ATP-dependent degradation of SulA, a cell division inhibitor, by the HslVU protease in *Escherichia coli*

Ihn Sik Seong, Ji Yeon Oh, Soon Ji Yoo[1], Jae Hong Seol[1], Chin Ha Chung*

Department of Molecular Biology and Research Center for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, South Korea

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Abstract HslVU is an ATP-dependent protease consisting of two multimeric components, the HslU ATPase and the HslV peptidase. To gain an insight into the role of HslVU in regulation of cell division, the reconstituted enzyme was incubated with SulA, an inhibitor of cell division in *Escherichia coli*, or its fusion protein with maltose binding protein (MBP). HslVU degraded both proteins upon incubation with ATP but not with its non-hydrolyzable analog, ATPγS, indicating that the degradation of SulA requires ATP hydrolysis. The pulse-chase experiment using an antibody raised against MBP-SulA revealed that the stability of SulA increased in *hsl* mutants and further increased in *lon*/*hsl* double mutants, indicating that SulA is an in vivo substrate of HslVU as well as of protease La (Lon). These results suggest that HslVU in addition to Lon plays an important role in regulation of cell division through degradation of SulA.

Key words: ATP-dependent protease; ATPase; HslVU; Protease La; SulA; Cell division

1. Introduction

One of the prominent events of protein breakdown in prokaryotes as well as in eukaryotic cells is the requirement of metabolic energy. A number of proteolytic enzymes responsible for the ATP-dependent process in *Escherichia coli* has been identified [1,2]. Of these, protease La (Lon) is a heat shock protein and plays an essential role in the hydrolysis of most abnormal proteins and certain short-lived regulatory proteins, such as SulA, which is an inhibitor of cell division [3]. Protease Ti (Clp), unlike Lon, consists of two different multimeric components, both of which are required for proteolysis [4,5]. While ClpA contains the ATP-hydrolyzing sites, ClpP, which is a heat shock protein, contains a serine active site for proteolysis. ClpP alone can rapidly cleave small peptides, although it absolutely requires ATP hydrolysis by ClpA for degradation of large polypeptides [6]. ClpA is a member of a family of highly conserved polypeptides, present in both prokaryotic and eukaryotic organisms [7]. In fact, the ClpP peptidase can function together with one of the homologous ATPases, ClpX, in degradation of certain regulatory proteins, such as λO protein [8,9] and the bacteriophage Mu vir repressor [10].

Like protease Ti, HslVU consists of two distinct multimeric components: a 19 kDa HslV and a 50 kDa HslU [11,12]. HslV harbors the peptidase activity, whereas HslU provides an essential ATPase activity, both of which can function together as a novel two-component protease [12–14]. HslU itself has ATPase activity, whereas HslV by itself is a weak peptidase that slowly degrades certain hydrophobic peptides, such as *N*-carbobenzoxy (Cbz)-Gly-Gly-Leu-7-amide-4-methyl-coumarin (AMC), and polypeptides, including insulin B-chain and casein [13,15]. In the reconstituted enzyme, HslU markedly stimulates the proteolytic activity of HslV (more than 20-fold), whereas HslV increases the rate of ATP hydrolysis by HslU several-fold.

All of the soluble ATP-dependent proteases in *E. coli* have been shown to exhibit protein-activated ATPase activity. Therefore, it has been suggested that hydrolysis of ATP and peptide bond occurs in a tightly coupled process. However, we have recently demonstrated that HslVU protease does not require ATP hydrolysis for its proteolytic activity, unlike proteases La and Ti. Non-hydrolyzable ATP analogs, such as ATPγS and β,γ-imido-ATP, have been shown to be capable of supporting the ability of HslVU protease in degrading insulin B-chain and the fluorogenic peptide even better than ATP [16,17]. Moreover, HslU/C287V, which is unable to hydrolyze ATP, could also support the HslV-mediated degradation of the substrates in the presence of ATP, although to a lesser extent than the wild-type HslU at its low protein concentration [18]. Therefore, the precise function of ATP hydrolysis by HslVU protease remains obscure.

The SulA protein of *E. coli* is encoded by the SOS-inducible *sulA* gene, which is also called *sfiA*. The target for SulA is the essential cell division protein FtsZ, which is a GTPase and plays a critical role in the initiation of cell division [19–21]. *sfiA* mutants were initially isolated based on suppression of mutation in *lon*, which encodes protease La [22,23]. Later, it has been demonstrated that the suppression is due to rapid degradation of SulA by protease La. The SulA protein has a high tendency to aggregate in vivo as well as in vitro and therefore, its purification has been facilitated by a genetic manipulation to fuse maltose binding protein (MBP) to SulA. This fusion protein has also been shown to retain the inhibitory function in vivo and to behave as a substrate for protease La in vitro [24].

Recently, Khattar [25] has reported that overexpression of the *hslVU* operon, which encodes HslV and HslU, suppresses SOS-mediated inhibition of cell division. This finding raised a possibility that HslVU protease can degrade SulA. In the present studies, therefore, we examined whether the SulA protein behaves as an in vivo as well as in vitro substrate for HslVU protease, in an attempt to determine the role of HslVU protease in regulation of cell division.

*Corresponding author. Fax: (82) (2) 872-1993.
E-mail: chchung@snu.ac.kr

[1] Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA.

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2. Materials and methods

2.1. Bacterial strains and plasmids

The plasmid pGEM/R9 carries *lonR9*, which has a single point mutation in the *lon* gene [26]. An internal portion of *lonR9* in pGEM/R9 (0.8 kb *Cla*I/*Nru*I fragment) was replaced by a 1.4 kb *Cla*I/*Sma*I fragment containing the tetracycline resistance (*tet*) gene of pBR322. The resulting plasmid was treated with an appropriate restriction enzyme and introduced into a JC7623 strain (*recBC*, *sbcB*) by selecting for tetracycline resistance. One of the transformants carrying *lon::tet* on the chromosome was transduced into MC1000 (*araD139 Δ(ara-leu)7679 galU galK Δ(lac)174 rpsL thi-1*) by phage P1 [27] and the resulting mutant was referred to as MC1000L. For constructing a MC1000H strain carrying *hslVU::kan*, pGEM-T/HslVU [13] was cut with *Tth*III1, gap-filled with Klenow fragment and ligated with a 1.5 kb *Sma*I fragment containing the kanamycin resistance (*kan*) gene of pUC4 (type KIXX, Pharmacia). The resulting plasmid was then introduced into JC7623 and one of the transformants was transduced into MC1000 as above. A MC1000LH strain (*lon::tet, hslVU::kan*) was obtained by P1 transduction of MC1000H grown in a JC7623 strain carrying *lon::tet*.

For in vivo experiments, these cells were transformed with pSulA5 [28], which carries the wild-type *sulA* gene under control of the *lacUV5* promoter. pMAL-p2-SulA was transformed into MC1000 cells for overproduction of MBP-SulA [29]. pMAL-p2-SulA and pSulA5 were obtained from Dr A. Higashitani (National Institute of Genetics, Japan).

2.2. Protein purification

The purified HslV and HslU were prepared as described previously [13]. The HslU mutant proteins, HslU/C287V, HslU/C287A and HslU/C287S, in which the Cys^{287} residue was replaced with Val, Ala and Ser, respectively, were purified as described [18]. MBP-SulA was purified from MC1000 cells carrying pMAL-p2-SulA by affinity chromatography on amylose resin (New England Biolabs) [24]. The protein concentration was determined as described by Bradford [30] using bovine serum albumin as a standard.

2.3. In vitro degradation of MBP-SulA and SulA by HslVU

Incubations were carried out at 37°C in the reaction mixtures (0.1 ml) containing 0.1 M Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 5 μg MBP-SulA, 0.5 μg HslV and 2 μg HslU in the absence or presence of various adenine nucleotides. After incubation, the reaction was stopped by adding 30 μl of 0.75 M Tris-HCl (pH 6.8) containing 7.5% SDS and 10% (v/v) 2-mercaptoethanol. They were then subjected to polyacrylamide gel electrophoresis (PAGE) on 13% (w/v) slab gels containing SDS and 2-mercaptoethanol [31]. The gels were stained with Coomassie blue R-250.

Since MBP-SulA has a unique cleavage site of factor Xa (FXa) (New England Biolabs) in the linker region between MBP and SulA, 5 μg of the fusion protein was first incubated with 0.6 μg of FXa for 30 min at 25°C. After incubation, the reaction was stopped by adding 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone (CALBIOCHEM), a specific inhibitor of FXa, to a final concentration of 2 μM. The SulA protein generated from MBP-SulA was then incubated with purified HslVU protease at 37°C for 2 h. The samples were subjected to SDS-PAGE as above.

2.4. In vivo degradation of SulA

Cells containing pSulA5 were grown at 30°C in M9 glycerol medium [32] supplemented with all of the amino acids except Met. After IPTG induction for 10 min, cells were pulse-labelled with [^{35}S]Met (10 μCi/ml, 1000 Ci/mmol) for 2 min and then chased for various periods. The resulting samples were immediately subjected to immunoprecipitation using an anti-MBP-SulA antibody [33], followed by SDS-PAGE. The gels were dried and exposed to X-ray films. The radioactivity in the band corresponding to SulA was quantified using a Phosphorimager (Fuji).

3. Results and discussion

3.1. Degradation of MBP-SulA by purified HslVU protease

In order to determine whether HslVU protease can degrade SulA, the enzyme was incubated with purified MBP-SulA fusion protein for various periods followed by electrophoresis under denaturing conditions (Fig. 1). The band corresponding to MBP-SulA (60 kDa) was diminished with a concomitant appearance of a new band with a size of about 45 kDa (marked with an arrowhead) upon incubation with ATP. The 45 kDa fragment is likely to be a degradation product, composed mainly of MBP (molecular mass about 42 kDa). Thus, it appears that HslVU protease specifically degrades the SulA portion of MBP-SulA.

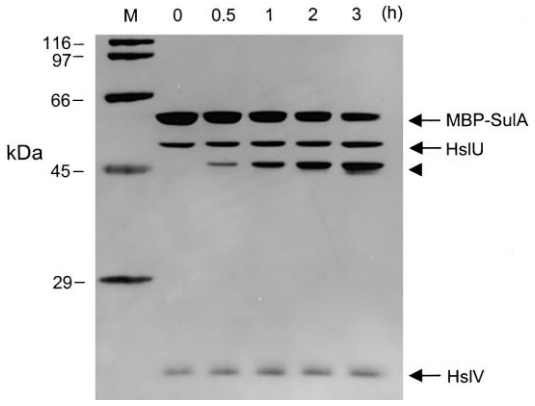

Fig. 1. Time course for degradation of MBP-SulA by purified HslVU protease. MBP-SulA (5 μg) was incubated with HslV (0.5 μg), HslU (2 μg) and 2 mM ATP for the indicated periods, followed by SDS-PAGE as described under Section 2. The numerals on top of the gel show the time of incubation and the arrowhead on the right indicates the degradation product from MBP-SulA. The letter M indicates the lane containing size markers.

On the other hand, little or no degradation of MBP-SulA by HslVU was observed when ATP was replaced with ATPγS, a non-hydrolyzable analog of ATP (Fig. 2A). Even dATP could not support the degradation of MBP-SulA, despite our previous finding that dATP is hydrolyzed and supports the cleavage of a peptide, Cbz-Gly-Gly-Leu-AMC, by HslVU protease approximately 30% as well as ATP [13]. This absolute requirement of ATP and its hydrolysis for the degradation of MBP-SulA is not consistent with the recent reports revealing that non-hydrolyzable ATP analogs can support the ability of HslVU protease to degrade insulin B-chain, casein and the peptide substrate and that HslU/C287V, which is unable to hydrolyze ATP, could also support the HslV-mediated degradation of the substrates in the presence of ATP [16–18].

In order to clarify the requirement of ATP hydrolysis further, we compared the ability of HslU mutant proteins in supporting the degradation of MBP-SulA by HslV. As shown in Fig. 2B, the wild-type HslU, HslU/C287A and HslU/C287S, but not HslU/C287V, supported the HslV-mediated degradation of MBP-SulA in the presence of ATP. As already reported [18], HslU/C287A and HslU/C287S, unlike HslU/C287V, can hydrolyze ATP and support the HslV-mediated hydrolysis of insulin B-chain nearly as well as the wild-type HslU. These results indicate that ATP hydrolysis is essential for the degradation of MBP-SulA by HslVU protease. Since insulin B-chain and casein are known to lack any appreciable secondary structure, it appears to be possible that ATP hydrolysis by HslVU protease is required for unfolding of MBP-SulA for channeling into the central cavity of HslV, where the active site is located [34]. In fact, protease La has been dem-

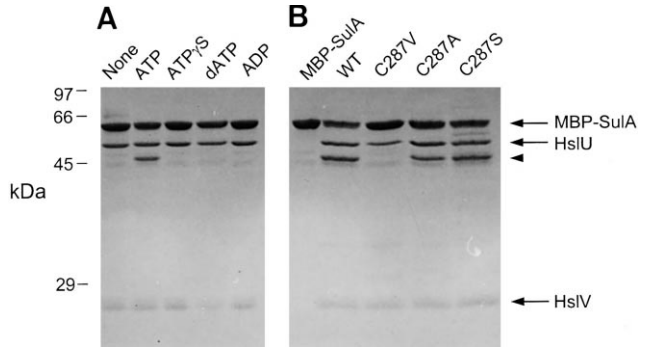

Fig. 2. Requirement of ATP hydrolysis for degradation of MBP-SulA by HslVU. (A) HslV (0.5 μg) was incubated with HslU (2 μg), MBP-SulA (5 μg) and the indicated adenine nucleotides (2 mM) for 2 h at 37°C. (B) HslV was incubated as above but with the various mutant forms of HslU in the presence of 2 mM ATP only. The samples were then subjected to SDS-PAGE. The arrowhead indicates the degradation product from MBP-SulA.

onstrated to degrade the unfolded form of CcdA, but not its native form, without ATP hydrolysis [35].

3.2. Degradation of SulA by HslVU in vitro and in vivo

To determine whether HslVU can degrade SulA as well as MBP-SulA, SulA was generated by treating MBP-SulA with FXa and then incubated with HslVU in the presence of ATP or its analogs. Fig. 3 shows that HslVU can degrade SulA in the presence of ATP but not with ATPγS or dATP. On the other hand, MBP generated from MBP-SulA by FXa remained intact under the same conditions. These results confirm that ATP hydrolysis is essential for the degradation of SulA, unlike that of insulin B-chain or the peptide substrate [16].

In order to determine whether SulA is also degraded in vivo by HslVU, the wild-type and *hsl* null mutant cells were pulse-labelled with [^{35}S]Met, followed by chasing with an excess of unlabelled Met. Since protease La (Lon) is known to rapidly degrade SulA [36], *lon* and *lon/hsl* mutant cells were also subjected to pulse-chase experiments for comparison. As previously reported [37], *lon* mutation markedly increased the half-life of SulA from 3 to 25 min (Fig. 4). SulA was further stabilized to a half-life of 65 min in a *lon/hsl* double mutant,

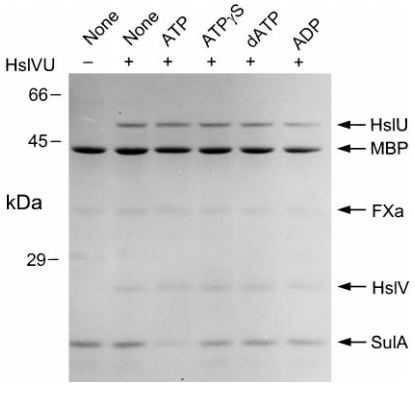

Fig. 3. In vitro degradation of SulA by HslVU. MBP-SulA (5 μg) was incubated with 0.6 μg of FXa for 30 min at 25°C. After incubation, the samples were treated with 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone. The mixture containing cleaved SulA and MBP was then incubated with purified HslVU protease at 37°C for 2 h in the absence or presence of various adenine nucleotides.

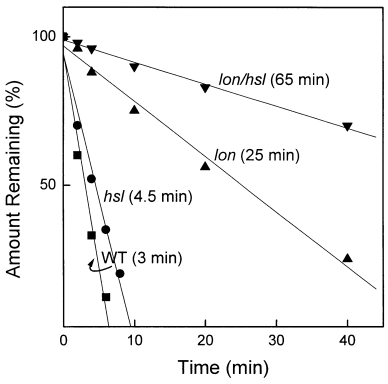

Fig. 4. Effects of *hsl* mutation on in vivo degradation of SulA. The wild-type (MC1000) and *hsl* (MC1000H), *lon* (MC1000L) and *lon/hsl* (MC1000LH) mutant cells were transformed with pSulA5. They were then subjected to pulse-chase analysis as described under Section 2. The resulting samples were immediately subjected to immunoprecipitation using an anti-MBP-SulA antibody, followed by SDS-PAGE. The gels were dried and exposed to X-ray films. The radioactivity in the band corresponding to SulA was then quantified using a Phosphorimager (Fuji). Similar data were obtained in three independent experiments.

although it was slightly stabilized by the *hsl* mutation alone. These results indicate that SulA behaves as an in vivo substrate of HslVU protease as well as of protease La.

It has recently been reported that overexpression of HslVU in *lon* mutant cells blocks SOS-mediated inhibition of cell division [25]. It has also been shown that constitutive expression of SulA prevents colony formation of *E. coli* [38]. In addition, we found that the cell growth was more severely arrested when overexpression of SulA was induced in a *lon/hsl* mutant than in the cells carrying a *lon* mutation alone (data not shown). Thus, it appears that HslVU protease, in addition to protease La, plays an important role in regulation of cell division through degradation of SulA.

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