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Binding of MG132 or Deletion of the Thr Active Sites in HslV Subunits Increases the Affinity of HslV Protease for HslU ATPase and Makes This Interaction Nucleotide-independent*

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Running title: Thr Active Site-mediated communication between HslV and HslU

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HslVU is an ATP-dependent protease in bacteria, consisting of HslV dodecamer and HslU hexamer. Upon ATP binding, HslU ATPase allosterically activates the catalytic function of HslV protease one to two orders of magnitude. However, relatively little is known about the role of HslV in the control of HslU function. Here we describe the involvement of the N-terminal Thr active sites (Thr1) of HslV in the communication between HslV and HslU. Binding of proteasome inhibitors to Thr1 led to a dramatic increase in the interaction between HslV and HslU with a marked increase in ATP hydrolysis by HslU. Moreover, carbobenzoxy-leucyl-leucyl-leucinal (MG132), could bind to Thr1 of free HslV and this binding induced a tight interaction between HslV and HslU with the activation of HslU ATPase, suggesting that substrate-bound HslV can allosterically regulate HslU function. Unexpectedly, the deletion of Thr1 also caused a dramatic increase in the affinity between HslV and HslU even in the absence of ATP. Furthermore, the increase in the number of the Thr1-deletion mutant subunit in place of HslV subunit in a dodecamer led to a proportional increase in the affinity between HslV and HslU with gradual activation of HslU ATPase. Although the molecular mechanism how the Thr1 deletion influences the interaction between HslV and HslU remains unknown, these results suggest an additional allosteric mechanism for the control of HslU function by HslV. Taken together, our findings indicate a critical involvement of Thr1 of HslV in the reciprocal control of HslU function and thus for their communication.

Key words: ATP-dependent protease, HslU ATPase, HslV protease, Proteasome inhibitors, Thr active site, Allosteric regulation

HslVU is a two-component ATP-dependent protease in bacteria, which comprises HslV protease and HslU ATPase (1-5). HslV, a homolog of the β-subunit of 20S proteasome, is a self-compartmentalized protease that has two stacked hexameric rings of identical subunits, each of which has an N-terminal Thr (Thr1) active site for proteolysis (6-12). The hexameric HslU ATPase, a member of AAA family (13, 14), binds to either one or both ends of an HslV dodecamer to form the HslVU complex. In the HslVU complex, the HslU and HslV central pores are aligned and the proteolytic active sites are sequestered in the internal chamber of HslV, with access to this chamber restricted to small axial pores (7-11).

Biochemical studies have shown that ATP binding and its subsequent hydrolysis by HslU play essential roles in controlling the proteolytic function of HslV and the interaction between HslU and HslV (10, 12, 15-17). Hexamerization of HslU itself is largely favored by the nucleotide binding to the ATPase (17). Moreover, HslV that by itself is a weak peptidase can be activated one to two orders of magnitude by ATP-bound HslU (15, 16). ATPγS, a nonhydrolyzable ATP analog, also supports HslV-mediated hydrolysis of small peptides but not that of native protein substrates, such as SulA, suggesting the role of ATP hydrolysis by HslU in unfolding of protein

substrates for their access to and subsequent degradation at the inner proteolytic chamber of dodecameric HslV (18). Importantly, chemical cross-linking analysis has shown that ATP-bound HslU interacts with HslV to form the HslVU complex, but ADP-bound HslU does not, implicating dynamic interaction between HslU and HslV during ATP hydrolysis cycles (17). However, it was unknown how the HslVU complex is maintained during threading of unfolded polypeptide from HslU into the inner chamber of HslV and subsequent cleavage of peptide bonds at the Thr1 active sites for the completion of a proteolytic cycle.

Unlike eukaryotic 20S proteasomes where substrate accessibility to proteolytic active sites are controlled by opening-and-closing the apical gates of α subunits (19-21), HslVU has been shown to utilize an allosteric mechanism whereby the active sites of HslV are switched on-and-off through the nucleotide-dependent interaction of HslU with HslV (12). Specifically, the C-terminal tails of HslU show a dramatic movement in a nucleotide-dependent manner (*i.e.*, they move toward HslV-HslV subunit interfaces from HslU-HslU subunit interfaces when ATP is bound) (10, 12, 22). Moreover, a synthetic HslU tail peptide of 10 amino acids could replace HslU in the activation of HslV-mediated peptide hydrolysis (23, 24). Thus, it appears that HslU allosterically regulates the proteolytic function of HslV in a nucleotide-dependent fashion. On the other hand, relatively little is known about the role of HslV in the control of HslU function except for its ability to stimulate the ATPase activity several fold.

In the present study, we demonstrate that binding of proteasome inhibitors to the Thr1 residues, which likely mimic the substrate-bound state of the active sites, dramatically increases the interaction between HslV and HslU. Significantly, MG132, unlike lactacystin or 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone (NLVS), could induce the interaction of HslV with HslU even in the absence of nucleotide or the sole presence of ADP. These findings provide a mechanism for the maintenance of stable HslVU complexes when substrates are bound to the Thr1 active sites for the completion of a proteolytic cycle. Surprisingly, deletion of the Thr1 residues was found to also cause a dramatic increase in the interaction between HslV and HslU in the absence of ATP. Collectively, our findings indicate that the N-terminal Thr active sites of HslV are involved in the communication between HslV and HslU, in addition to its role in the catalysis of peptide bond cleavage.

EXPERIMENTAL PROCEDURES

Materials **—** Enzymes for DNA cloning were purchased from Takara, New England Biolab, and Stratagene. Carbobenzoxy-Gly-Gly-Leu-amidomethyl coumarin (Z-GGL-AMC) was purchased from Bachem. MG132, lactacystin, and NLVS were obtained from A.G. Scientific, Cayman Chemical, and Calbiochem, respectively. Other reagents were purchased from Sigma, unless otherwise indicated.

Strains and Cloning **—** BW21135 *ΔhslVU::kan* strain was generated from MG21135 strain by using λRed system (25). Two primers (forward, gat gaa aat gat tga acg cga tta tag gat aaa acg gct cac tgg gct atc tgg aca agg; reverse, ccc cat cta taa ttg cat tat gcc ccg tac ttt tgt acg gcg tcc cgg aaa acg att ccg: the *hslVU* homologous regions are underlined) and pKD13 as a template were used to produce a PCR product for homologous recombination. The deletion of *hslVU* operon was confirmed by PCR and immunoblot analysis.

pBR-PL was constructed from pBR322 by substituting the HindIII-NruI segment of the vector with a polylinker (aa gct tAC TAG TTA CCG CGG TCG ACA TCC ATG GAG CTC GGG CCC cga: the lower cases are vector sequences). pV-1 expressing only HslV was generated by deleting the NruI-BglII segment of *hslU* gene in pGEM-T/HslVU vector. Site-directed mutagenesis (QuickChange, Stratagene) was performed to insert His_6 tag at the C-terminus of HslV (SYKAHHHHHH: the HslV sequence is underlined), resulting in pVH-1 vector. Mutations in start codons and Thr1 deletion were also generated by site-directed mutagenesis. All mutations were confirmed by DNA sequencing. Vectors for the production of mixed dodecamers consisting of HslV and Thr1-deletion mutant (T1Δ) subunits (Table 1) were constructed by sequential insertions of *hslV* and *t1Δ* genes (restriction fragments of pV-1 or pVH-1) into the polylinker site of pBR-PL.

Protein Expression and Purification **—** HslU

and HslV were purified as described previously (2, 16). pETDuet-1 vectors (Novagen) were used for co-expression of HslU and His-tagged HslV proteins. BL21 (DE3) *ΔhslVU* cells transformed with the vectors were cultured at 37°C to an optical density of 0.5-0.6 at 600 nm, and then treated with 0.1 mM IPTG for 30 min for protein induction.

To express HslV mixed dodecamers, BW21135 *ΔhslVU::kan* cells harboring appropriate vectors were grown overnight at 37°C in Luria broth supplemented with ampicillin. Proteins were purified by using Ni^{2+}-nitrillotriacetic acid (NTA)-agarose columns according to the manufacturer's instruction (Qiagen) with some modifications: *i.e.*, imidazole was used at 50-60 mM for washing and at 450 mM for elution. Purified proteins were dialyzed against 20 mM Tris-HCl buffer (pH 7.8) containing 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 10% glycerol, and stored at -70°C for further uses. Protein concentration was measured by Bradford method using BSA as a standard.

NTA Pull-down Analysis — Reaction mixtures (0.5 ml) containing HslU (150 nM) and His-HslV (75 nM) in 50 mM HEPES buffer (pH 8) containing 150 mM NaCl, 5% glycerol, and 0.04% Triton X-100 were incubated at 4°C for 1 h in the absence or presence of 2 mM adenine nucleotides and 5 mM $MgCl_2$. After incubation, the mixtures were supplemented with 10 μl of 1 M imidazole and 20 μl of Ni^{2+}-NTA resins, and rocked at 4°C for 1 h. The resins were washed four times with 0.5 ml of 50 mM HEPES buffer (pH 8) containing 300 mM NaCl, 5 mM $MgCl_2$, 60 mM imidazole, 5% glycerol, 0.04% Triton X-100, and 2 mM adenine nucleotides. Proteins bound to NTA resins were eluted by SDS sampling buffer, subjected to SDS-PAGE, and stained with Coomassie blue R-250.

Assays — ATP hydrolysis was measured using an enzyme-coupled assay (26). HslU (0.2 μM) and HslV (0.2 μM) in 100 mM Tris-HCl buffer (pH 8) containing 150 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, and 0.5 mM EDTA were incubated at 37°C with 2 mM ATP, 3 mM phosphoenolpyruvate, 0.5 mM NADH, 20 U/ml of pyruvate kinase, and 20 U/ml of lactic dehydrogenase. Absorbance at 340 nm was continuously recorded using a spectrophotometer (Ultrospec2000, Pharmacia) equipped with a temperature controller. The rate of ATP hydrolysis was calculated from the slope within a linear range, based on the extinction coefficient of NADH ($\varepsilon_{340nm} = 6.22 \times 10^3$).

Peptide hydrolysis was assayed by incubation of HslU (10 nM) and HslV (5 nM) in 100 mM Tris-HCl buffer (pH 8) containing 5 mM $MgCl_2$, 0.5 mM EDTA, and 2 mM ATP with 0.1 mM Z-GGL-AMC at 37°C (27). Fluorescence ($\lambda_{excitation}$ = 355 nm, $\lambda_{emission}$ = 460 nm) of released AMC was continuously measured using a fluorometer (FluoStar, BMG) equipped with a temperature controller. The rate of peptide hydrolysis was then calculated from the slope within a linear range.

Gel Filtration by Spin-column — Spin-columns were prepared by packing Sephadex G-25 (0.2 ml) in 0.5 ml Eppendorf tubes that have a hole at the tip of their bottoms. After equilibration with 20 mM Tris-HCl buffer (pH 7.8) containing 100 mM NaCl and 5% glycerol, they were loaded with HslV incubated with proteasome inhibitors, sit on 1.5 ml Eppendorf tubes, and spun for 10 sec. The samples eluted from the columns were then used for further analysis.

RESULTS

Effects of Proteasome Inhibitors on the Interaction between HslV and HslU — To facilitate the purification of HslV and to assay the interaction between HslV and HslU by NTA pull-down analysis, poly-His (His_6) was tagged to the C-terminal end of HslV. The resulting protein (referred to as HslV-His) was purified to apparent homogeneity and subjected to assay for its ability to cleave Z-GGL-AMC in the presence of HslU. We also examined the ability of HslV-His to promote ATP hydrolysis by HslU. HslV-His cleaved the peptide and stimulated the ATP hydrolysis by HslU as well as HslV (Fig. 1*A*). In the presence of HslU and ATP, HslV-His could also degrade protein substrates, including α-casein and MBP-SulA, as well as HslV (data not shown). These results indicate that the C-terminal His tag does not interfere with the interaction between HslV and HslU.

HslV and HslU interact with each other (*i.e.*, form the HslVU complex) in the presence of ATP, and this interaction is required for their mutual activation (1-3, 17). In an attempt to determine

how HslV allosterically activates HslU ATPase, we first monitored the interaction between HslV and HslU in the presence of each of three well-known proteasome inhibitors: MG132, lactacystin, and NLVS. MG132 is known to reversibly react to the N-terminal Thr active sites of the 20S proteasome β-subunits, while lactacystin and NLVS irreversibly modify them (22, 28-30). HslV-His was incubated with each of the inhibitors in the absence or presence of adenine nucleotides. The samples were then subjected to NTA pull-down analysis. In the presence of ATP, all three inhibitors caused a dramatic increase in the amount of HslU co-precipitated with His-HslV as compared to DMSO that was used as a control (Fig. 1*B*, *upper panel*). Under the same experimental conditions, these inhibitors abolished the peptidase activity of HslV, indicating that these inhibitors efficiently block the Thr1 active sites of HslV (Fig. 1*C*, *upper panel*). These results demonstrate that the binding of proteasome inhibitors to the Thr1 active sites leads to a marked increase in the interaction between HslV and HslU. Unexpectedly, MG132, but not lactacystin or NLVS, could increase the interaction of HslV-His with HslU even in the presence of ADP or the absence of any nucleotide. Thus, it appears that MG132 can bind to free HslV (*i.e.*, HslV uncomplexed with HslU) and this binding induces tight interaction between HslV and HslU even in the absence of ATP. On the other hand, lactacystin and NLVS appear to bind only to HslV that is complexed with ATP-bound HslU, resulting in a further stabilization of the HslVU complex. Taken together, these results suggest that the Thr1 active sites of HslV protease are involved in the interaction between HslV and HslU.

To determine whether MG132, unlike lactacystin and NLVS, could indeed bind to free HslV, each of the inhibitors was subjected to incubation with HslV-His alone followed by gel filtration to remove unbound inhibitors by using Sephadex G-25-filled spin-columns. Eluted HslV proteins were then subjected to incubation with HslU in the presence of ATP followed by NTA pull-down analysis. Gel filtration abrogated the stimulatory effect of lactacystin or NLVS on the interaction between HslV and HslU, but showed little or no influence on that of MG132 (Fig. 1*B*, *lower panel*). Consistently, gel filtration abolished the inhibitory effects of lactacystin and NLVS on the peptidase activity of HslV, but not that of MG132 (Fig. 1*C*, *lower panel*). These results indicate that MG132 binding to the Thr1 active sites of free HslV is responsible for the induction of tight interaction between HslV and HslU in the absence of ATP or the presence of ADP. However, it remains unclear how MG132, unlike lactacystin and NLVS, can bind to free HslV. Although both MG132 and NLVS have the same tri-leucine peptide backbone, they have different N-terminal capping groups and C-terminal reactive groups. Therefore, one possibility is that, due to different steric or chemical properties of reactive groups, MG132, but not NLVS, is capable of reacting with or stably binding to the Thr1 active sites of free HslV. However, this differential interaction of MG132 is not due to the reactive aldehyde group itself, because N-acetyl-DEVD-aldehyde, a caspase-3 inhibitor, did not show any of the properties exhibited by MG132 (data not shown).

Since lactacystin or NLVS promoted the interaction between HslV and HslU only when ATP was present, we examined whether the presence of ATP might be persistently required for maintaining the stable interaction of HslU with the inhibitor-bound HslV. HslV-His and HslU were incubated with ATP in the absence or presence of lactacystin or NLVS, and the HslVU complexes formed were pulled down by NTA resins. Precipitates were then subjected to extensive washing with buffers containing ATP, ADP, or none of the nucleotides, followed by SDS-PAGE. In the absence of the inhibitors, HslU was dissociated from HslVU complexes by washing with buffers containing ADP or no nucleotide (Fig. 1*D*). In their presence, however, HslU remained stably associated with HslV under all washing conditions tested, indicating that the stability of inhibitor-bound HslVU complex is no longer influenced by the presence or absence of any adenine nucleotide. These results also indicate that ATP-bound HslU is required for the initial step where the inhibitors bind to the Thr1 residue of HslV, probably through a covalent modification of the hydroxyl group of the Thr1 residue. This notion is consistent with previous reports that HslV requires association with ATP-bound HslU to allosterically activate the Thr1 residue of HslV (15, 22, 31).

Since HslV is known to stimulate the ATPase activity of HslU by 2 to 3 fold (2, 16; see Fig. 1*B*)

and since all three tested proteasome inhibitors markedly increase the interaction between HslV and HslU when ATP is present, we examined whether this increased interaction leads to a further enhancement of HslV-stimulated ATPase activity of HslU. All of the inhibitors dramatically enhanced the HslV-stimulated ATPase activity of HslU, although to different extents (Fig. 1*E*, *left panel*). In addition, removal of unbound inhibitors by gel filtration on spin-columns abolished the stimulatory effect of lactacystin or NLVS, not that of MG132, on ATP hydrolysis by HslU (Fig. 1*E*, *right panel*). These results again reveal that MG132 can bind to free HslV and this binding induces tight association of HslV with HslU, resulting in a dramatic activation of HslU ATPase. Collectively, these results implicate a role of the Thr1 active sites of HslV in the interaction between HslV and HslU and thereby in the control of HslU function.

Effect of the Deletion of N-terminal Thr on the Interaction between HslV and HslU — To clarify the involvement of Thr1 active sites in the interaction between HslV and HslU, we generated a HslV mutant lacking the Thr1 residue, tagged poly-His to its C-terminus, and purified by using NTA-agarose columns. Unexpectedly, the Thr1-deletion mutant (referred to as T1Δ-His), unlike HslV-His, was co-purified with HslU from NTA-agarose column (Fig. 2*A*), despite the experimental conditions that the buffer used for the affinity chromatography was not supplemented with ATP. These results indicate that, like MG132-bound HslV, T1Δ can form stable complexes with HslU even in the absence of ATP. To confirm this finding, T1Δ-His was purified to apparent homogeneity (*i.e.*, separated from HslU) and then incubated with HslU in the absence or presence of ADP or ATP. NTA pull-down analysis reveals that HslU co-precipitates with T1Δ-His under all conditions tested (Fig. 2*B*), indicating that T1Δ interacts with HslU regardless of the binding of either adenine nucleotide to HslU. Moreover, the amount of HslU co-precipitated with T1Δ-His was much greater than that with HslV-His, indicating that the deletion of Thr1 leads to a marked increase in the interaction between HslV and HslU. We next compared the ability of T1Δ to that of HslV in stimulating the ATPase activity of HslU. Fig. 2*C* shows that T1Δ is capable of activating HslU ATPase much better than HslV, despite the fact that T1Δ-His does not show any proteolytic activity (data not shown). In addition, T1Δ-His stimulated the ATP hydrolysis by HslU nearly as well as T1Δ, indicating that the His tag does not interfere with the interaction between T1Δ and HslU. These results indicate that the increase in the interaction between T1Δ and HslU is responsible for the increase in the ATPase activity of HslU.

Effects of MG132 and Thr1 Deletion on HslVU Complex Formation — ATP binding is required not only for the HslVU complex formation by HslU hexamer and HslV dodecamer but also for the hexamerization of HslU subunits themselves. Noteworthy, however, was the finding that MG132-bound HslV (referred to as mHslV) could interact with HslU even in the absence of ATP. To confirm this finding further, HslV was incubated with MG132, extensively dialyzed to remove unbound inhibitor, and further incubated with HslU in the absence or presence of ATP. The samples were then subjected to gel filtration on a Superose-6 column. In the presence of ATP, HslU alone behaved as a hexamer (~300 kDa) and mHslV by itself ran a dodecamer (~250 kDa) (Fig. 3*A*, *left panel*). When HslU were incubated with HslV or mHslV in the presence of ATP, HslU with either combination of the HslV proteins were recovered in the fractions corresponding to a size of the HslVU complex (~600 kDa). These results indicate that the binding of MG132 to HslV shows little or no effect on the dodecameric structure of HslV or the formation of the HslVU complex. Without ATP, HslU ran as a monomer (~50 kDa) whether HslV was present or not, but was recovered in the fractions corresponding to a size of the HslVU complex (~600 kDa) in the presence of mHslV (Fig. 3*A*, *right panel*). These results indicate that mHslV is capable of interacting with HslU to form a stable HslVU complex in the absence of ATP.

Like mHslV, T1Δ could interact with HslU even in the absence of ATP. To confirm this finding also, T1Δ was subjected to incubation with HslU in the absence or presence of ATP followed by gel filtration chromatography as above. T1Δ alone ran as a dodecamer whether or not ATP was present (Fig. 3*B*). Upon incubation with HslU, T1Δ was recovered in the fractions corresponding to a size

of the HslVU complex regardless of the presence of ATP. These results indicate that deletion of the Thr1 residue shows little or no effect on the dodecameric structure of HslV or the formation of the HslVU complex. These results also indicate that T1Δ, like MG132-bound HslV, can interact with HslU to form stable HslVU complexes in the absence of ATP.

Requirements of HslV C-terminal Tails in HslVU Complex Formation — The C-terminal tails of HslU move toward the HslV-HslV subunit interfaces when ATP is bound to HslU, otherwise they are buried at the HslU-HslU subunit interfaces (10, 12, 22). Moreover, the deletion of the C-terminal 7 amino acids of HslU has been shown to abrogate the ability of HslU to interact with HslV (24). Therefore, it has been suggested that the ATP-dependent movement of the C-terminal tails contributes to the interaction between HslU and HslV and thus to the HslVU complex formation. Then, the question is how mHslV and T1Δ can interact with HslU to form the HslVU complex in the absence of ATP. In order to clarify this issue, we examined whether the C-terminal tails are required for the interaction of HslU with mHslV or T1Δ. An HslU mutant (termed CΔ7) that lacks the C-terminal 7 amino acids was incubated with mHslV-His or T1Δ-His in the presence of ATP, and then subjected to NTA pull-down analysis. Like HslV, mHslV and T1Δ were unable to interact with CΔ7 (Fig. 4*A*). Consistently, none of HslV, mHslV, or T1Δ could stimulate the basal ATPase activity of CΔ7 (Fig. 4*B*). These results indicate that the C-terminal tails of HslU are required for the interaction of HslU with mHslV or T1Δ as with HslV and thus for the stimulation of its ATPase activity by the HslV proteins.

A synthetic HslU tail peptide of 10 amino acids (termed C10-peptide) can replace HslU in supporting the hydrolysis of Z-GGL-AMC by HslV, suggesting that C10-peptide binds to the HslV-HslV subunit interface in a similar way where the C-terminal tails of HslU bind to HslV (23, 24). To confirm whether the C-terminal tails of HslU are required for the interaction of mHslV or T1Δ with HslU in the absence of ATP, HslU with either mHslV-His or T1Δ-His were incubated with C10-peptide. After incubation, the samples were subjected to NTA pull-down analysis. The interaction of HslU with mHslV or T1Δ was gradually decreased by increasing the concentration of C10-peptide, but not by that of a control peptide with scrambled sequence (Fig. 4*C*). These results support that the C-terminal tails of HslU are required for the interaction of HslU with mHslV or T1Δ. However, the dissociation of the HslVU complex by C10-peptide was not complete even at the highest concentration of the peptide tested. This observation could be due to lower affinity of C-10 peptide to HslV compared to that of HslU, considering that, unlike C10-peptide that individually interacts with HslV, hexameric HslU may interact with HslV in a cooperative manner where multiple C-terminal tails simultaneously make contact with HslV. Alternatively, secondary interacting sites between HslU and HslV may contribute additional stability in binding, which would prevent complete dissociation of the HslVU complex even at high concentrations of C10-peptide.

Effect of Increasing Number of T1Δ Subunit in an HslV Dodecamer on the Interaction between HslV and HslU — Of surprise was the finding that deletion of the Thr1 residue dramatically increased the interaction between HslV and HslU as the binding of MG132 to the active sites did. To clarify this finding further, we attempted to generate mixed dodecamers consisting of increasing number of T1Δ subunit in place of HslV subunit as shown in Fig. 5*A*. Since the efficiency of translation initiation in bacteria depends on the start codon of a gene (in the order of ATG>GTG>TTG in *E. coli*) (32, 33), a series of vectors that contain multiple *hslV* genes with different combinations of start codons were constructed for the control of relative expression level of each subunit (Fig. 5*B*). In addition, we expressed T1Δ-His with HslV in *ΔhslVU::kan* cells to facilitate the purification of mixed dodecamers and to determine the molar ratio of their subunits in each mixed dodecamer based on the difference in the subunit size due to the His tag. Six different mixed dodecamers containing increasing number (*i.e.*, from about 2 to 10) of T1Δ subunit were purified and termed a-f (Fig. 5*C*). These results indicate that HslV and T1Δ subunits were co-assembled in a dodecamer *in vivo* in a random manner without preference. In addition, N-terminal sequence analysis by Edman

degradation of HslV and T1Δ proteins in the gels (i.e., HslV from the lanes c, d and f and T1Δ from the lane d) reveal that their N-terminal Met residues were completely processed and that the proteins have the same anticipated N-terminal sequences whether GTG or TTG was used as the starting codon (i.e., TTIVS for HslV and TIVS for T1Δ).

We then examined the effect of increasing number of T1Δ subunit in an HslV dodecamer on the interaction between HslV and HslU. Each of purified mixed dodecamers containing increasing number of T1Δ-His subunit was subjected to incubation with HslU followed by NTA pull-down analysis. The amounts of HslU co-precipitated with mixed dodecamers gradually increased upon increasing the number of T1Δ subunit in a dodecamer (Fig. 6*A*). Consistently, the increase in the number of T1Δ subunit in a dodecamer led to a gradual increase in the ATPase activity of HslU (Fig. 6*B*). In addition, the sinusoidal shape of the curve indicates that there is positive cooperativity among T1Δ subunits with regard to stimulation of the ATPase activity of HslU. Under the same conditions, we also assayed peptide hydrolysis by incubation with Z-GGL-AMC in the presence of ATP. Significantly, the peptidase activity rather increased upon increasing the number of T1Δ subunit up to 5-6 in a dodecamer and then gradually fell down upon further increase in that of T1Δ subunit (Fig. 6*C*). We have previously shown that ATPγS, a non-hydrolysable ATP analog, increases the interaction between HslV and HslU, and thereby supports the peptide hydrolysis by the HslVU complex much better than ATP (18). Thus, it appears likely that the increased peptidase activity of mixed dodecamers containing up to 5-6 T1Δ subunits is due to the T1Δ-mediated increase in the interaction between HslV and HslU, despite the fact that T1Δ subunits in the dodecamers are catalytically inactive. However, further increase in the number of catalytically inactive T1Δ subunit led to a gradual decrease in the peptide hydrolysis, due to the limitation of the Thr1 active sites.

DISCUSSION

The present study demonstrates that the N-terminal Thr active sites of HslV are involved in the communication between HslV and HslU. Binding of proteasome inhibitors to the Thr1 active sites in the presence of ATP were found to markedly increase the interaction between HslV and HslU. Moreover, this inhibitor-mediated tight binding led to a dramatic increase in HslV-stimulated ATPase activity of HslU. Considering that the binding of inhibitors (particularly of peptide inhibitors) to the Thr1 active sites likely mimics the protein substrate-bound state of HslV, these findings suggest that allosteric mechanism can also work in a reverse manner: *i.e.*, substrate-bound HslV can allosterically regulate the function of HslU. Likewise, it has recently been shown that binding of proteasome inhibitors to the proteolytic active sites of the 20S proteasome core particle (CP) increases the interaction of CP with the 19S regulatory particle (RP) (34). A similar phenomenon has also been reported in another bacterial ATP-dependent Clp protease where DFP-bound ClpP has an increased affinity toward ClpX and ClpA (35). Thus, the allosteric mechanism by which the functions of ATPase and protease components are mutually regulated appears universal among two-component ATP-dependent proteases despite their different architectures.

Noteworthy was the finding that MG132-bound HslV (mHslV) could tightly interact with HslU even in the absence of ATP, under which condition HslU hexamers tend to dissociate into monomers. Moreover, structural studies have shown that the C-terminal tails of HslU are inserted at the HslV-HslV subunit interfaces when ATP is bound, otherwise they are buried at the HslU-HslU subunit interfaces (10, 12, 22). Therefore, we initially suspected if mHslV might be able to bind to HslU without the need of ATP-dependent movement of the C-terminal tails. However, the interaction of the HslU C-terminal tails with mHslV was essential for the interaction of mHslV with HslU, as evidenced by the observations that C10-peptide competes with HslU for the interaction with mHslV and that neither HslV nor mHslV can interact with HslU lacking the C-terminal 7 amino acids (CΔ7). Thus, it appears that conformational transmission from MG132-bound Thr1 active sites to HslU is strong enough for the movement of the HslU C-terminal tails to the HslV-HslV subunit interfaces as well as for keeping HslU subunits in a hexamer even under conditions without ATP. These findings again

suggest that the allosteric mechanism for the activation of ATP-dependent HslVU protease operates in a reciprocal fashion (*i.e*., through mutual communication between HslV and HslU) but not in a unidirectional manner.

For a complete proteolytic cycle, HslVU should remain as a stable complex at least for the period of substrate threading from HslU to the inner chamber of HslV, and its subsequent binding to and cleavage at the Thr1 active sites. Since ATP binding to HslU is required for the interaction between HslV and HslU, the nucleotide-dependent movement of the HslU C-terminal tails to the HslV-HslV subunit interfaces may initially participate in keeping HslV and HslU in a stable complex during substrate threading. As the threaded polypeptide reaches the inner chamber of HslV, its binding to the Thr1 active site(s) induces a further increase in the affinity between HslV and HslU for the cleavage of peptide bonds and thus for the completion of a proteolytic cycle. Recently, Kleijnen *et al.* (34) proposed a similar model for eukaryotic proteasomes, based on the findings that proteasome inhibitors stabilize association between 20S CP and 19S RP. They also have suggested that allosteric regulation induced by substrate binding at the active sites of the CP could be an effective mechanism for preventing dissociation of the CP-RP complexes during substrate proteolysis, based on their findings that certain inhibitors protect CP-RP complexes from disassembly even under ATP-depleted conditions. Moreover, Rabl *et al*. (36) have recently shown that archeal proteasome ATPase PAN and some of eukaryotic 19S RP subunits contain a common C-terminal tail motif and their tails play an important role in gate opening at the α rings of the CP and in PAN-CP or RP-CP complex association. Therefore, it is conceivable that the C-terminal tails of PAN and 19S RP, like those of HslU, might mediate allosteric communications between the active sites of the CP and proteasomal ATPases.

In the case of HslVU, the increase in the rate of HslU-mediated ATP hydrolysis by the increased affinity between HslV and HslU may further stimulate substrate unfolding and translocation. ATP hydrolysis by HslU is known to be stimulated by protein substrates (16, 37), thus acting in favor of providing mechanical energy for substrate unfolding. Conversely, the increased ATP hydrolysis also leads to the generation of ADP, which causes the reverse movement of the HslU C-terminal tails back to the HslU-HslU subunit interfaces and thereby weakening the interaction between HslV and HslU. However, our finding that mHslV with HslU can form a stable HslVU complex regardless of the presence of any adenine nucleotide indicates that the increase in the affinity between HslV and HslU by substrate binding to the Thr1 active site can occur independently of nucleotide-bound state of HslU. Collectively, our findings provide a mechanism for the maintenance of the stable HslVU complexes when substrates are bound to the Thr1 active sites for the completion of a proteolytic cycle.

Of particular interest was the finding that deletion of the Thr1 residue of HslV also causes a dramatic increase in the affinity between HslV and HslU and thereby a marked enhancement in the HslV-stimulated ATP hydrolysis by HslU. Furthermore, T1Δ could form a stable complex with HslU even in the absence of ATP, and yet this complex formation required the C-terminal tails of HslU. Thus, it appears that elimination of the Thr1 residue from HslV and blockade of the active sites by MG132 or other proteasome inhibitors cause the same allosteric effects on the interaction between HslV and HslU. However, the molecular mechanism how deletion of the Thr1 residue influences the interaction between HslV and HslU remains totally unclear, since none of currently available structural information on HslV has revealed a significant movement of the Thr1 residue upon the interaction with HslU (22). Although the structure of MG132-bound HslV is not available, X-ray structural analysis has shown that the covalent binding of NLVS to the Thr1 residue is accompanied by conformational movement of the upper segment (Ala47 to Thr51) in the substrate binding cleft (22). Thus, binding of proteasome inhibitors including MG132 to the substrate pockets may transmit a conformational change through this loop to the interface of the HslU docking sides, leading to an increased affinity toward HslU. It is unlikely, however, that the Thr1 deletion induces the same conformational movement of the substrate-binding cleft, which is accompanied with peptide inhibitors, because the Thr1 residue and the upper segment of the substrate-binding cleft neither are close enough to

interact directly nor seem to be conformationally linked. Nevertheless, our findings with T1Δ suggest that there might be other conformational states of the Thr1 residue (for example, a distorted conformation of the Thr1 residue upon covalent linkage to substrates), which largely affects the interaction between HslV and HslU, thus providing an additional allosteric mechanism for controlling the mutual activation of HslV and HslU besides substrate binding to the active sites.

Noteworthy was the finding that the peptidase activity of HslVU rather increased upon increasing the number of T1Δ subunit up to 5-6 in an HslV dodecamer, although it gradually fell down upon further increase in that of T1Δ subunit. These results suggest an intriguing relationship between the number of Thr1 active sites and the overall peptidolytic activity of HslV. That is, up to 5-6 Thr1 active sites in an HslV dodecamer might be sufficient for its proteolytic function. This notion is an interesting coincidence with the eukaryotic 20S CP, since only 6 N-terminal Thr residues are catalytically active among 14 β-subunits of 20S CP. It is possible that during evolutionary process 8 β-subunits became catalytically inactive while gaining other regulatory or accessory functions, perhaps because the remaining active sites are sufficient for fulfilling the necessary proteolytic function of 20S CP.

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FOOTNOTES

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The abbreviations used are: MG132, carbobenzoxy-leucyl-leucyl-leucinal; NLVS, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone; Z-GGL-AMC, carbobenzoxy-Gly-Gly-Leu-7-amido-4-methyl coumarin

LEGENDS TO FIGURES

FIGURE 1. **Effects of proteasome inhibitors on the interaction between HslV and HslU.** ***A,*** peptide hydrolysis was assayed by incubation of HslU with 0.1 mM Z-GGL-AMC in the presence of HslV (●) or HslV-His (○) at 37°C (*left panel*). ATP hydrolysis was also assayed by incubation of HslU with 2 mM ATP in the absence (▲) or presence of HslV (●) or HslV-His (○) at 37°C (*right panel*). ***B,*** HslU (150 nM) and His-HslV (75 nM) were incubated with 20 μM proteasome inhibitors and 5 mM $MgCl_2$ at 4°C for 1 h in the absence (–) or presence of 2 mM ATP (*T*) or ADP (*D*) (*upper panel*). HslV alone was also incubated with proteasome inhibitors at 4°C for 1 h, followed by gel filtration on a spin-column filled with Sephadex G-25. HslV proteins eluted from the columns were incubated with HslU and 5 mM $MgCl_2$ at 4°C for 1 h in the absence (–) or presence of 2 mM ATP (*T*) or ADP (*D*) (*lower panel*). The samples were then subjected to NTA pull-down analysis. ***C,*** HslU and HslV were incubated with 20 μM proteasome inhibitors, 2 mM ATP, and 5 mM $MgCl_2$ at 4°C for 1 h (*upper panel*). HslV alone was also incubated with the inhibitors at 4°C for 1 h, followed by gel filtration as in ***B***. HslV proteins eluted from the columns were incubated with HslU, 2 mM ATP, and 5 mM $MgCl_2$ at 4°C for 1 h (*lower panel*). The

samples were then subjected to assay for peptide hydrolysis by incubation with 0.1 mM Z-GGL-AMC at 37°C for 15 min. The peptidase activities seen with DMSO (*i.e.*, in the absence of proteasome inhibitors) were expressed as 1.0 and the others were as their relative values. ***D,*** HslU (450 nM) and HslV-His (225 nM) were incubated with 2 mM ATP, 5 mM $MgCl_2$, and 0.2% DMSO at 4°C for 1 h (*left panel*). HslU (150 nM) and HslV-His (75 nM) were incubated as above but in the presence of 20 μM proteasome inhibitors (*right panel*). The samples were added with 20 μl of NTA resins and further incubated at 4°C for 1 h. Precipitates were washed extensively buffers containing ATP (*T*), ADP (*D*), or none of the nucleotides (–). ***E,*** ATP hydrolysis was assayed by incubation of HslU and HslV in the presence of increasing concentrations of MG132 (●), lactacystin (○), or NLVS (▲) at 37°C for 15 min (*left panel*). HslV alone was subjected to incubation with increasing concentrations of proteasome inhibitors followed by gel filtration as in ***B***. Eluted HslV proteins were then assayed for ATP hydrolysis by incubation with HslU, 2 mM ATP, and 5 mM $MgCl_2$ at 37°C for 15 min (*right panel*). The ATPase activities seen with DMSO were expressed as 1.0 and the others were as their relative values. The data are representatives or the averages of three independent experiments, and the standard deviations in ***A, C,*** and ***E*** are shown as error bars.

FIGURE 2. **Effect of the deletion of Thr1 on the interaction between HslV and HslU.** ***A,*** HslV-His (*left panel*) or T1Δ-His (*right panel*) was co-expressed with HslU in BW21135 *ΔhslVU::kan* cells. Cell lysates (*lanes L*) were prepared and mixed with NTA resins. After collecting unbound proteins (*lanes U*), NTA resins was washed with 50 mM imidazole. Bound proteins (*lanes B*) were then eluted with 450 mM imidazole. The same aliquots of the fractions were subjected to SDS-PAGE followed by staining with Coomassie blue R-250. ***B,*** purified HslV-His or T1Δ-His was incubated with HslU and 5 mM $MgCl_2$ at 4°C for 1 h in the absence (–) or presence of 2 mM ATP (*T*) or ADP (*D*). The samples were then subjected to NTA pull-down analysis. ***C,*** ATP hydrolysis was assayed by incubation of HslU with HslV (○), HslV-His (●), T1Δ (△), or T1Δ-His (▲) for increasing periods at 37°C. The data are representatives of three independent experiments, and the standard deviations in ***C*** are shown as error bars.

FIGURE 3. **Effects of MG132 and Thr1 deletion on HslVU complex formation.** ***A,*** HslV (0.3 mg) was incubated in the absence or presence of MG132 (20 μM) at 4°C for 1 h. After incubation, the sample was extensively dialyzed against 20 mM Tris-HCl buffer (pH 7.8) containing 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 10% glycerol. The resulting HslV and mHslV proteins (0.2 mg) were then incubated with HslU (0.2 mg) in the absence or presence of 2 mM ATP and 5 mM $MgCl_2$ at 4°C for 1 h. HslV, mHslV, and HslU by themselves were also incubated as above. After incubation, each sample was subjected to gel filtration on a Superose-6 column (1 x 30 cm) equilibrated with the dialysis buffer that contains both 5 mM $MgCl_2$ and 2 mM ATP (*left panels*) or $MgCl_2$ only (*right panels*). Fractions of 0.5 ml were collected, and 50 μl of them were subjected to SDS-PAGE followed by staining with Coomassie blue R-250. ***B,*** HslV or T1Δ (0.2 mg) was incubated with HslU (0.2 mg) in the absence or presence of 2 mM ATP and 5 mM $MgCl_2$ at 4°C for 1 h. The samples were then treated as above. The size markers used were thyroglobulin (*T*, 669 kDa), apoferritin (*F*, 443 kDa), catalase (*C*, 232 kDa), and bovine albumin (*A*, 66 kDa). The data are representatives of two independent experiments.

FIGURE 4. **Requirements of HslU C-terminal tails in HslVU complex formation.** ***A,*** HslV-His was incubated 4°C for 1 h in the absence or presence of 20 μM MG132. T1Δ-His was also incubated as above but without MG132. The samples were further incubated by themselves (–) or with HslU or CΔ7 in the presence of ATP and $MgCl_2$ at 4°C for the next 1 h. They were then subjected to NTA pull-down analysis. ***B,*** the samples prepared as above were also assayed for ATP hydrolysis. The ATPase activity seen with HslU or C7 alone was expressed as 1.0 and the others were as its relative values. ***C,*** HslV-His incubated with MG132 was subjected to gel filtration on Sephadex G25-filled spin-column. Eluted mHslV-His protein and T1Δ-His were incubated with HslU in the presence of increasing amounts of C10-peptide or a

nonspecific decapeptide (*scrambled*) at 4°C for 1 h. The samples were then subjected to NTA pull-down analysis. The data are representatives or the averages of three independent experiments, and the standard deviations in ***B*** are shown as error bars.

FIGURE 5. **Generation of mixed dodecamers consisting of varied numbers of HslV and T1Δ subunits.** ***A,*** schematic diagram shows mixed dodecamers consisting of varied numbers of HslV (*H*) and T1Δ subunits (*Δ*). ***B,*** each of the *hslV* gene segments contains its original promoter and Shine-Dalgano (*SD*) sequence. Underlines indicate the nucleotides mutated from the original sequences to generate different start codons. T1Δ was generated by deletion of AAC, the codon for Thr1. All combinations used for the generation of mixed dodecamers are listed in Table 1. ***C,*** each (2 μg) of purified mixed dodecamers was subjected to SDS-PAGE in 12% gels. The intensity of each band was scanned by using a densitometer for the estimation of the number of HslV (indicated by the numerals below the gel panel) in each dodecameric complex (*lanes a-f*). The *lane M* indicates size markers.

FIGURE 6. **Effect of increasing number of T1Δ subunit in a HslV dodecamer on the interaction between HslV and HslU.** HslV-His, T1Δ-His, and each of mixed dodecamers were incubated with HslU and 2 mM ATP at 4°C for 1 h. The incubation mixtures were then subjected to NTA pull-down analysis (***A***). Aliquots of the same mixtures were assayed for the hydrolysis by ATP (***B***) and Z-GGL-AMC (***C***) by further incubation at 37°C for 15 min. The data in ***B*** and ***C*** are the averages of three independent experiments, and the standard deviations are shown as error bars.

Table 1. Vector constructs used for in vivo generation of HslV mixed dodecamers

Constructs	Gene 1	Gene 2	Gene 3
a	*hslV*[a]	*hslV*[a]	*t1Δ*
b	*hslV*[a]	*t1Δ*	none
c	*hslV*[a]	*t1Δ*	none
d	*t1Δ*	*hslV*	none
e	*t1Δ*	*t1Δ*	*hslV*
f	*t1Δ*	*t1Δ*	*hslV*[b]

The start codons used in the constructs were GTG in *hslV*, ATG in *hslV*[a], TTG in *hslV*[b], and GTG in *t1Δ* (see Fig. 5*B*).

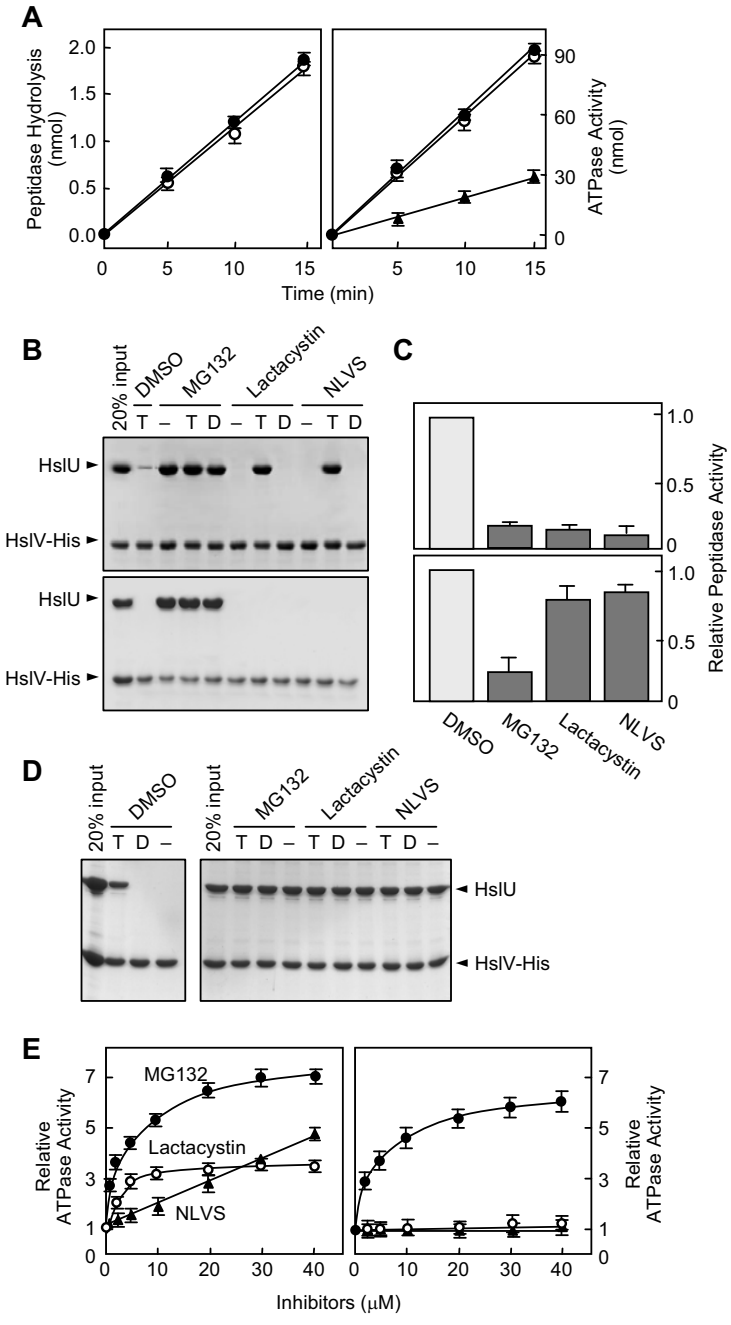

Fig. 1 (Park et al.)

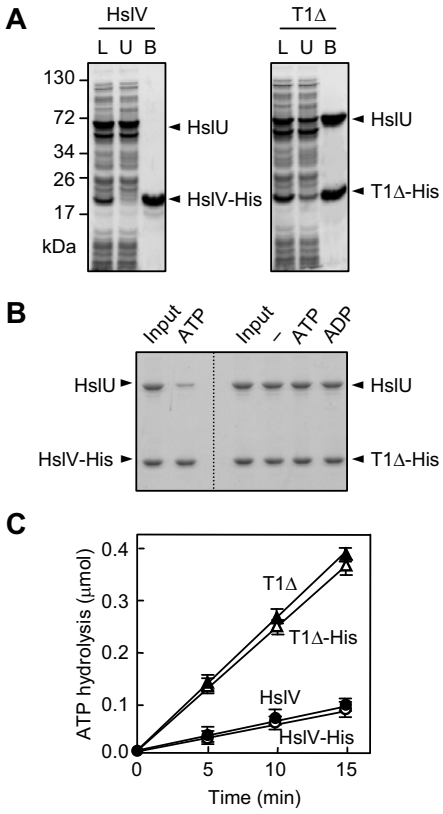

Fig. 2 (Park et al.)

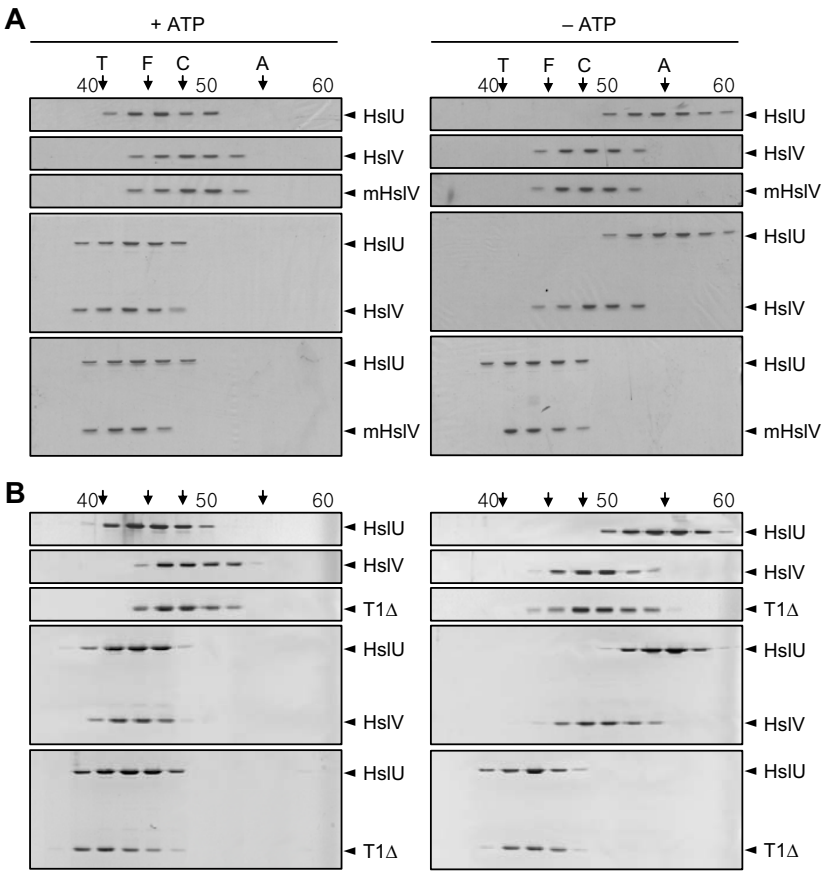

Fig. 3 (Park et al.)

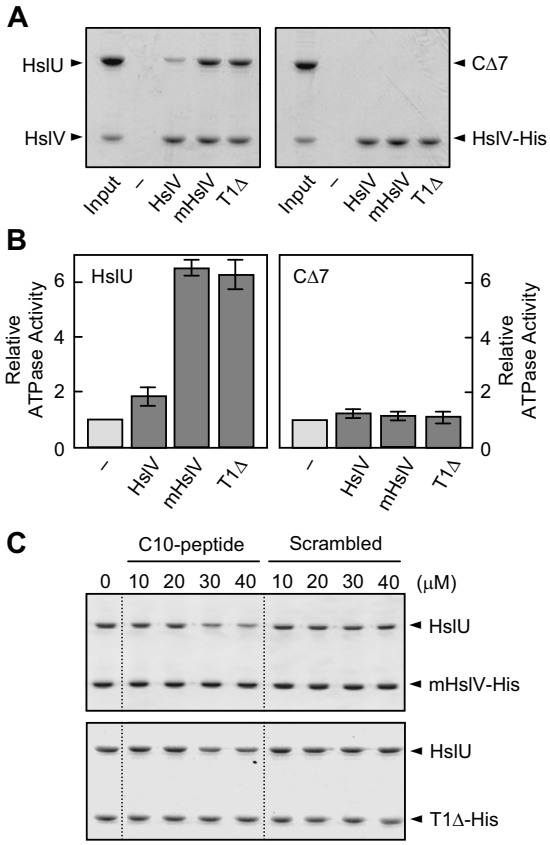

Fig. 4 (Park et al.)

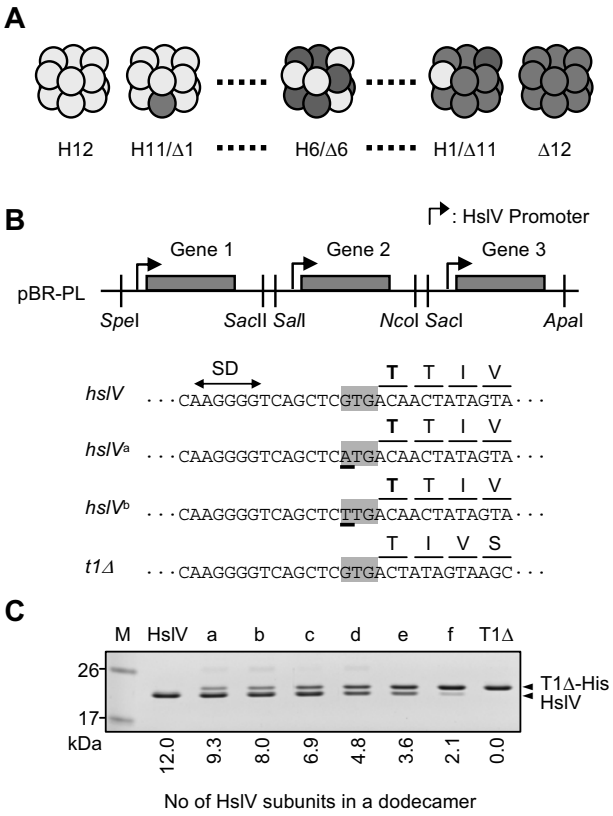

Fig. 5 (Park et al.)

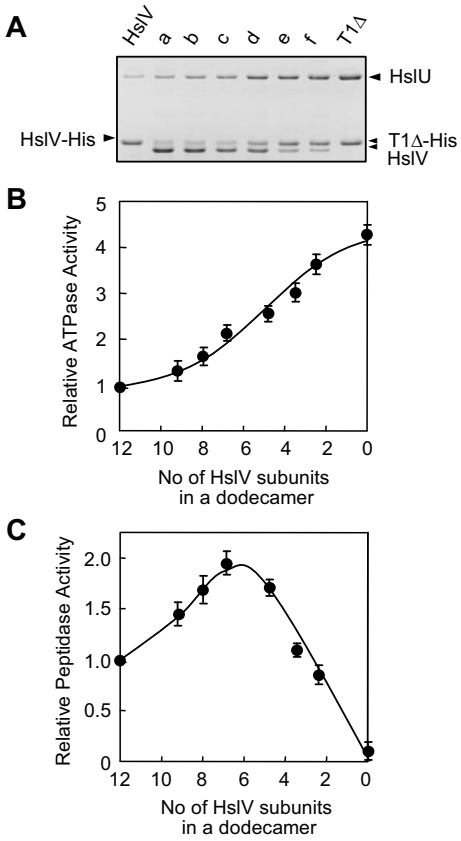

Fig. 6 (Park et al.)