or MBP-LacZα was incubated with Lon protease without ATP, applied to an amylose resin column, and then eluted with 10 mM maltose, and then the eluate was analyzed by SDS-PAGE and subsequent Coomassie Brilliant Blue staining as described under "MATERIALS AND METHODS." The left lane contained protein size markers, and the right one the standard proteins used.

TABLE II. **Kinetic parameters for the interaction between LonS679A and substrates.**

Substrate	k_{ass} (M^{-1} S^{-1})	k_{diss} (S^{-1})	k_{ass}/k_{diss} (M^{-1})
α-Casein	2.13×10^4	1.10×10^{-3}	1.93×10^7
MBP-SulA	7.88×10^4	1.10×10^{-3}	7.16×10^7
SA20	1.39×10^4	4.02×10^{-3}	3.46×10^6
SA8	9.77×10^3	13.4×10^{-3}	7.29×10^5
BSA	No binding		

The kinetic parameters were determined using the BIAcore system, as described under "MATERIALS AND METHODS."

μM (Fig. 6, A and B). However, it showed no effect on the degradation of casein, a well known *in vitro* substrate for Lon protease (Fig. 6B). These results show that SA8 selectively inhibits the degradation of SulA by Lon protease but not that of casein, suggesting that SA8 interacts with Lon in a way somewhat specific to Lon-SulA complex formation but not in general to the Lon-substrate complex.

Effect of the SA8 Peptide on the Degradation of MBP-SulAC161 by Lon Protease—We investigated whether or not the SA8 peptide induces the degradation of MBP-SulAC161 by Lon protease. In the presence of 400 μM SA8, MBP-SulAC161 was not degraded by Lon protease (Fig. 7). This result suggests that SA8 alone is not able to restore the impaired susceptibility of C-terminal deleted SulA to degradation by Lon protease.

Effects of Amino Acid Substitutions in SA8 on the Degradation of MBP-SulA by Lon Protease—To determine whether or not there is a specific arrangement of the 8 amino acids of SA8 for the inhibition of SulA-degradation by Lon protease, we prepared 4 mutant peptides, KAHSNLYH (SA8I163A; Ile at 163 changed to Ala), KIASNLYH

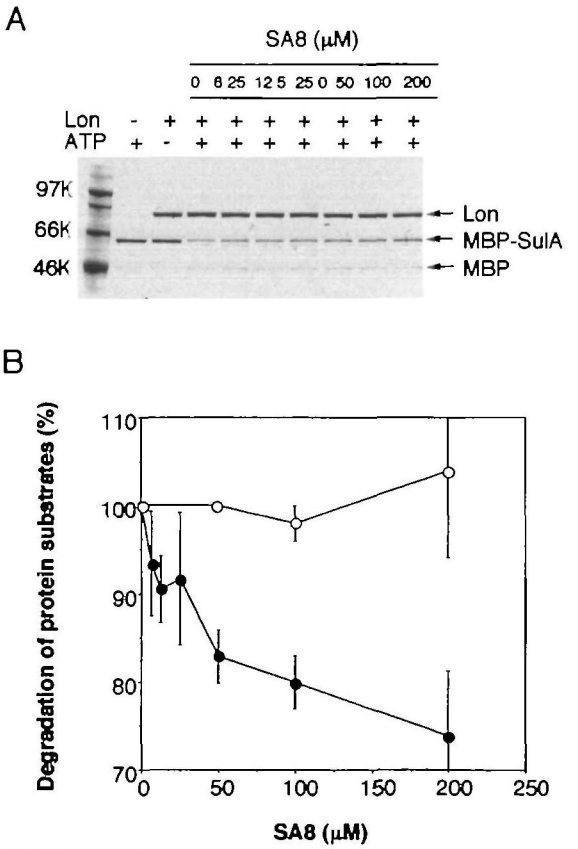

Fig. 6. **Effect of SA8 on the degradation of MBP-SulA or casein by Lon.** Reaction mixtures containing 0.25 μM Lon protease (as the monomer) and various concentrations of SA8 in 7.5 mM $MgCl_2$ and 50 mM Tris-HCl, pH 8.0, were pre-incubated at 37°C for 10 min, and then 0.25 μM MBP-SulA or resolfin-labeled casein was added together with 4 mM ATP to each reaction mixture. After incubation at 37°C for 30 min, the degradation of MBP-SulA was analyzed by SDS-PAGE (panel A), and the results were subjected to quantitative analysis of MBP-SulA by means of NIH image (panel B; closed circles). The degradation of resolfin-labeled casein was measured with a spectrophotometer, as described under "MATERIALS AND METHODS" (panel B; open circles). The results are the means ± SD for three independent experiments as relative values to the degradation of substrates in the presence of ATP and Lon but without SA8 in each experiment.

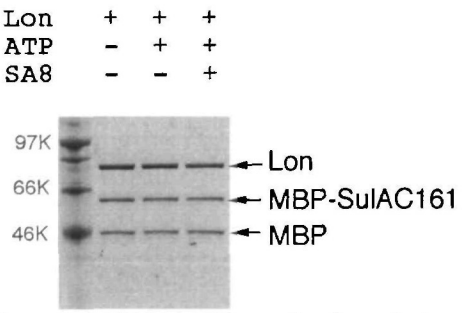

Fig. 7. **Effect of the SA8 peptide on the degradation of MBP-SulAC161 by Lon protease.** A mixture containing 0.25 μM Lon, 400 μM SA8, 0.25 μM MBP-SulAC161, and 4 mM ATP was incubated for 30 min, followed by SDS-PAGE analysis and Coomassie Brilliant Blue staining.

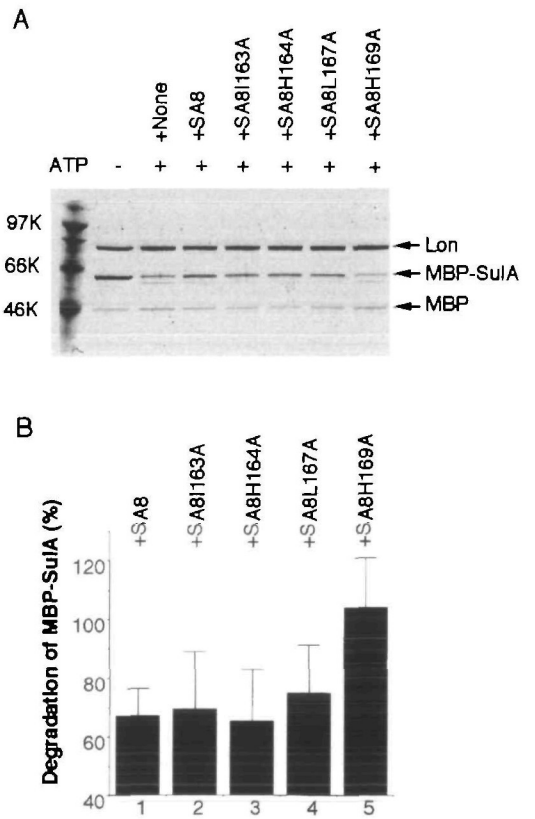

Fig. 8. **Effect of a mutated SA8 on the degradation of MBP-SulA by Lon protease.** SA8, SA8I163A, SA8H164A, SA8L167A, or SA8H169A was pre-incubated at 400 μM with 0.25 μM purified Lon protease (as the monomer) in 50 μl of a reaction buffer composed of 7.5 mM $MgCl_2$ in 50 mM Tris-HCl, pH 8.0, at 37°C for 10 min, and then 0.25 μM MBP-SulA and 4 mM ATP were added to start the ATP-dependent proteolysis. After incubation for 30 min at 37°C, the reaction was terminated and the degradation of MBP-SulA was analyzed by SDS-PAGE (panel A). The results were quantitatively analyzed by means of NIH image and are presented as the means ± SD for three independent experiments relative to the degradation of MBP-SulA in the presence of ATP and Lon but without SA8 or a mutant peptide in each experiment (panel B).

(SA8H164A; His at 164 changed to Ala), KIHSNAYH (SA8L167A; Leu at 167 changed to Ala), and KIHSNLYA (SA8H169A; His at 169 changed to Ala). SA8I163A, SA8H164A, and SA8L167A inhibited the degradation of MBP-SulA to similar extents to in the case of SA8 (Fig. 8). However, SA8H169A did not show significant inhibition. These results suggest that residue 169, *i.e.* histidine, at the SulA C-terminus is important enough for the interaction with Lon protease to inhibit the ATP-dependent degradation of SulA.

DISCUSSION

It is well known that SulA is induced by DNA damage in *E. coli* to inhibit cell division by suppressing FtsZ ring formation, and then is degraded by Lon protease *in vivo* as a SOS-response of the cells (*2*, *3*, *10–12*). However, the degradation and molecular interaction of SulA *in vitro* have been poorly investigated because of its toxicity and instability in *E. coli* cells. We succeeded in the purification of SulA using an MBP-fusion system, which enabled us to study the degradation of SulA by Lon *in vitro* (*22*). Moreover, we showed that the SulA protein has a specific region in the C-terminal 20 amino acid residues (SA20) which is necessary for SulA to only interact with Lon, but it is clearly different from cleavage sites; SulA lacking SA20 was not degraded by Lon protease (*23*). Furthermore, it was shown that *lon*$^+$ cells containing a mutant SulA lacking the C-terminal 8 amino acid residues (SA8) was also more lethal than ones containing the native SulA (*23*). These results imply the possibility that SA8 is the recognition site of SulA for Lon protease. In this study, we focused on the function of SA8 out of SA20.

We investigated the accumulation and stability of both SulA and a deletion mutant of SulA devoid of SA8 (SulAC-161) *in vivo* by Western blotting with anti-SulA IgG. SulAC161 was accumulated and stable more than native SulA in wild type cells (Fig. 2). On the other hand, the levels of accumulation and stability of SulA and SulAC161 were the same in *lon*$^-$ cells. Moreover, we prepared a deletion mutant of SulA devoid of SA8 as a MBP-fusion protein (MBP-SulAC161), and then examined whether or not this deletion mutant of SulA was digested by Lon protease *in vitro*. This deletion SulA was not degraded by Lon protease (Fig. 3), probably because it reduced the ability to enhance the ATPase and peptidase activities of Lon protease (Fig. 4). Furthermore, MBP-SulAC161 did not form a complex with Lon protease (Fig. 5). These results suggest that SA8 of SulA contains a recognition site for Lon protease. It is possible that deletion of SA8 causes a conformational change of SulA, and thus Lon protease does not recognize the deletion SulA. However, the deletion SulA was found to act as a cell division inhibitor *in vivo* (Fig. 1), and SA8 directly interacted with Lon (Table II). In addition, SA8 alone showed no effect on the degradation of MBP-SulAC-161 by Lon protease (Fig. 7), and did not have the ability to enhance the ATPase or peptidase activity of Lon protease (data not shown). Therefore, SA8 was found to contain a recognition site for Lon protease rather than a region simply related to a non-specific change of the SulA conformation or an allosteric effector–like domain.

Figure 6 shows that SA8 inhibited the degradation of SulA, probably due to blocking of the interaction between Lon and SulA. However, interestingly, SA8 did not inhibit the degradation of casein by Lon (Fig. 6B). These results indicate the specificity of SA8 as to inhibition of the degradation of SulA *in vitro*. *lon*$^+$ cells containing SA8 fused to MBP were more sensitive to UV-irradiation than ones containing the LacZα fusion protein (unpublished data). In addition, SA8 seems not to be effective for the degradation of Lon substrates in general except SulA *in vivo*, because mucoid formation was not observed in *lon*$^+$ cells in spite of that they contained the overproduced SA8 fusion protein, which should otherwise stabilize the RcsA protein, another major Lon substrate (data not shown). Taken together, these *in vitro* and *in vivo* study results imply that SA8 specifically regulates the degradation of SulA by Lon protease.

The sequence of SA8 of SulA was shown to be conserved among enterobacteria species (*26*). We investigated whether or not the sequence of SA8 was specific to the interaction with Lon protease. Judging from the results of experiments involving alanine-substitution mutations, the SulA degradation by Lon protease was little affected on substitution of the isoleucine at position 163, that of the histidine at 164 or that of the leucine at 167. However, the substitution of histidine at 169 abolished the biological activity of SA8 (Fig. 8). We have also observed that an alanine-substituted mutant of SulA as to histidine 169 is as stable as SulAC161 *in vivo* (Ishii, Y. and Amano, F., manuscript in preparation). These results suggest that the extreme C-terminal histidine of SA8 affects the interaction with Lon protease.

Overall, this study has revealed that SA8 of SulA is a recognition site for Lon protease with a specific sequence for the interaction with Lon protease, and that SA8 specifically stabilizes the degradation of SulA by Lon protease *in vivo* under some experimental conditions. This information seems to reflect the mechanism underlying the regional regulation of the SulA-Lon interaction in cell physiology.

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