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Structural Alteration in the Pore Motif of the Bacterial 20S Proteasome Homolog HslV Leads to Uncontrolled Protein Degradation

Eunyong Park[1]†, Jung Wook Lee[1]†, Hee Min Yoo[1]†, Byung Hak Ha[1], Jun Yop An[2], Young Joo Jeon[1], Jae Hong Seol[1], Soo Hyun Eom[2] and Chin Ha Chung[1]

*1 - **School of Biological Sciences,** Seoul National University, Seoul 151-742, South Korea*
*2 - **School of Life Sciences,** Steitz Center for Structural Biology, Gwangju Institute of Science and Technology, Gwangju 500-712, South Korea*

***Correspondence to Soo Hyun Eom and Chin Ha Chung:** eom@gist.ac.kr; chchung@snu.ac.kr*
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Abstract

In all cells, ATP-dependent proteases play central roles in the controlled degradation of short-lived regulatory or misfolded proteins. A hallmark of these enzymes is that proteolytic active sites are sequestered within a compartmentalized space, which is accessible to substrates only when they are fed into the cavity by protein-unfolding ATPases. HslVU is a prototype of such enzymes, consisting of the hexameric HslU ATPase and the dodecameric HslV protease. HslV forms a barrel-shaped proteolytic chamber with two constricted axial pores. Here, we report that structural alterations of HslV's pore motif dramatically affect the proteolytic activities of both HslV and HslVU complexes. Mutations of a conserved pore residue in HslV (Leu88 to Ala, Gly, or Ser) led to a tighter binding between HslV and HslU and a dramatic stimulation of both the proteolytic and ATPase activities. Furthermore, the HslV mutants alone showed a marked increase of basal hydrolytic activities toward small peptides and unstructured proteins. A synthetic peptide of the HslU C-terminal tail further stimulated the proteolytic activities of these mutants, even allowing degradation of certain folded proteins in the absence of HslU. Moreover, expression of the L88A mutant in *Escherichia coli* inhibited cell growth, suggesting that HslV pore mutations dysregulate the protease through relaxing the pore constriction, which normally prevents essential cellular proteins from random degradation. Consistent with these observations, an X-ray crystal structure shows that the pore loop of L88A-HslV is largely disordered. Collectively, these results suggest that substrate degradation by HslV is controlled by gating of its pores.

Introduction

ATP-dependent proteases, including the eukaryotic 26S proteasome, play essential roles in the degradation of the majority of cellular proteins, including regulatory proteins and misfolded or damaged proteins.[1–3] By using energy from ATP hydrolysis, these enzymes unfold and translocate substrate proteins into an enclosed proteolytic chamber for degradation. Their proteolytic active sites are spatially sequestered from the cytosol, and this feature is potentially important for preventing uncontrolled access of cytosolic proteins to the active sites.

Among such ATP-dependent proteases, HslVU is a bacterial two-component protease complex, which consists of the HslV peptidase and the HslU ATPase. HslV, a homolog of the β-subunit of eukaryotic 20S proteasomes,[4–6] forms a hollow, barrel-shaped dodecameric complex with subunits arranged into two stacked hexameric rings.[7] Each HslV subunit has an N-terminal threonine (Thr1 or T1) proteolytic active site residing on the inner surface of the HslV chamber.[7–11] Based on its crystal structures, the two axial openings on the apical surfaces of the HslV complex seem to serve as the only gates for substrate proteins to access the

proteolytic active sites, and their narrowness (~13 Å) probably precludes folded proteins from entering the HslV chamber.[7,10–12] The HslU ATPase, a hexameric AAA+ (*A*TPases *a*ssociated with diverse cellular *a*ctivities) protein, is required to unfold substrate proteins and subsequently thread them into the HslV complex. Based on studies of HslU[13,14] and other similar AAA+ enzymes,[15–17] it appears that HslU unfolds proteins by mechanically pulling substrate polypeptides through the central pore of its hexameric ring using energy from ATP hydrolysis. HslU binds to either one or both ends of an HslV dodecamer to form an HslVU complex (i.e., $U_6V_6V_6$ or $U_6V_6V_6U_6$; U and V denote HslU and HslV protomers, respectively).[11,12,18] In the HslVU complex, the pores of HslU hexamer and HslV dodecamer are aligned axially so that a substrate protein unfolded by HslU can be immediately channeled into the interior of the HslV complex through the apical openings.[11,12]

The operation of the HslVU protease is highly regulated by bidirectional allosteric interactions between HslV and HslU. First, the active sites of HslV seem to switch "on" and "off" through a nucleotide-dependent interaction between HslU and HslV.[11,13,19] HslV alone shows a very weak hydrolytic activity toward small peptide substrates, but its activity dramatically increases when it binds to HslU in the presence of ATP.[20,21] This stimulation of HslV's proteolytic activity has been suggested to be mediated by a conformational change of the proteolytic active-site cleft, which would be allosterically induced upon docking of HslU on the apical surface of HslV.[19] Crystal structures of the HslVU complexes have shown that in the ATP-bound state, HslU extends its C-terminal tails from the HslU–HslU subunit interface to intercalate them into an HslV–HslV inter-subunit crevice on the apical surface.[11,13] The insertion of the C-terminal tails into HslV is likely a major mechanism of the HslU-mediated proteolytic activation, since a synthetic HslU tail peptide alone can dramatically stimulate peptide hydrolysis by HslV.[22,23] Through this mechanism, the Thr1 sites of HslV might be activated only when HslV is engaged with the HslU ATPase. Secondly, the Thr1 active sites of HslV can conversely affect HslU's function. We have previously shown that binding of proteasome inhibitors, such as MG132 and lactacystin, to the Thr1 active sites dramatically increases the interaction between HslU and HslV, concomitant with a marked stimulation of ATP hydrolysis.[24] Given that these inhibitors likely mimic proteolytic substrates, it appears that the active sites of HslV can sense a substrate engagement and propagate such information to HslU. Taken together, these reciprocal allosteric activations between HslU and HslV seem to be important in controlling substrate degradation and perhaps are necessary for achieving optimal proteolytic and ATPase activities of the HslVU complex.

On the other hand, gating of constricted pores of proteolytic chambers has been suggested as a major mechanism by which the activity of eukaryotic and archaeal 20S proteasomes is regulated.[25–27] According to this model, the active sites inside the proteolytic chamber always remain "on", and yet substrates cannot access the active sites because the pores into the chamber are normally closed. The pore opening of 20S proteasomes requires an interaction with a partner ATPase complex (also called regulatory particle), of which the C-terminal tail motifs insert into pockets formed on the apical α-subunit rings of the 20S proteasome.[27] An analogous gating mechanism has also been suggested for the bacterial ATP-dependent protease ClpP complexes (ClpP forms a proteolytic chamber, and ClpX and ClpA are ATPases).[28–31] Deletion or mutation of ClpP's natural pore motif has been reported to increase the degradation rates of certain short peptide substrates and of unstructured proteins.[28,29,31] Furthermore, ATPase-independent gate opening of the ClpP complex induced by the small acyldepsipeptides (ADEPs) leads to a toxic effect on bacterial cells,[32,33] supporting the physiological importance of the gating mechanism for ClpP proteases. Nevertheless, it remains unclear whether such gating mechanisms are general among ATP-dependent proteases. For example, previous studies suggest that HslVU operates more likely by allosteric regulation of the proteolytic active sites than by a gating of the constricted pores.[19,22,23]

In the present study, we provided evidence for pore gating as an important mechanism in regulating HslVU's activities. We showed that mutation of the highly conserved Leu88 residue (number for the *Escherichia coli* protein) of HslV's pore motif to Ala, Gly, or Ser results in dramatic reinforcement of the interaction between HslV and HslU and a consequent increase of the proteolytic and ATPase activities of the HslVU complex. Furthermore, these HslV mutants themselves exhibited a remarkably elevated hydrolytic activity toward naturally unfolded α-casein as well as a small peptide substrate. Surprisingly, in the presence of the HslU C-terminal tail peptide, the HslV mutants were even capable of degrading certain folded proteins, suggesting unregulated substrate entry through the structurally altered pores. Supporting this idea, a crystal structure of an HslV mutant, in which Leu88 was replaced by Ala (denoted L88A), shows that the pore loops are largely disordered. In addition, the expression of this mutant in *E. coli* cells significantly inhibited cell growth. Collectively, these results indicate that the pore motif of HslV is not only involved in allosteric communication between HslV and HslU but also critical for controlling substrate access to the proteolytic chamber. Together with observations in similar enzymes, such as 20S proteasomes and ClpP, our findings suggest that

gating mechanisms are widely employed among ATP-dependent proteases to protect cellular proteins from undesirable hydrolysis.

Results

Structural and sequence analysis of the HslV pore region

HslV of *E. coli* contains the "Arg^{86}-Met-Leu-Arg^{89}" ("RMLR"; numbering for *E. coli* HslV) sequence in the innermost region of its pore, restricting the pore size (Fig. 1a). Viewed from the side, the pore motifs constitute the upper and lower entrances of the proteolytic chamber of HslV (Fig. 1a, right panel). Crystallographic data have shown that the apical surface of HslV (residues 47–94) is composed of three α-helices (α1, α2, and α3) separated by very short loops, among which the α3 helix contains the RMLR motif (Fig. 1b).[7] The α3 helix is aligned in a way that the side chains of the Arg86 and Arg89 residues are directed inward to the axis, constituting a positively charged pore lining (Fig. 1c). On the other hand, the side chain of Leu88 is retained on the opposite side, between α1 and α2 in the hydrophobic crevice formed by the side chains of Leu56, Leu59, Trp82, and Leu91 residues (Fig. 1b).

The amino acid sequences of the HslV pore motif are highly conserved throughout bacteria. Positions 86 and 89 are predominantly occupied by basic amino acids, Arg or Lys (Fig. 1d). Interestingly, Leu88 is absolutely conserved among 93 species examined (Fig. 1e). Crystal structures of the *Haemophilus influenzae* complex showed that upon binding to HslU, the apical pore of HslV is aligned with the central pore of HslU and undergoes noticeable conformational changes: the α3 helix of HslV is converted into a less-defined loop structure.[11] At the same time, this loop is placed in close proximity (~6 Å) to a short segment between the ATP-binding Walker B (or DExx) motif and the αN8 helix in HslU. This suggests that the RMLR pore motif might be involved both in the interaction between HslV and HslU and in gate opening of the HslV chamber.

Mutations in the pore motif of HslV alter peptide cleavage and HslU interaction

In order to examine the potential involvement of the RMLR pore motif in HslVU's function, we first introduced single-alanine substitutions into *E. coli*

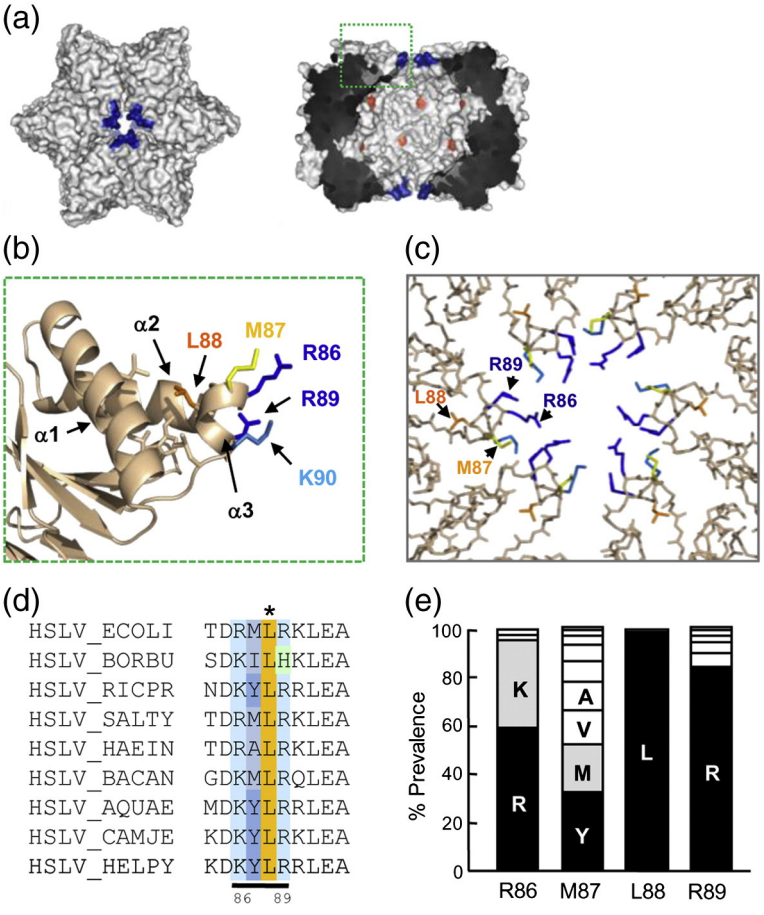

Fig. 1. Structure and sequence alignment of the HslV pore region. (a) Top and side views of the pore region of *E. coli* HslV are shown (PDB ID: 1E94).[34] The RMLR pore motif of HslV is marked in blue. The Thr1 active sites in the inner chamber of HslV dodecamer are marked in orange. The detailed structure of the region in the green dotted box is shown in (b). (b) The structure of the RMLR pore motif is presented in a ribbon diagram. The side chains of the RMLR motif (in blue, yellow, and orange) and hydrophobic amino acids near the L88 residue are displayed as sticks. (c) A close-up view of the HslV pore region from the top is shown in stick presentation. The color scheme is the same as in (b). (d) The amino acid sequences of HslV pore regions from different species are aligned. The underline indicates the RMLR pore motif, in which the conserved Leu88 residue was marked with an asterisk. (e) The prevalence of each residue in the RMLR pore motif was expressed as a percentage of the total 93 references.

HslV by mutating the conserved Arg86, Leu88, and Arg89 residues. We also made a mutant (denoted "A4") in which the entire RMLR motif is replaced by four alanines. The HslV mutants attached with a His-tag at their C-terminus were expressed in *E. coli* cells as stable, soluble proteins and purified to apparent homogeneity. It has previously been shown that the C-terminal His-tag interferes neither with the activity of HslV nor with its ability to interact with HslU ATPase.[24,35] Gel-filtration analysis showed that all purified HslV mutants behave as stable dodecamers in solution, similar to the wild-type (WT) protein (Supplementary Fig. 1).

To determine the effects of the mutations in the pore motif on the hydrolysis of small peptides, we incubated WT-HslV and its mutant forms with the fluorogenic peptide substrate Z-GGL-AMC (carbobenzoxy-Gly-Gly-Leu-7-amido-4-methyl coumarin) in the presence of HslU and ATP and monitored the increase in fluorescence over time. The Arg86-to-Ala (R86A) and Arg89-to-Ala (R89A) mutants showed little peptidolytic activity compared to WT-HslV, suggesting that the Arg residues of the RMLR pore motif are required for normal functions of the HslVU complex (Fig. 2a). Consistent with these results, the A4 mutant was also inactive in peptide hydrolysis. On the other hand, the activity of the Leu88-to-Ala (L88A) mutant was strikingly higher (>7-fold) than WT-HslV. Similar results were obtained when the Leu88 residue was replaced by Gly (L88G) or Ser (L88S). Taken together, these data suggest that the RMLR pore motif is involved in regulating the activity of HslVU.

Next, we tested the ability of the HslV pore motif mutants to stimulate the ATPase activity of HslU. L88A, L88G, and L88S mutants all showed much greater stimulation of ATP hydrolysis by HslU than WT-HslV (i.e., 15- to 17-fold *versus* 2- to 3-fold), whereas R86A, R89A, and A4 did not stimulate at all (Fig. 2b). Given that both the peptidase activity of HslV and the ATPase activity of HslU are largely stimulated by complex formation between HslV and HslU,[20,21,24,35] these data suggest that a mutation on the RMLR pore motif greatly influences the ability of HslV to interact with HslU.

To directly test whether mutations of the HslV pore motif affect the interaction between HslV and HslU, we performed nitrilotriacetic acid (NTA) pull-down assays utilizing the His-tag attached to the C-terminus of HslV. In the presence of ATP, the amount of HslU co-precipitated with the L88A, L88G, or L88S mutant was 4- to 5-fold higher than that with WT-HslV (Fig. 3a), indicating their markedly increased affinity toward HslU. In contrast, little or no HslU was co-precipitated with the R86A, R89A, or A4 mutants, indicating their lack of interaction with HslU. The inability of these mutants to bind to HslU could not be rescued by the non-hydrolyzable ATP analog ATPγS, which allows tighter association between HslU and WT-HslV[36] (Fig. 3b). These results show again that the RMLR pore motif is intimately involved in the interaction between HslV

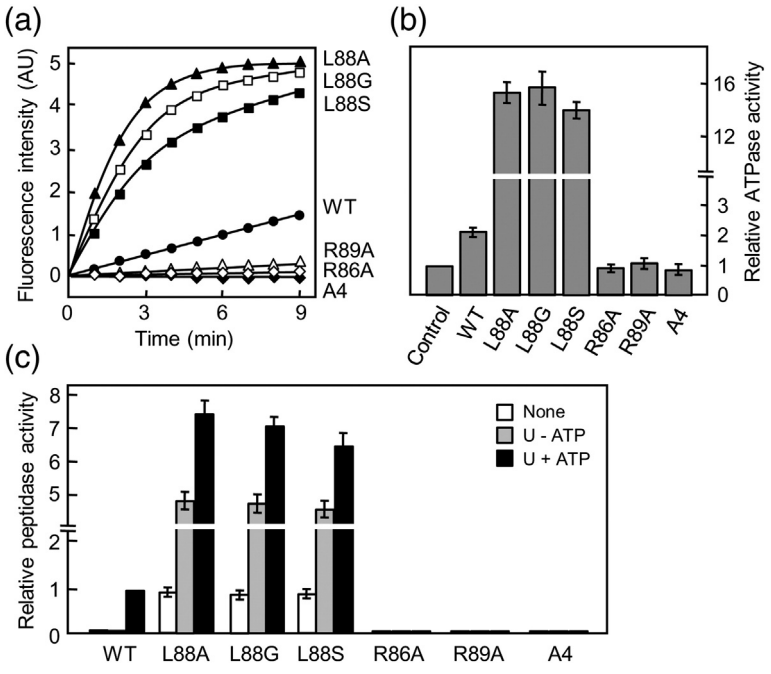

Fig. 2. Effects of mutations in the HslV pore motif on the hydrolysis of peptide and ATP by HslVU. (a) Each (2.5 nM) of the WT-HslV proteins and its mutant forms (L88A, L88G, L88S, R86A, R89A, and A4) was incubated with HslU (5 nM) in the presence of 2 mM ATP and 0.1 mM Z-GGL-AMC at 37 °C for increasing periods. A single representative experiment is shown. Averages of initial rates measured from three independent experiments are shown in (c). (b) ATP hydrolysis was assayed by incubation of HslU with or without HslV (WT) and its mutant forms for 10 min at 37 °C. The ATPase activity of HslU alone (control) was expressed as 1.0 and the others were expressed as its relative values. (c) Each of the HslV proteins was incubated alone or with HslU in the presence or absence of ATP for 2 min. The peptidase activity seen by incubation of HslV with HslU and ATP was expressed as 1.0 and the others were expressed as its relative values. The data are representatives of three independent experiments, and the SD in (b) and (c) is shown as error bars.

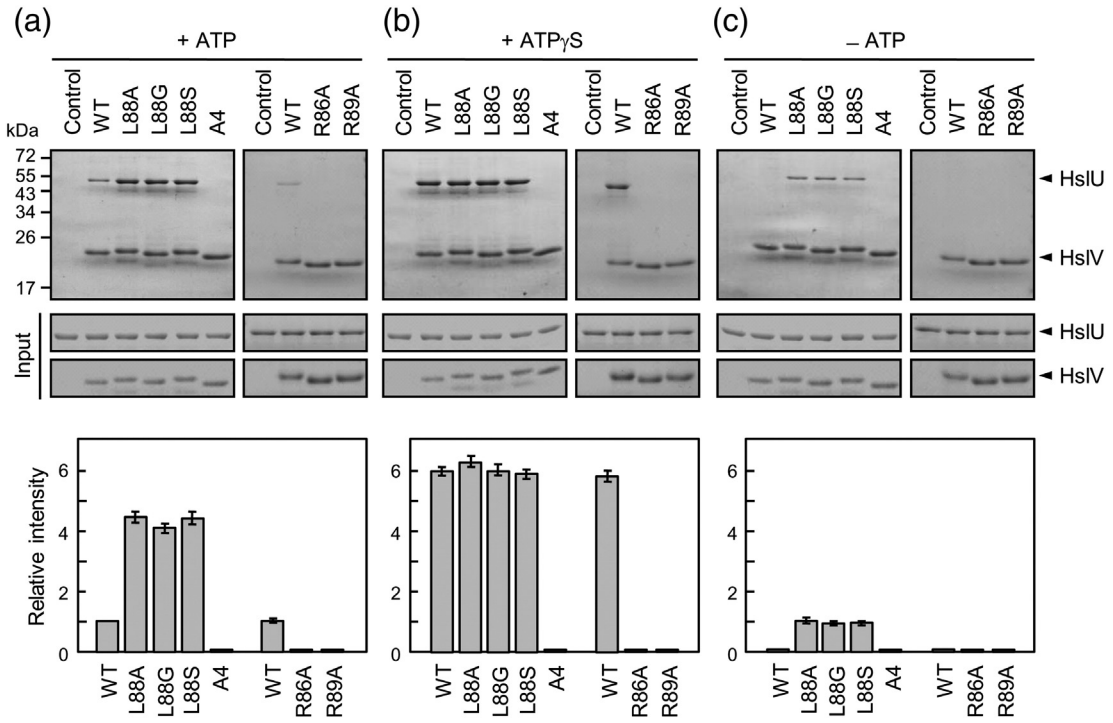

Fig. 3. Effects of mutations in the HslV pore motif on the formation of the HslVU complex. Each (75 nM) of the His-tagged HslV (WT) proteins and its mutant forms was incubated at 4 °C for 20 min with HslU (150 nM) in the presence of 2 mM ATP (a) or 0.5 mM ATPγS (b) or in the absence of any nucleotide (c). The samples were then subjected to NTA pull down. The input materials ("Input") and resin-bound proteins were analyzed by SDS-PAGE followed by Coomassie staining (top panels). The amounts of co-precipitated HslU were quantified by densitometry of Coomassie-stained gels, and their relative values are shown (bottom panels). The values are normalized with respect to the amount of HslU co-precipitated with WT-HslV in the presence of ATP. The data were means from three independent experiments (error bars, SD).

and HslU, likely through direct contact with the loop between the Walker B motif and αN8 helix of an HslU subunit.[11] As a similar case seen in the ClpP–ClpX complex,[37] this interaction, in addition to binding of HslU's C-terminal tails to HslV, may provide an important mechanism for communications between HslV and HslU.

Surprisingly, the L88A, L88G, and L88S mutants, unlike WT-HslV, could co-precipitate HslU even in the absence of ATP, although the amounts of co-precipitated HslU were less than those seen in its presence (Fig. 3c). Therefore, we examined whether HslU might be able to support peptidase activity of the HslV mutants even in the absence of ATP. Indeed, the peptidase activities of the Leu88 mutants were stimulated by HslU in the absence of ATP; the stimulation was 4- to 5-fold higher than that seen with WT-HslV in the presence of both HslU and ATP (Fig. 2c, gray bars).

We noted that the L88A, L88G, and L88S mutants, without addition of any other components, could degrade the peptide substrate with efficiency comparable to that of WT-HslV in the presence of HslU and ATP (Fig. 2c, white bars). Under the same condition, WT-HslV showed negligible peptidase activity, as previously reported.[20,21] These results suggest that Leu88 of HslV is important not only for the interaction between HslV and HslU but also in regulating its own proteolytic activity. This observation could be explained if mutation of Leu88 largely bypasses the requirement of HslU for allosteric activation of the Thr1 active sites. Alternatively, more rapid diffusion of the substrate through the potentially enlarged pores of the Leu88 mutants might contribute to the increased basal peptidolytic activity of the mutants.

Finally, replacement of Leu88 by Phe (L88F) or Trp (L88W) also increased the peptidolytic activity of HslV in both the absence and presence of HslU and stimulated the ATPase activity of HslU more than WT-HslV (Supplementary Fig. 2). However, the extents were substantially less than the other Leu88 mutants. These results suggest that mutation of Leu88 to Phe or Trp partly induces a conformational change in HslV similar to that caused by the L88A, L88G, or L88S mutation, probably due to inability of these amino acids to form a proper interaction with the neighboring α1 and α2 helices.

The C-terminal tails of HslU are required for its interaction with Leu88 HslV mutants

The results from NTA pull-down assays indicate that L88A, L88G, and L88S HslV (simply "Leu88 mutants", hereafter) can stably associate with HslU even in the absence of ATP (Fig. 3c). In the WT complex, the C-terminal tails of HslU move toward the HslV–HslV subunit interfaces when ATP binds HslU; otherwise, they are buried at the HslU–HslU subunit interfaces.[13,38] This ATP-dependent movement of the C-terminal tails seems to be required for the interaction between HslU and HslV, since the deletion of the seven C-terminal amino acids of HslU abrogates its ability to interact with HslV, although the mutant still forms a hexamer as the WT enzyme.[23] Then, the question is how the Leu88 mutants can interact with HslU in the absence of ATP. In an attempt to clarify this issue, we examined whether the C-terminal tails are required for the interaction of HslU with the Leu88 mutants. An HslU mutant that lacks the seven C-terminal amino acids (termed HslU/CΔ7) was incubated with the HslV mutants in the absence and presence of ATP and then subjected to NTA pull-down analysis. None of the Leu88 mutants could interact with HslU/CΔ7 whether ATP was present or not (Fig. 4a). Consistent with this observation, HslU/CΔ7 could not further increase the basal peptidase activity of the Leu88 mutants (Fig. 4b). These results suggest that the C-terminal tails of HslU are still required for its interaction with the Leu88 mutants and for the stimulation of the peptidase activity. These results also imply that binding of the Leu88 mutants to HslU may induce the movement of the C-terminal tails toward the HslV–HslV subunit interfaces even in the absence of ATP.

A synthetic peptide of 10 amino acids corresponding to the C-terminus of HslU (termed "C10") can replace HslU in supporting the hydrolysis of Z-GGL-AMC by HslV, indicating that C10 binds to the HslV–HslV subunit interface in a similar way in which the C-terminal tails of HslU bind to HslV.[22,23] Thus, we examined whether C10 can also replace HslU in stimulating peptide hydrolysis by the Leu88 mutants. Indeed, C10 markedly increased (7- to 8-fold) the

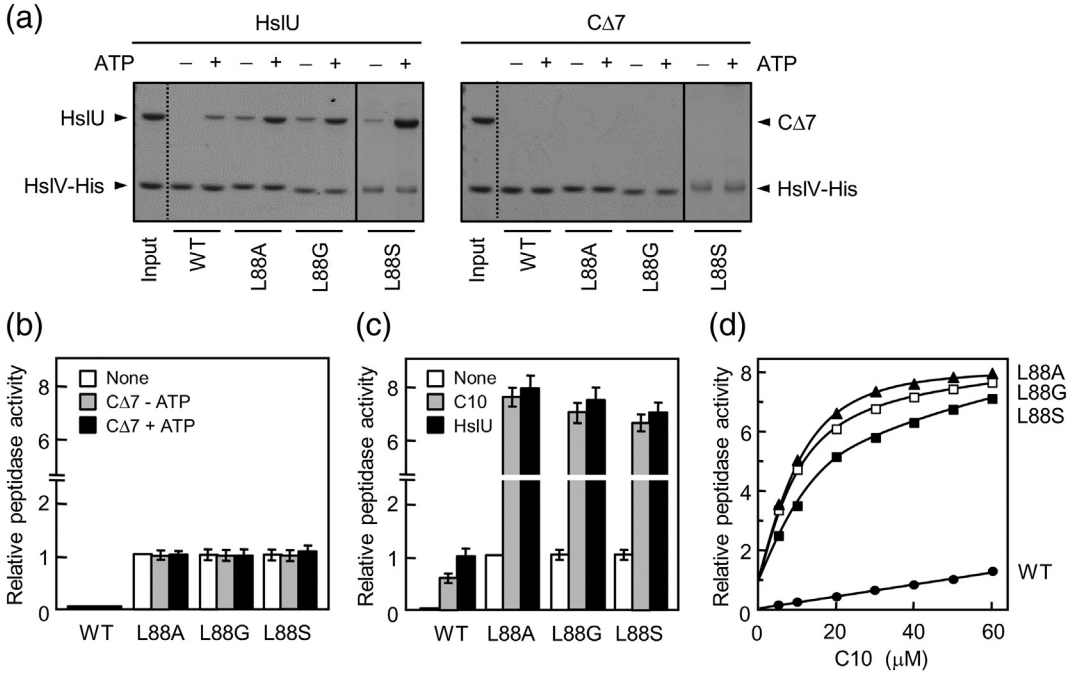

Fig. 4. Requirement of the C-terminal tails of Leu88 mutants for their interaction with HslU. (a) Each (75 nM) of the HslV (WT) proteins and its mutant forms (L88A, L88G, and L88S) was incubated with HslU or HslU/CΔ7 (150 nM) in the absence or presence of 2 mM ATP at 4 °C for 20 min. The mixtures were then subjected to NTA pull-down analysis followed by SDS-PAGE. (b) Each (2.5 nM) of the HslV (WT) proteins and its mutant forms was incubated with HslU/CΔ7 (5 nM) and 0.1 mM Z-GGL-AMC in the absence or presence of 2 mM ATP at 37 °C for 2 min. The peptidase activity seen with L88A alone was expressed as 1.0 and the others were expressed as its relative values. (c) Each (2.5 nM) of the HslV (WT) proteins and its mutant forms was incubated with 0.1 mM Z-GGL-AMC in the absence or presence of 20 μM C10 or HslU (5 nM) and 2 mM ATP. The peptidase activity seen with L88A alone was expressed as 1.0 and the others were expressed as its relative values. The data are representatives of three independent experiments, and the SD in (b) and (c) are shown as error bars. (d) Each (2.5 nM) of the HslV (WT) proteins and its mutant forms was incubated with 0.1 mM Z-GGL-AMC in the presence of increasing concentrations of C10.

basal peptidase activity of the Leu88 mutants (Fig. 4c). The C10-stimutated peptidase activity of the mutants was comparable to that seen in the presence of both HslU and ATP. Under the same conditions, C10 increased the peptidase activity of WT-HslV only to approximately 60% of the level seen in the presence of HslU and ATP. Moreover, the concentrations of C10 required to reach half-maximal peptidase activity of the Leu88 mutants were much lower than those for WT-HslV (Fig. 4d). These results suggest that the Leu88 mutants have a substantially higher affinity for the C-terminal tails of HslU than WT-HslV.

Leu88 mutations of HslV alter its ability to degrade protein substrates

To determine the effect of mutations in the HslV pore motif on protein degradation by the HslVU and HslV complexes, we used two different types of substrates: SulA and Arc as folded protein substrates, and α-casein and Arc/I37A as unstructured or loosely structured protein substrates.[39] SulA (~19 kDa) is a bacterial cell division inhibitor protein,[40–42] and Arc is a small (~6 kDa) protein from the bacteriophage P22.[43] Arc normally folds as a dimer (~12 kDa), but the I37A mutation disrupts the

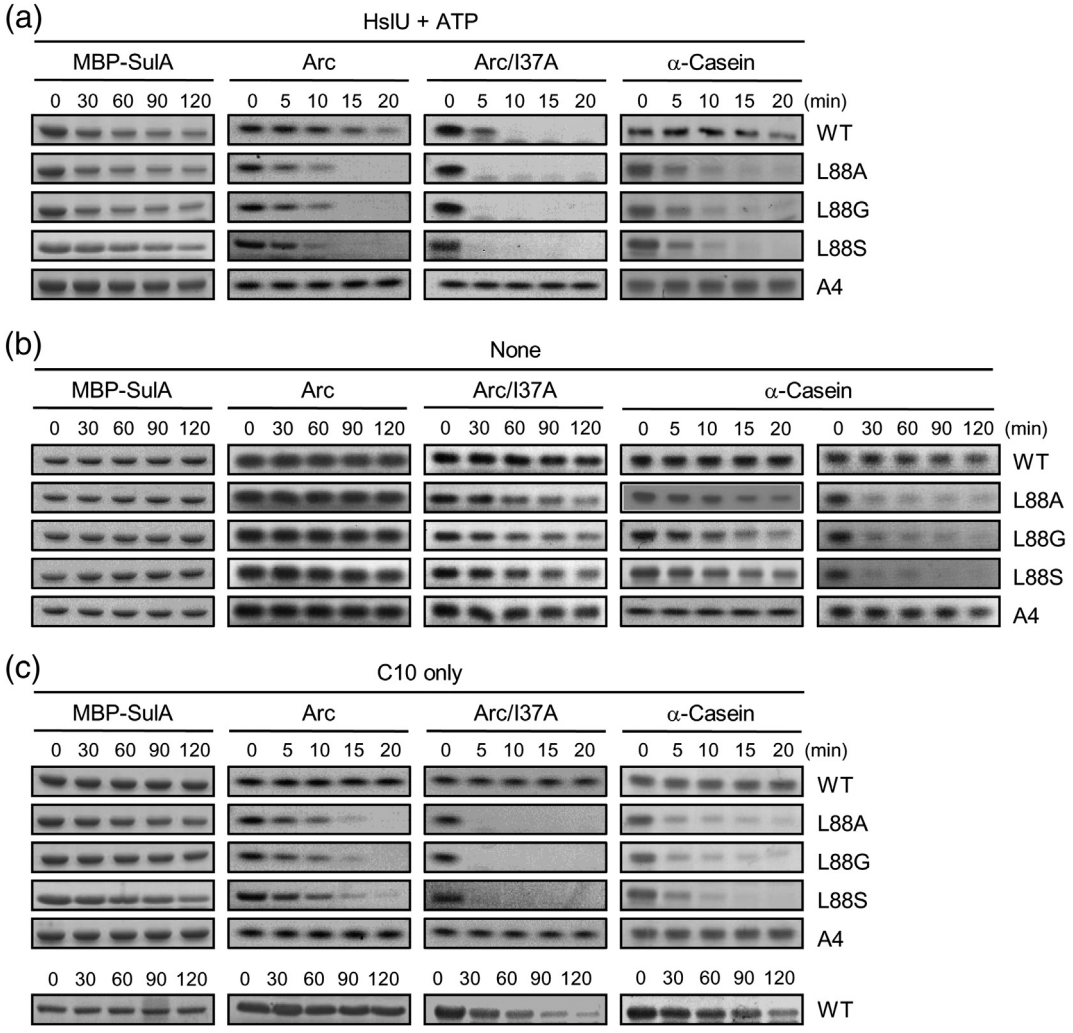

Fig. 5. Effects of mutations in the HslV pore motif on protein degradation. (a) Each (0.4 μM) of the HslV (WT) proteins and its mutant forms (L88A, L88G, L88S, R86A, R89A, and A4) was incubated at 37 °C with MBP-SulA (1 μM), Arc (10 μM), Arc/I37A (10 μM), or α-casein (5 μM) in the presence of HslU (0.4 μM). The mixtures also contained an ATP-regenerating system consisting of 2 mM ATP, 5 mM $MgCl_2$, 20 mM phosphocreatine, and 10 U/ml of creatine kinase. The reaction was stopped by adding SDS sampling buffer after the indicated periods of incubation. The samples were then subjected to SDS-PAGE followed by staining with Coomassie blue R-250. (b) Incubations were performed as in (a), but without any of the following: HslU, ATP-regenerating system, or C10. (c) Incubations were performed as in (a), but in the presence of 20 μM C10 in place of HslU and ATP-regenerating system. See Supplementary Fig. 2 for the quantified data obtained by three independent experiments performed as in this figure.

dimerization and causes it to behave as a molten globule.[44] Both SulA and Arc require HslU for their ATP-dependent unfolding and translocation into the interior of the proteolytic chamber of HslV.[41,42,44] On the other hand, degradation of α-casein or Arc/I37A by HslVU does not strictly depend on ATP hydrolysis as they can be substantially degraded in the presence of ATPγS.[35] They are also slowly degraded over time by WT-HslV alone in the absence of HslU and ATP (see Fig. 5b). In the presence of HslU and ATP, the Leu88 mutants degraded all the protein substrates tested more rapidly than WT-HslV (Fig. 5a and Supplementary Fig. 3a). It is possible that the increased affinity of the Leu88 mutants toward HslU and the ensuing higher rate of ATP hydrolysis by HslU stimulate substrate unfolding and translocation. On the other hand, the A4 mutant could not degrade any of the protein substrates under the same conditions (Fig. 5a and Supplementary Fig. 3a) in agreement with its inability to cleave Z-GGL-AMC and interact with HslU (see Figs. 2 and 3).

We next examined whether the HslV mutants can support protein degradation without HslU (Fig. 5b and Supplementary Fig. 3b; also see Supplementary Fig. 2d for L88F and L88W). To our surprise, L88A, L88G, and L88S by themselves could efficiently hydrolyze α-casein and Arc/I37A, whereas WT-HslV displayed very low activity toward these unstructured proteins. Rapid degradation of α-casein and Arc/I37A in the absence of HslU might be attributable to an increased basal catalytic activity of the Thr1 sites in the Leu88 mutants. However, another possibility would be that an altered structure in the axial pores (e.g., enlarged pores) of these mutants facilitates entry of unfolded polypeptide substrates into the HslV chamber.

To distinguish between these possibilities, we compared degradation rates of the substrates under different conditions. If an enlarged pore size is responsible for increased proteolytic activity of the Leu88 mutants, one may expect that the activity increases more for α-casein and Arc/I37A than for the smaller Z-GGL-AMC substrate. We found that the Z-GGL-AMC substrate was degraded in the presence of C10 by WT-HslV at a rate comparable (~70%) to those seen with Leu88 mutants (L88A, L88G, and L88S) in the absence of C10 (see Fig. 4c). Therefore, the proteolytic activity of HslV toward small peptide substrates appears to be similar under these two conditions. However, we found that under the same condition, the Leu88 mutants without C10 can cleave α-casein and Arc/I37A much faster than WT-HslV with C10 (Fig. 5b *versus* c). These results suggest that an altered pore structure is a major factor causing the efficient hydrolysis of α-casein and Arc/I37A by the Leu88 mutants themselves.

Unlike α-casein and Arc/I37A, MBP-SulA (SulA is fused with a maltose-binding protein tag) and WT Arc could not be degraded by WT-HslV or any of the Leu88 mutants in the absence of HslU (Fig. 5b and Supplementary Fig. 3b). This finding would be expected if the sizes of folded SulA and Arc are larger than the pore size of the Leu88 mutants. Interestingly, however, even native Arc as well as SulA (although a lesser extent than Arc) became susceptible to degradation when C10 was added to the Leu88 mutants, whereas WT-HslV could still not hydrolyze either Arc or SulA under the same conditions (Fig. 5c and Supplementary Fig. 3c). Since substrate entrance into the interior of HslV through its pores is a prerequisite for subsequent degradation, these results suggest that binding of C10 to the Leu88 mutants further expands their pores, making them wide enough to allow the passage of at least a part of these folded proteins into the HslV chamber. C10 might also enlarge the pores of WT-HslV to some extent. Even so, however, the intact RMLR motif appears to maintain the pores narrow enough to block the passage of native Arc or MBP-SulA, since C10-stimulated WT-HslV could not degrade them at all.

Leu88 mutations of HslV inhibit cell growth

During the preparation of HslV proteins, we noticed that cells overexpressing all Leu88 mutants grew substantially slower than those overexpressing WT-HslV. This result raised a possibility that these mutants are toxic to cells. To confirm this observation, we generated expression vectors, in which the coding sequence for WT-HslV or the L88A mutant, either in conjunction with HslU or not, was placed after a weak *lac* promoter in a low-copy plasmid (the plasmid contains the p15A origin of replication derived from pACYC184). *E. coli ΔhslVU* cells transformed with each plasmid were cultured and induced for synthesis of HslV and HslU proteins by addition of IPTG (Fig. 6a). Cells expressing the L88A mutant, regardless of the expression of HslU, grew more slowly than those expressing WT-HslV alone or WT-HslV and HslU together (Fig. 6a). At the same time, filamentation of *E. coli* cells was observed when the L88A mutant was expressed (Fig. 6b), indicating that the production of the L88A mutant causes cellular stress. SDS-PAGE and immunoblotting analysis of cell lysates showed that the expression levels of WT-HslV and of the L88A mutant were nearly identical (Fig. 6c). As expected, a lysate from L88A-HslV-expressing cells hydrolyzed Z-GGL-AMC much faster than a lysate from cells expressing WT-HslV, and a lysate from cells expressing both L88A and HslU cleaved the peptide much more rapidly than a lysate expressing WT-HslV and HslU (Fig. 6d). Since the extent of cell growth inhibition by L88A is not affected by co-expression of HslU, the observed toxicity seems solely due to expression of the HslV mutant.

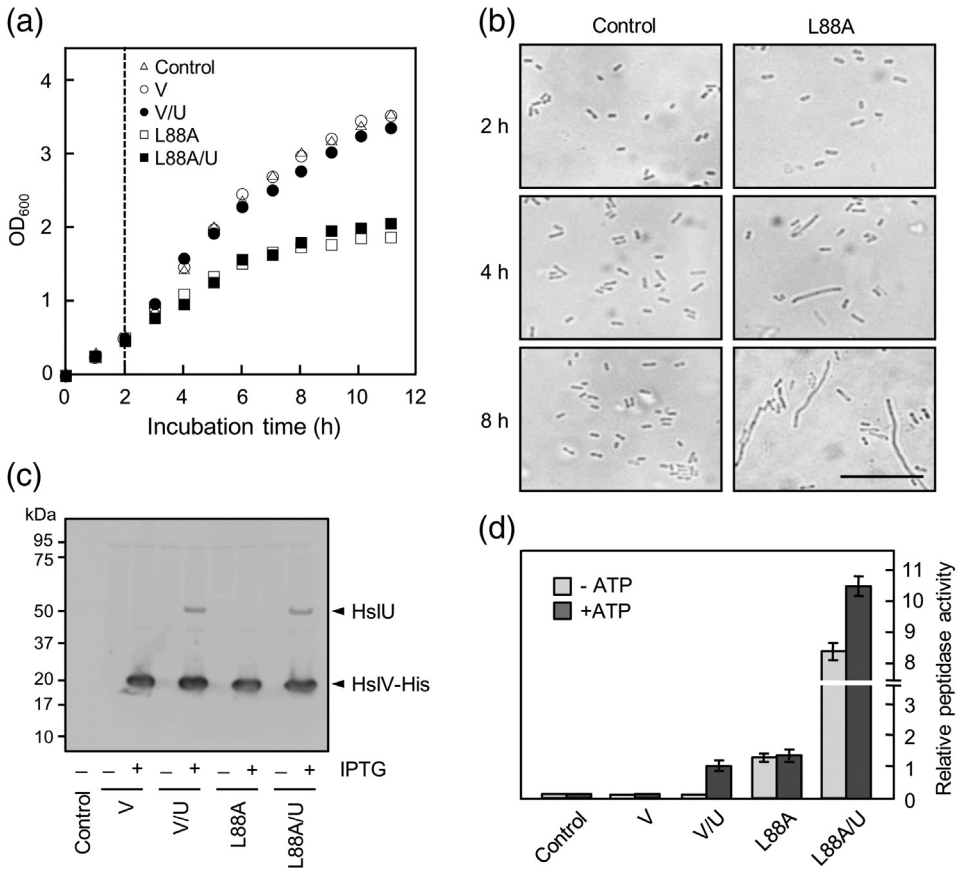

Fig. 6. Effect of Leu-to-Ala mutation in HslV on cell growth. (a) The plasmid encoding HslV or its mutant form (L88A) with or without HslU under a *lac* promoter was introduced into *hslVU*-null *E. coli* cells (BW21135 *ΔhslVU*::*kan*). Control indicates the *E. coli* cells transformed with an empty plasmid. The cells were grown in LB medium and growth was monitored by measuring $OD_{600\ nm}$. Expression of HslU and HslV proteins was induced by addition of 0.1 mM IPTG, when the cultures reached ~0.5 of $OD_{600\ nm}$ (at $t = 2$ h). (b) *E. coli* cells expressing WT-HslV (control) or L88A mutant were cultured as in (a). At each indicated time of culture, aliquots of cells were diluted with Luria broth and applied onto slide glasses covered with 1% agar. They were then observed under a microscope. The scale bar indicates 10 μm. (c) After incubation for 8 h, cell lysates were prepared and subjected to SDS-PAGE followed by immunoblotting with anti-HslV and anti-HslU antibodies. (d) Aliquots (20 μg) of the lysates in (c) were assayed for peptide hydrolysis by incubation with 0.1 mM Z-GGL-AMC for 5 min at 37 °C in the absence or presence of 2 mM ATP.

Structure of the L88A mutant and comparison with other structures of HslV

In order to investigate how the L88A mutation would bring about conformational changes in the HslV complex, we crystallized the L88A mutant and determined its structure at 2.9 Å resolution by molecular replacement (Supplementary Table 1). Comparison with X-ray structures of WT-HslV alone [blue, Protein Data Bank (PDB) ID: 1E94] and WT-HslV in complex with HslU (green, PDB ID: 1G3I) shows that the overall structure of the L88A mutant (magenta, PDB ID: 4G4E) is very similar to that of the WT-HslV complex (Fig. 7a and c). However, electron density corresponding to the pore region (approximately residues 83 to 89) of the L88A mutant was distinctly weak (Fig. 7b and Supplementary Fig. 4), suggesting that the pore motif of HslV becomes largely disordered. In addition to the pore motif, the region involved in the contact with HslU, α1, α2, and β-turn 5 of the L88A mutant also show relatively weak electron density and high *B*-factors (Supplementary Fig. 5).

Widening of HslV's axial openings by disordered pore motifs likely explains why L88A-HslV displays increased proteolytic activity toward several tested substrates, such as α-casein, Arc/I37A, and small peptides. In fact, when measured by distance between the backbones of opposing HslV's pore motifs, the pore size of the L88A mutant is ~20% larger than that of WT-HslV (Supplementary Fig. 4). Considering that in WT-HslV, a substantial area of the pore is covered by the radially inward-pointing side chains of the Arg86 and Arg89 residues (see

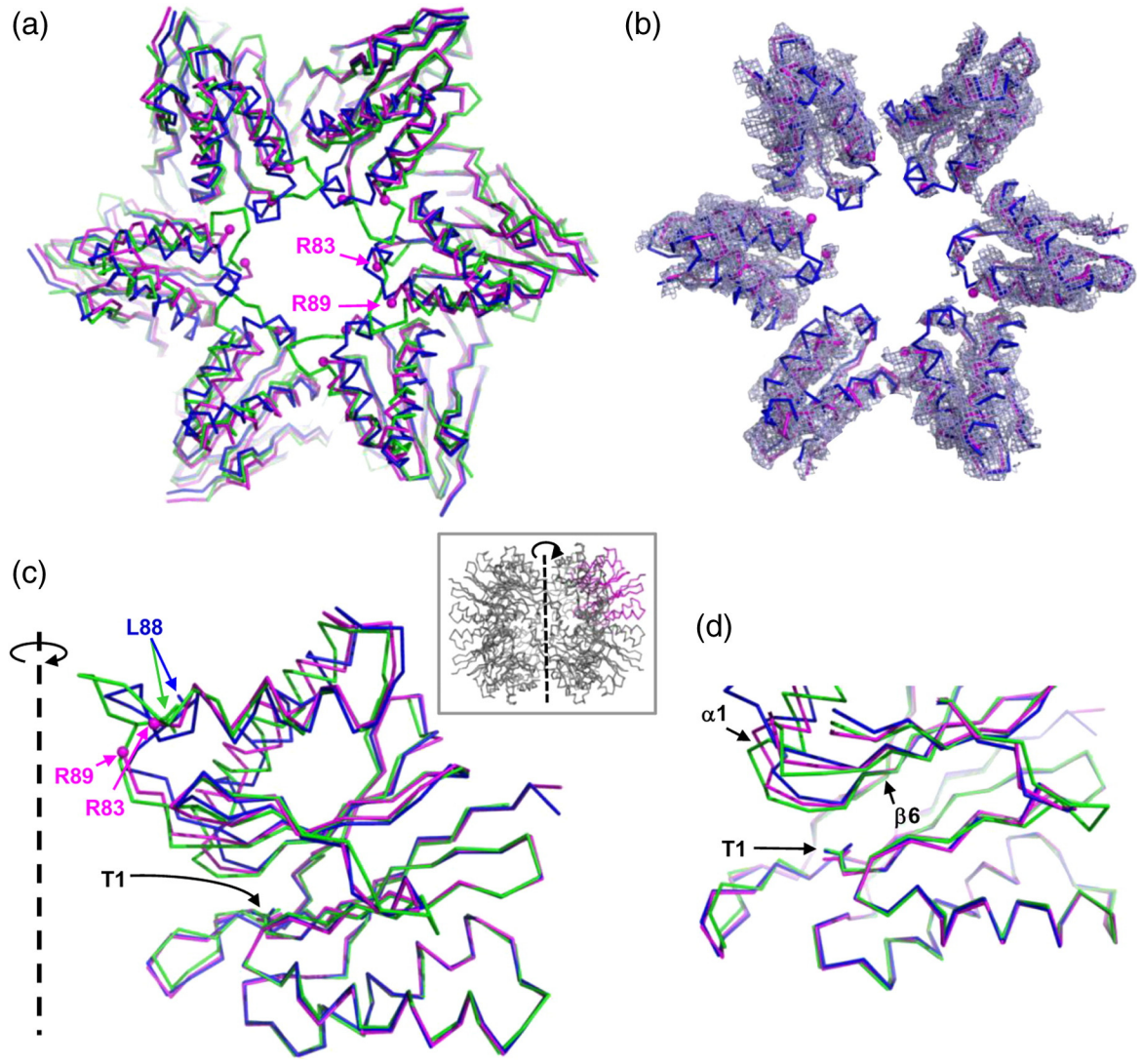

Fig. 7. Comparison of the structures of L88A and HslV. (a) The hexameric ring structures of L88A mutant (magenta, PDB ID: 4G4E), WT-HslV (blue, PDB ID: 1E94), and WT-HslV in complex with HslU (green, PDB ID: 1G3I) are viewed from the top along with 6-fold axis. In the L88A mutant, the pore region with weak electron density was omitted in the model and each of its ends is shown as a sphere. (b) $2F_o - F_c$ map, contoured at 1.0 σ, was presented in the pore region, α1, and α2 of the L88A mutant. The backbones of WT-HslV and the L88A mutant are shown in blue and magenta, respectively. (c) A side view of superposition among the L88A mutant (magenta), WT-HslV (blue), and WT-HslV in complex with HslU (green). The inset shows the position of the drawn HslV protomer (magenta) in an HslV dodecamer. The 6-fold axis is indicated as a broken line. (d) As in (c), but the Thr1 active site was visualized from a slightly different angle.

Fig. 1c), the disordered pore motifs of the mutant likely lead to an even larger increase in effective pore size. The crystal structure also hints at how the L88A mutation makes HslV bind to HslU with higher affinity. From previous crystal structures, it has been noticed that the α3 helix of HslV containing the RMLR motif becomes a less-defined loop upon forming a complex with HslU.[7,11,13] Conversely, it is conceivable that loss of the helical structure in the α3 region of HslV is a part of conformation transitions required for docking with HslU, perhaps for binding of the C-terminal tails of HslU. Therefore, the Leu88 mutations may provide a thermodynamic advantage in complex formation between HslV and HslU, assuming that the flexible pore loops of the mutants resemble those of the HslU-bound HslV complex.

Previously, crystal structures of the HslVU complex have suggested that association with HslU allosterically activates the Thr1 sites through transmitting conformational changes from the apical surfaces of HslV to the active-site clefts.[19] Although a dramatic stimulation is accompanied with the Leu88 mutations in substrate degradation by HslV itself, the active-site cleft in the crystal structure of

L88A mutant shows only a marginal (1–1.5 Å) conformational movement by comparison with the apo form of the WT-HslV complex (Fig. 7d). Thus, our data suggest that the Thr1 active sites still retain substantial catalytic activity without allosteric stimulation and that the increased proteolytic activity of the Leu88 mutants largely originates from widening of HslV's pores. The conformation of the active sites without the HslU-mediated allosteric activation would not be optimal for catalysis, as indicated by the fact that even for the Leu88 mutants, the peptidase activity can be further activated 6- to 8-fold by HslU or C10 peptide. However, the basal activity appears enough to support reasonable degradation of protein substrates such as α-casein and Arc/I37A, as long as substrates can reach the active sites. This idea is consistent with the previous observations that even in the absence of HslU or the C10 peptide, WT-HslV is still active toward certain unfolded polypeptide[45] and proteasome inhibitors such as MG132.[24]

Discussion

In the present study, we first showed that the conserved RMLR pore motif of HslV plays an important role in the interaction between HslV and HslU and thereby for the proteolytic function of the HslVU complex. This conclusion is based on our findings that replacement of Leu88 by Ala, Gly, or Ser leads to a dramatic increase in the affinity between HslV and HslU and of the proteolytic activities of the HslVU complex, whereas mutation of Arg86 or Arg89 to Ala abolishes the interaction with HslU and interferes with the proteolytic activity. More importantly, mutations in the pore motif not only affect the interaction between HslV and HslU but also dramatically influence the proteolytic activities of HslV complex in the absence of HslU. The Leu88 mutants (L88A, L88G, and L88S) of HslV by themselves efficiently degrade several tested substrates, while WT-HslV poorly hydrolyzes these substrates. Thus, these results provide strong evidence that the RMLR pore motif normally prevents access of proteins into the cavity of the HslV complex. Disruption of the intact pore structure by mutation of the Leu88 residue seems to allow entrance of loosely folded protein substrates into the HslV complex without requirement of translocation by the HslU ATPase.

Previous structural analysis of HslV complexes in the absence of HslU indicates that Leu88 in the α3 helix is in contact with hydrophobic residues in the α1 and α2 helices.[7,10,46,47] Upon docking with HslU, however, the α3 helix becomes an unstructured loop, resulting in a significant movement of the Leu88 side chain.[11] Thus, the structural changes in the α3 helix region seem conformationally linked to the interaction of HslV with HslU. Our data show that the replacement of Leu88 by other amino acids (e.g., Ala, Gly, or Ser) can induce loss of structure in the α3 region, a similar conformational transition observed upon docking with an HslU hexamer in the presence of ATP.[11] Our data also show that the Leu88 mutations in turn render the interaction between HslV and HslU much more favorable. The increased interaction between HslV and HslU is strong enough to be maintained even in the absence of ATP, at conditions where HslU hexamers tend to dissociate into monomers. Furthermore, HslU could activate peptide hydrolysis by the Leu88 mutants in the absence of ATP. The C-terminal tails of the HslU complex are likely involved in this process as the Leu88 mutants showed higher affinity toward the C10 peptide. Taken together, in addition to the previous observations,[24] the present study adds another mechanism on reciprocal allosteric communications between HslU and HslV complexes: (1) ATP binding to HslU ↔ (2) movement of the HslU C-terminal tails and (3) a structural change of HslV's pore motif ↔ (4) activation of the Thr1 sites in HslV.

The structural change in the RMLR motif by L88A mutations appears to result in an effective enlargement of HslV's pores. In fact, previous structural studies have suggested that upon association with HslU, the overall pore diameter (with side chains taken into account) increases from ~13 Å to ~20 Å.[11,13] Such pore widening probably facilitates channeling of polypeptide substrates from HslU into the interior of HslV, as seen with the Leu88 mutants. Although it is unclear exactly how docking with an HslU hexamer leads to opening of HslV's pore, positively charged arginines in the RMLR pore loop might be involved in the process. When not associated with HslU, these arginines are likely pointing their side chains toward the central axis, generating a highly basic environment at the center of the pore. Perhaps, these pore loops are repulsive against each other but maintain their positions by virtue of the helical structure of the α3 segment; binding of HslU on the apical surface of HslV or a mutation of Leu88 (e.g., L88A) would destabilize the α3 helix, and this would cause the RMLR motifs to be repelled from each other, thereby expanding the pore. This might also explain why the A4 mutant, which lacks two arginines, does not show an increased proteolytic activity (i.e., no effective pore enlargement) despite a Leu-to-Ala mutation at position 88.

Of surprise was the finding that in the presence of the C10 peptide, the Leu88 mutants were capable of degrading not only unstructured proteins but also certain folded proteins, such as MBP-SulA and native Arc proteins. In the case of WT-HslV, these proteins are only degraded when unfolded and translocated by HslU ATPase. Since the entry through the pore is obligatory for degradation, the

C10-bound Leu88 mutant complexes must have pores that are large enough to accommodate at least a portion of these proteins. It is also interesting that the Thr1 active sites can efficiently cleave peptide bonds in folded proteins. Such ability to pass folded segments through the open pores of HslV and subsequently degrade at the Thr1 sites might be important in the WT-HslVU complex, as substrate polypeptides may start to refold after moving through the central pore of the HslU hexamer. These data also indicate that prior unfolding of protein substrates is not essential for hydrolysis at the Thr1 sites, as long as substrates can enter into the interior of the HslV complex. The ATP hydrolysis-dependent unfolding process by HslU seems to be employed mainly to confer substrate selectivity, rather than to facilitate hydrolysis of peptide bonds. In the future, it would be interesting to know whether the inner surface of the HslV complex plays a role in loosening folded structures of substrate proteins to promote peptide bond hydrolysis.

It appears that substrate access to the interior of HslV is largely controlled by an HslU-mediated gating mechanism. A gating mechanism seems to be a major way to prevent nonspecific degradation of cellular proteins by eukaryotic 20S proteasomes.[25–27] In crystal structures of eukaryotic 20S proteasomes,[25,48] the pores are completely occluded by the N-terminal segments of the α-subunits. HslV had been believed to operate differently, likely controlled by allosteric activation of the Thr1 sites, as it has pores with a considerable opening (~13 Å).[11,13,19] The findings in the present study suggest that HslV also has a gating mechanism. It appears that narrow pores in a free HslV complex efficiently prevent even unstructured proteins from entering the interior, while artificial widening of these pores by the Leu88 mutations dramatically accelerates degradation of α-casein and Arc/I37A. Interestingly, ClpP proteases are also suggested to have a gating mechanism despite having a different architecture from HslV and the 20S proteasomes.[28–33] Alterations in the N-terminal portion of ClpP forming the axial pores have been shown to allow the degradation of certain unstructured proteins and peptides without association with the ClpX or ClpA ATPases.[28–31] Thus, gating mechanisms seem to be present in many ATPase-dependent proteases.

Finally, it should be pointed out that expression of the Leu88 mutants in *E. coli* significantly inhibited cell growth. This observation is most likely explained by the degradation of endogenous proteins that are required for normal cellular functions, especially, those containing extended polypeptide segments or small folded domains and those being synthesized by ribosomes. A similar case has been reported for the ClpP protease.[32,49] Like HslV, ClpP by itself cannot degrade protein substrates (e.g., casein), and hydrolyzes small peptide substrates at only slow rates.[50] In the presence of ClpA or ClpX, the activity of ClpP toward both peptides and proteins is greatly increased.[50,51] Remarkably, binding of novel small molecules called ADEPs (e.g., ADEP1 and ADEP2) was shown to enable ClpP to degrade natively unstructured casein[32] and ribosome-associated nascent chains[49] in the absence of the Clp ATPases. Moreover, these molecules show antibacterial activity, despite the fact that ClpP is not essential for viability of *E. coli* cells at normal growth conditions.[32,49] Therefore, it has been suggested that ADEPs dysregulate ClpP activity by relaxing the control mechanism that normally safeguards cytoplasmic proteins from random degradation by ClpP.[32,49] Indeed, a structural study has recently shown that binding of ADEP to ClpP causes a disorder in the pores of ClpP, increasing the effective pore size,[33] as we observed in the structure of the L88A mutant of HslV. Therefore, we speculate that many ATP-dependent proteases may be able to degrade cellular proteins nonspecifically if the pores can be open independently of their ATPase proteins. This property of ATP-dependent proteases can probably be exploited in developing new classes of therapeutic drugs. For example, it might be possible to obtain antibiotics that act by binding to the apical surface of HslV to induce gate opening. A similar approach is also conceivable for eukaryotic 20S proteasomes in searching for compounds with novel cellular activities.

Materials and Methods

Materials

Enzymes necessary for DNA cloning were purchased from New England BioLabs, Stratagene, and Takara. Z-GGL-AMC was purchased from Bachem. MG132 was obtained from A.G. Scientific. Other reagents were purchased from Sigma, unless otherwise indicated. Site-directed mutagenesis was performed as recommended by the manufacturer (QuikChange). All mutations were confirmed by DNA sequencing.

Protein expression and purification

BW25113 *ΔhslVU*::*kan* cells[24] harboring appropriate vectors were grown overnight at 37 °C in Luria broth supplemented with ampicillin to express each of the HslV mutants. HslV mutants, Arc, and Arc/I37A were purified by using Ni^{2+}-NTA-agarose columns as previously described.[24] Purified proteins were dialyzed against 20 mM Tris–HCl buffer (pH 7.8) containing 100 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, and 10% glycerol, and stored at −70 °C for further use. HslU and MBP-SulA were purified as previously described.[6,21] Protein concentration was measured via the Bradford method using bovine serum albumin as a standard.

Ni^{2+}-NTA pull-down analysis

Reaction mixtures (0.5 ml) that have HslU (150 nM) and HslV-His (75 nM) in 50 mM Hepes buffer (pH 8) containing 150 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, and 0.04% Triton X-100 were incubated at 4 °C for 1 h in the presence or absence of 2 mM ATP or 0.5 mM ATPγS [adenosine 5′-*O*-(thiotriphosphate)]. After incubation, the mixtures were added with 10 μl of 1 M imidazole and 20 μl of 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 ATP. Proteins bound to NTA resins were eluted by SDS sampling buffer, subjected to SDS-PAGE, and stained with Coomassie blue R-250. Where indicated, band intensities were quantified using "Image J" software.

Assays

ATP hydrolysis was measured by using an enzyme-coupled assay.[52] 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, GE Healthcare) 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) with 0.1 mM Z-GGL-AMC in 100 mM Tris–HCl buffer (pH 8) containing 5 mM $MgCl_2$, 0.5 mM EDTA, and 2 mM ATP at 37 °C. Fluorescence (λ_{ex} = 355 nm, λ_{em} = 460 nm) of released AMC was continuously measured by 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.

The degradation of protein substrates was assayed by incubation of HslU (0.4 μM) and HslV (0.4 μM) at 37 °C for appropriate periods with 100 mM Tris–HCl buffer (pH 8) containing 5 mM $MgCl_2$, 2 mM ATP, 20 mM phosphocreatine, and 10 U/ml of creatine kinase. Reactions were stopped by treatment with SDS sampling buffer. The samples were then subjected to SDS-PAGE followed by staining with Coomassie blue R-250. For assaying the hydrolysis of Arc proteins, SDS-PAGE was performed using Tris–Tricine buffer and discontinuous 4%, 10%, and 16% slab gels to improve the resolution. For others, SDS-PAGE was routinely performed using 12% gels with Tris–glycine buffer.

Crystallization and data collection

Purified L88A protein was concentrated to 9 mg/ml and crystallized at 21 °C using the hanging drop vapor diffusion method. Crystals were obtained using a reservoir solution consisting of 0.1 M Tris–HCl (pH 8.0), 28% (v/v) PEG monomethyl ether 550, and 0.2 M ammonium formate. X-ray diffraction data were collected on beam line 6C1 of the Pohang Accelerator Laboratory in Korea. Crystals were immersed in paratone and cryo-cooled in a liquid nitrogen stream at 110 K. Data were processed with HKL2000 software (HKL Research Inc.).

Structure determination and refinement

The structure of the L88A mutant was determined by molecular replacement using the PHENIX software at 2.9 Å resolution. Monomer of HslV (PDB ID: 1E94)[34] was used as a search model and 12 molecules in an asymmetric unit could be found. Structures of HslV dodecamer were subjected to many cycles of manual rebuilding using the Coot program and refinement using PHENIX. Non-crystallographic symmetry restraints with 12 non-crystallographic symmetry groups were used in refinement. After a series of simulated annealing, rigid-body, group *B*-factor, individual *B*-factor, and TLS refinements, the final structure was obtained with R_{work} = 0.258 and R_{free} = 0.293. Five residues in the pore region (amino acids 84–88) were not modeled in the final structure because of the unclear electron density. The statistics for the data collection and structure refinement are summarized in Supplementary Table 1.

Accession number

Coordinates and structure factors of L88A have been deposited in the PDB with accession number 4G4E.

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Supplementary Data

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† E.P., J.W.L., and H.M.Y. contributed equally to this work.

Abbreviations used:
AAA, ATPases associated with diverse cellular activities; ADEP, acyldepsipeptide; EDTA, ethylenediaminetetraacetic acid; MBP, maltose-binding protein; NTA, nitrilotriacetic acid; PDB, Protein Data Bank; WT, wild type; Z-GGL-AMC, carbobenzoxy-Gly-Gly-Leu-7-amido-4-methyl coumarin.

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