Dynamic flexibility of the ATPase p97 is important for its interprotomer motion transmission

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The hexameric protein p97, a very abundant type II AAA ATPase (ATPase associated with various cellular activities), is involved in a diverse range of cellular functions. During its ATPase cycle p97 functions as an ATP motor, converting the chemical energy released upon hydrolysis of ATP to ADP into mechanical work, which is then directed toward the proteins that serve as substrates. A key question in this process is: How is the nucleotide-induced motion transmitted from the C-terminal ATPase domain (the D2 domain) of p97 to the distant N-terminal substrate-processing domain? We have previously reported the surprising finding that motion transmission between the two ATPase domains (the D2 and D1 domains) is mediated by the D1-D2 linker region of its neighboring protomer. In this study we report efforts to better understand this process. Our findings suggest that the amino acid sequence containing Gly-Gly that is located at the C terminus of the D1-D2 linker functions as a pivoting point that allows the dynamic movement of the D1-D2 linker. Furthermore, we found that locking the D1-D2 linker to the D2 domain by introducing disulfide bonds significantly impaired the motion-transmission process. These results support our previous model for interprotomer motion transmission, and provide more detailed information on how the motion transmission between the two ATPase domains of p97 is relayed by the flexible movement of the D1-D2 linker from its neighboring protomer.

disulfide bond block | hexaric ATPase associated with various cellular activities

Our long-term interests are in the biosynthesis of glycoproteins and the processes involved in their degradation when they do not correctly fold. An important protein in this process is the ATPase p97. AAA (ATPase associated with various cellular activities) ATPases represent a large group of structurally similar but functionally diverse enzymes that harness chemical energy from ATP hydrolysis to process substrate proteins. These proteins comprise conserved ATPase domains of 200–250 amino acids, referred to as AAA modules (1), which contain Walker A/B motifs (2) and motifs of the so-called second region of homology (SRH) (3). Characterized by the presence of one or two AAA modules, AAA ATPases can be further classified as type I and type II, respectively. Central questions to fully understanding the action of AAA ATPase are determining how ATP hydrolysis in the ATPase domain is coordinated with the conformational changes and how the conformational changes are transmitted to the substrate proteins. Although the mechanism for ATP-driven conformational changes is relatively well understood (4), the second question, the mechanism of motion transmission (especially that in type II AAA ATPases) remains unclear.

As one of the best-studied AAA ATPases, p97 (called Cdc48 in yeast and VCP in mammals), offers a unique opportunity to study the motion-transmission process. Protein p97 is involved in a multitude of very important cellular activities, ranging from protein degradation, cell-cycle regulation and homotypic membrane fusion, to programmed cell death, as well as B- and T-cell activation (5–14). Moreover, because there are many uncharacterized cofactors that bind to p97, this protein may be involved in many yet-to-be identified cellular functions (15, 16). This potential diversity of cellular activities implicates p97 in various human diseases, such as Alzheimer's (17) and Parkinson diseases, and cancer (18). Recently, it has been shown that certain single amino acid mutations on p97 can cause a severe degenerative disorder, known as inclusion body myopathy associated with Paget disease of bone and frontal temporal dementia (19).

As is the case with many other AAA ATPases, p97 has a hexameric tertiary structure. Each p97 protomer contains three major domains: the N-terminal domain (N domain) and two highly conserved ATPase domains in tandem (designated the D1 and D2 domains). The N and D1 domains, and the D1 and D2 domains, are linked together by two 20-aa linker regions, called the N-D1 linker and the D1-D2 linker, respectively. The N domain of p97 is the major substrate-processing domain, which binds a number of cofactors that participate in the functions of p97 (18, 20–22). The D1 domain has been shown to be a degenerate ATPase domain that is responsible for stability of the hexameric state stability (23), and the C-terminal D2 domain is the primary ATPase domain of p97 at physiological temperature (24).

It is well established that during the process of ATP hydrolysis, p97 undergoes dramatic conformational changes (25). In this manner, p97 functions as an ATP motor, harnessing the chemical energy released by ATP hydrolysis and converting it into mechanical energy. The major ATPase domain (the D2 domain) and the principal substrate-processing domain (the N domain) of p97 reside at opposite ends of the protein, which implies the ATP hydrolysis-induced motion must be transmitted through the length of the entire molecule, from the D2 domain to the D1 domain, and then to the distant N domain.

Structural studies suggested that the D1-D2 linker might be involved in communication between the D2 and D1 domains (26, 27). In a previous study, we have demonstrated a unique interprotomer motion-transmission pathway between the D2 and D1 domains of p97: the ATP hydrolysis-induced motion is transmitted from the D2 domain to the D1 domain through the D1-D2 linker of its neighboring protomer, rather than that of the same protomer (28). We have now extended our studies into the detailed mechanism of how the D1-D2 linker relays the motion transmission. Sequence analysis reveals that there are two consecutive glycine residues (G480 and G481) residing at the C-terminal end of the D1-D2 linker that can potentially provide the D1-D2 linker with dynamic properties. In fact, it was estimated by Wang et al. (29) that this linker travels greater than 48 Å during the ATP hydrolysis cycle. These observations suggested that the conformational changes induced by ATP hydrolysis may be propagated through the dynamic movement of the D1-D2 linker. To test this idea, we mutated the two glycine residues at positions 480 and 481 to proline and found that this caused a dramatic defect on the in vivo endoplasmic reticulum-associated degradation (ERAD) activity of p97. Based on the crystal structure of p97, we introduced a double-cysteine mutation into p97 at positions that would be expected to result in a disulfide bond that would "lock" the D1-D2 linker of its neighboring protomer to the D2 domain through disulfide bonds.

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We found that the biological function of p97 was greatly impaired upon loss of the motion flexibility of the D1-D2 linker. These findings lead us to propose a detailed mechanism that the motion transmission between the D2 and D1 domains of p97 is mediated by the flexible movement of the D1-D2 linker from the neighboring protomer.

Additionally, during the course of constructing the disulfide bond locked form of p97, we identified that the residue G610 is essential for ATP hydrolysis in p97, although it resides relatively far from the nucleotide binding site. Based on this finding we propose that the region near residue G610 plays a critical role in mediating coordinated ATP hydrolysis between adjacent p97 protomers.

Results

Mutation of the Flexible C-Terminal Sequence Gly-Gly on the D1-D2 Linker Abolishes ERAD and ATPase Activities of p97. We have previously shown that the D1-D2 linker of the AAA ATPase p97 relays the motion transmission between the two ATPase domains of its neighboring protomer (28). Amino acid sequence analysis of the D1-D2 linker, as shown in Fig. 1*A*, reveals that there are two adjacent glycine residues (the most flexible of the 20-aa residues), at the C terminus of the linker (G480 and G481). Strikingly, these two adjacent glycine residues are conserved from yeast to human (Fig. 1*A*), suggesting that they might be of biological importance. In principle, these two glycine residues could impart flexibility to the D1-D2 linker because they can serve as a flexible hinge (30, 31). We reasoned that the dynamic nature of the D1-D2 linker might play a critical role in maintaining its function as a motion relay. To test this idea, we mutated both Gly residues to the less flexible Pro residues, assuming that this change should greatly impair the dynamic property of the D1-D2 linker. We then tested the mutational effect on ERAD activities using the assay system previously described (28). Briefly, the plasmids carrying wild-type or mutant p97 were transfected into a cell line stably expressing an ERAD substrate T-cell receptor (TCR)-α-GFP and the amount of the ERAD substrate TCR-α-GFP was measured. As expected, mutations of the two glycine residues to prolines resulted in dramatic substrate accumulation. In this mutant substrate accumulated ~70% more than that of the wild-type protein (Fig. 1*B*). This value was found to be comparable to deletion of the entire D2 domain, as reported previously (28). The dramatic defect in ERAD upon mutation clearly demonstrated the biological significance of the two highly conserved glycine residues in the D1-D2 linker.

Interestingly, for the N-D1 linker that connects the D1 and N domains, there are also two consecutive glycine residues (G207 and G208) present at the C terminus of the N-D1 linker (Fig. 1*A*). This identity to the D1-D2 linker therefore suggests that these two glycine residues may also play a role similar to their counterpart in the D1-D2 linker. In fact, the "Gly-Gly" sequence in the N-D1 linker has been proposed to form a flexible hinge that allows the N-D1 linker to rotate and relocate (32), playing the same role as our data demonstrated for the "Gly-Gly" sequence in the D1-D2 linker shown here. However, as shown in Fig. S1*B*, mutations of the two glycine residues in the N-D1 linker to proline residues showed no defect in the ERAD of p97. This finding suggests that either the "Gly-Gly" sequence in the N-D1 linker plays a different role, or the flexibility of the N-D1 linker is of no functional significance for p97.

Because the gene encoding p97 is one of the most conserved genes ranging from archaebacteria to yeast and to humans (33–35), we also studied the effect of mutation of the two glycine residues in its yeast ortholog, Cdc48. As described previously (28), the corresponding glycine residues were mutated to prolines in yeast Cdc48 and the growth ratio was analyzed. As shown in Fig. 1*C*, mutations of glycine at position 490 and 491 to proline in yeast Cdc48 resulted in severe growth defects at both 30 and 37 °C, indicating that the importance of these two consecutive glycine residues is conserved in yeast.

We then tested the effect of mutant GG480-481PP on ATPase activity of p97. To do this, the wild-type p97 full-length fragments were subcloned into a pET28a vector and overexpressed in *Escherichia coli*, as described previously (28). The well-studied Walker B mutant E578Q, which has been shown to cause dramatic loss in ATPase activity of p97 (36), was used as control. As shown in Fig. 1*D*, mutant GG480-481PP exhibited about 50% loss in ATPase activity, indicating the importance of the D1-D2 linker flexibility in the ATPase activity.

Taken together, the above results suggest that the dynamic flexibility of the D1-D2 linker plays a critical role in the motion transmission process and that the impaired motion transmission process affects ATPase activity of p97.

Rationale of Selection of Mutational Sites for Introducing Disulfide Bond Lock. To test this proposal we designed a mutant of p97 in which two residues that are in close proximity are replaced by cysteine residues, with the objective being to tightly lock the D1-

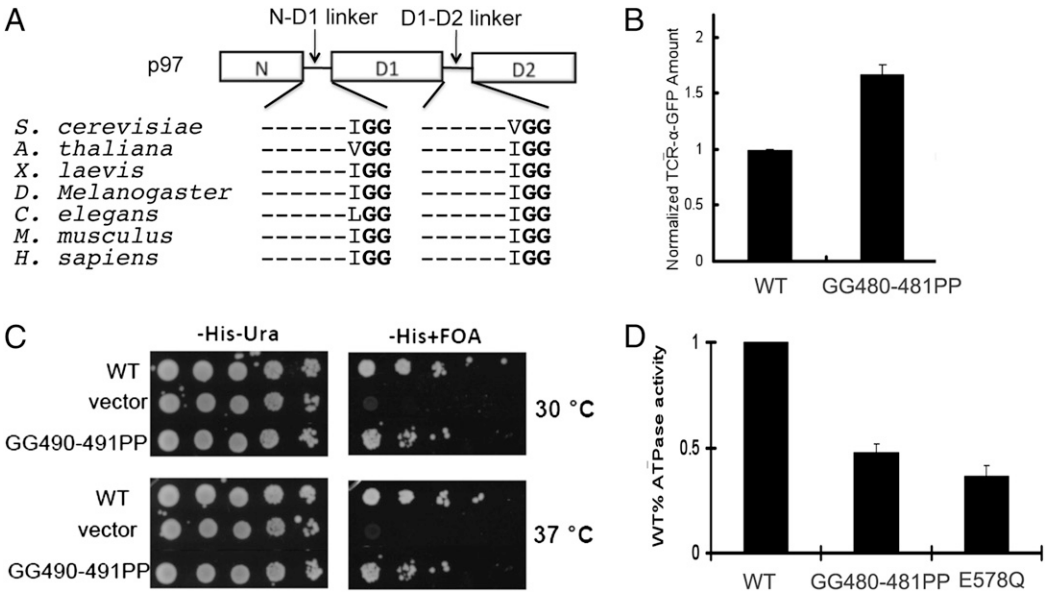

Fig. 1. Mutational effects of p97 in the highly conserved Gly-Gly sequence at the C terminus of D1-D2 linker. (*A*) Domain sketch of p97 protein. Sequence alignment of the N-D1 and D1-D2 linker region from different eukaryotic species shows the V/IGG sequence in the C terminus of linker region is highly conserved. (*B*) Effects of mutation in the Gly-Gly sequence on degradation of the ERAD substrate TCR-α-GFP. (*C*) Spotting assay of Cdc48 mutant strains showing the effects of mutation to the Gly-Gly sequence of yeast Cdc48. The same amount of L126 carrying Cdc48 variants were collected at log phase. The cells was spotted on –Ura-His or –His supplemented with fluoroorotic acid (–His+FOA) plates and incubated at 30 or 37 °C. (*D*) Effects of mutation in the Gly-Gly sequence on ATPase activity.

D2 linker to the D2 domain via a covalent disulfide bond formed between these introduced cysteine residues. We reasoned that this disulfide bond would greatly hamper the flexibility of the D1-D2 linker, and thereby result in a defect in motion transmission, as measured by a significant loss in ERAD.

As shown in Fig. 2*A*, analysis of the crystal structure of p97 shows that residue G610 in the D2 domain and R465 on the D1-D2 linker of its neighboring protomer are in close proximity, providing ideal candidates for introducing a disulfide bond lock. It should be noted that, for wild-type p97, each protomer contains 12 cysteine residues; no interprotomer disulfide bond has been reported. As shown in Fig. 2*A*, all 12 cysteine residues reside relatively far away from R465 and G610. We assumed that this would greatly reduce the chance of formation of an undesired disulfide bond between the mutationally introduced cysteine residue and the other cysteine residues present in wild-type p97. We previously showed that the mutant R465A exhibited no loss in ERAD (28), indicating that this residue is not involved in the motion transmission process. Therefore, we chose to mutate the residues R465 and G610 to cysteine residues.

Confirmation of Formation of the Designated Interprotomer Disulfide Bond. We first confirmed that the disulfide bond had indeed formed between the residues 465 and 610, as designed. The double-cysteine mutant of p97 was overexpressed in *E. coli*, and the purified mutant p97 was subjected to disulfide bond analysis by mass spectrometry to verify the formation of the disulfide bond between the two newly introduced cysteines. Protein samples were treated by trypsin digestion, and wild-type p97 was used as control. Peaks that appear under unreducing conditions, but not under reducing conditions, should indicate disulfide bond-containing peptides. As shown in Fig. 2*B*, a few peaks corresponding to disulfide bond-containing peptides were observed. Among them, a peak with a molecular weight of 5,325.3 matched the predicted peptide $M+H^+$ value of disulfide bond C465-C610 by trypsin digestion in R465C/G610C double-cysteine mutant sample (the theoretical value for the protein fragment molecular weight is 5,322.6), This peak was eliminated by DTT treatment and was absent in the wild-type sample. These observations confirmed the existence of disulfide bond C465-C610 in R465C/G610C double-cysteine mutant.

Mutation of G610 Causes Defect in ATPase Activity of p97. After confirming the formation of the disulfide bond between the D1-D2 linker and the D2 domain of its neighboring protomer, we then asked if these mutations affected the ATPase activity of p97. As shown in Fig. 3*A*, although single-cysteine mutant R465C showed no effect on ATPase activity, both the single-cysteine mutant G610C and the double-cysteine mutant R465C/G610C exhibited a significant loss in the ATPase activity of p97. We further prepared mutants G610A and G610V and tested the effects of these mutations. Mutant G610A exhibited a defect similar to that of G610C, and G610V exhibited a slightly lesser effect (Fig. 3*A*). The physiological importance of residue G610 was also confirmed by yeast-growth phenotype experiments. In agreement with the above ATPase activity assay data, a yeast strain bearing an R475C mutation grew similarly to the wild-type, whereas both single-mutation G620C and double-mutation G620C/R475C in yeast Cdc48 exhibited severe growth defects at both 30 and 37 °C (Fig. 3*B*).

Disulfide Bond-Locked D1-D2 Linker Caused a Significant Defect in ERAD. As shown above, mutation of G610 to cysteine caused a dramatic defect in ATPase activity of p97. Because ERAD activity of p97 is dependent on both ATPase activity and motion transmission, we reasoned that both G610C and R465C/G610C should show an activity loss in ERAD activity. Further analysis revealed that both of the mutants of G610C and R465C/G610C exhibited an ATPase activity loss to almost the same extent (approximately eightfold less than wild-type p97) (Fig. 3*A*). This finding suggests the defect in ATPase activity upon mutation of G610 should have the same impact on the ERAD activity for both mutants of G610C and R465C/G610C. If the flexibility of the D1-D2 linker in motion transmission process is indeed critical for the motion-transmission process, the double-cysteine mutant R465C/G610C might exhibit a more severe ERAD defect, and the difference in their ERAD activity could be attributed to the difference of the flexibility of D1-D2 linker in the single mutant G610C and double-cysteine mutant R465C/G610C.

Next, we tested the effects of the mutation on ERAD. As shown in Fig. 3*C*, compared with the wild-type p97, the single-cysteine mutant R465C showed no effect on ERAD, consistent with our previous finding that residue R465 of p97 is not biological significant in the ERAD process (28). As expected, both the single-cysteine mutant G610C and double-cysteine mutant R465C/G610C exhibited defects in ERAD activity. The single-

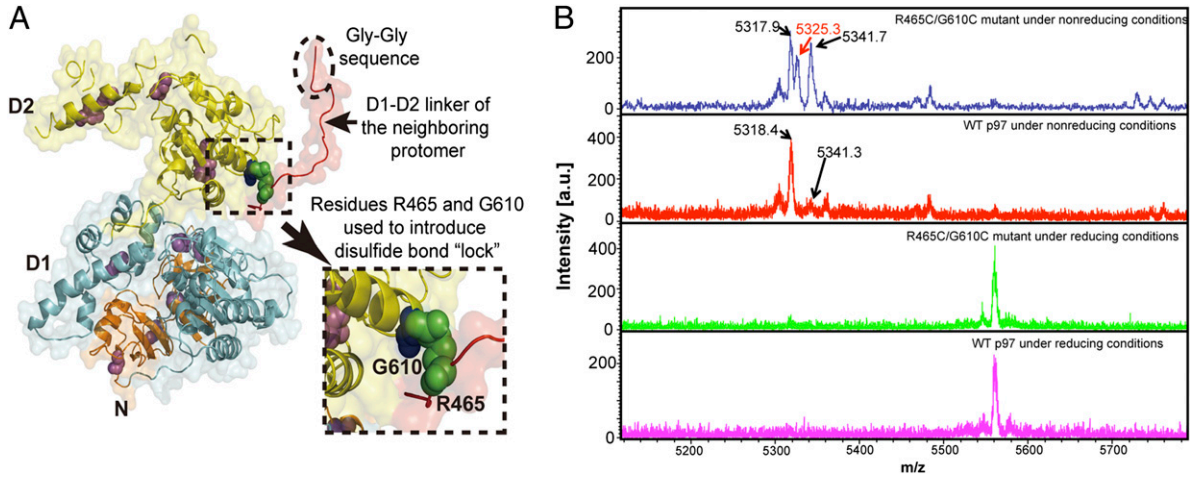

Fig. 2. Introduction of a disulfide bond to limit flexibility of the D1-D2 linker. (*A*) Rationale of selection of G610 and R465 for introducing a disulfide bond lock between the D1-D2 linker and its neighboring protomer. Residues G610 and R465 are shown in blue and green spheres, respectively. All cysteine residues present in wild-type p97 are located far from residues 610 and 465, shown in purple spheres. (*Lower Right*) A close-up view of the corresponding region of the overview figure. (*B*) Mass spectrum of trypsin-digested wild-type p97 and R465C/G610C double-cysteine mutant under nonreducing or reducing conditions. A peak at *m/z* of 5325.3 was found in R465C/G610C double-cysteine mutant but not in wild-type protein. This peak, which was eliminated by DTT treatment, corresponds to the potential digested protein fragment containing C465-C610 (theoretical value is 5,322.6). These results confirmed formation of disulfide bond between C465 and G610.

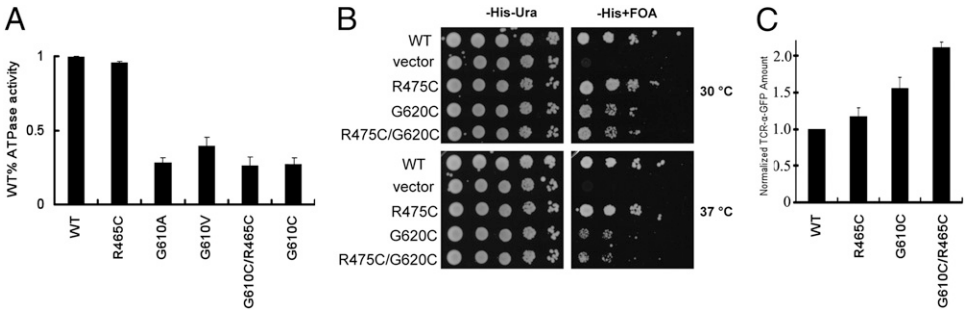

Fig. 3. Mutational effects in p97 upon losing flexibility of the D1-D2 linker. (*A*) Mutational effects of residues R465 and/or G610 on ATPase activity of p97. (*B*) Spotting assay of Cdc48 mutant strains showing the effects of mutation to residue R475 or G620 of yeast Cdc48. (*C*) Effects of mutation in residues R465 and/or G610 on degradation of the ERAD substrate TCR-α-GFP.

cysteine mutant of G610C showed a moderate defect in ERAD (about 50% more substrate accumulation than wild-type p97). This finding may be attributed to the loss of ATPase activity upon mutation of G610. In contrast, the double-cysteine mutant R465C/G610C showed a much more significant defect in ERAD (more than 110% more substrate accumulation). We assume that in addition to the loss of ATPase activity, as the case in the single-cysteine mutant G610C, there is another factor that accounts for the ERAD defect in the double-cysteine mutant R465C/G610C: that is, impaired motion-transmission pathway. As a result, despite bearing the same amount of loss in ATPase activity, the difference in the mobility of the D1-D2 linkers in G610C and R465C/G610C mutants resulted in distinct defects in ERAD activity: the tightly locked D1-D2 linker in the R465C/G610C mutant exhibited a much more severe defect in ERAD activity than G610C. This finding is consistent with our proposal that the flexibility of the D1-D2 linker plays an essential role in the motion-transmission process.

Discussion

A characteristic feature of AAA ATPases is that they often assemble into hexamers, implying the functional importance of interprotomer communication. Interprotomer communication is generally related to a coordinated ATP hydrolysis process between adjacent protomers. At the interprotomer interfaces, a conserved arginine residue, known as an "arginine finger," senses the γ-phosphate of ATP in the adjacent promoter and regulates its hydrolysis (37–40). We recently demonstrated that the motion-transmission process in protein p97 also behaved in a transacting manner (28), and this finding provided another explanation for the structural requirement for hexamerization of AAA ATPase.

In this study, we showed that mutation of the residue G610 caused dramatic ATPase activity loss in p97. In fact, residue G610 was reported to be located in a conserved motif within the classical AAA clade of proteins called the intersubunit signaling (ISS) motif. This motif, which is located proximal to the arginine finger, has been shown to play an essential role in coordinated ATP hydrolysis between adjacent protomers in the yeast AAA ATPase *m*-AAA protease (41). Our results therefore confirm the functional importance of this motif and suggest that it might play a role in protein p97 similar to that reported in the *m*-AAA protease. Based on the model presented in *m*-AAA protease (41), we propose a model of how the ISS motif in p97 mediates the interprotomer-coordinated ATPase hydrolysis in p97. As shown in Fig. 4, the ISS motif interacts with the arginine finger R635 through direct contact between ISS motif residue D609 and SRH motif residue R638, as observed in the crystal structure of p97 (27). In this manner, protein conformational changes induced by ATP hydrolysis in the D2 domain causes movement of the ISS motif, which further drives the movement of the arginine finger and thus regulates the ATP hydrolysis in its neighboring protomer. Mutation to the residue G610 might disrupt the local structure and dynamics of the ISS motif, therefore interfering with the ATPase activity of its neighboring protomer. In support this model, it has been reported that mutation of the SRH motif residue R638 or the arginine finger R635 caused a dramatic defect in ATPase activity of p97 (42). Interestingly, in yeast *m*-AAA protease mutations to the ISS motif resulted in an apparent increase in its ATPase activity, indicating an inhibitory effect of one protomer on the ATPase activity of the adjacent protomer. In striking contrast, disruption of the interprotomer signaling by mutations to the ISS motif of p97 caused dramatic loss in its ATPase activity. This result clearly demonstrated a positive cooperativity with respect to ATPase activity in p97, which is in agreement with the results from the kinetic studies of p97 by others (36, 42, 43).

In this study, we demonstrated that mutation of the two consecutive glycine residues to prolines resulted in loss in ATPase activity (Fig. 1*D*). There are several possible explanations for this finding. First, the Gly-Gly sequence in the N-terminal D1-D2 linker might be directly involved in ATPase hydrolysis process. Analysis of the crystal structure of p97 shows that although the N-terminal D1-D2 linker region is located relatively far away from that of its neighboring protomer, it is indeed adjacent to the ATP hydrolysis center on the same protomer. However, as shown previously, mutations to the neighboring residues of the Gly-Gly sequence, E477- I579, which is also in close proximity to the ATP hydrolysis site, have no effect on ERAD activity in p97 (28). Therefore, we argue that it is unlikely that the Gly-Gly sequence plays a direct role in ATP hydrolysis reaction. Instead, we favor another explanation: the flexibility of the D1-D2 linker plays an indirect regulatory role in ATP hydrolysis of p97. We propose that loss in ATP hydrolysis upon mutations to the D1-D2 linker is a result of the impaired motion-transmission system. In other words, ATP hydrolysis and motion transmission are mutually coupled: ATP hydrolysis initiates motion transmission

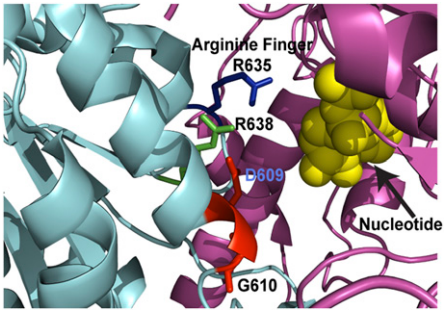

Fig. 4. Proposed role of ISS motif in regulating ATP hydrolysis of the neighboring protomer. Two neighboring protomers are colored in purple and cyan, respectively. ISS motif residues G609 and D610 (red) regulates the interprotomer ATP hydrolysis through interactions with the SRH motif residue R638 (green) and the arginine finger residue R635 (blue).

and motion transmission can also have a feedback effect on the ATP hydrolysis process. In support of this proposal, although it was recently reported that mutation of the D1-D2 linker residue L464—the motion relay between the D1 and D2 domains—to glutamate did not affect ATPase activity of p97 (28), further studies show that mutant L464P demonstrated a significant loss in ATPase activity (Fig. S1*A*). Residue L464 is located far away from the nucleotide-binding site and should not be directly involved in ATP hydrolysis. We reason that the loss in ATPase activity upon mutation of this residue could be attributed to the impaired motion transmission system. Further studies are needed to address the relationship between the processes of motion transmission and ATP hydrolysis of p97.

As mentioned earlier, because the major ATPase domain and the substrate-processing domain reside at the opposite end of p97 (i.e., at the C and N termini), the nucleotide-induced motion must be transmitted across the entire molecule. In a previous study, we proposed a unique interprotomer motion-transmission pathway for p97, indicating that the motion transmission between the D2 and D1 domains are mediated by a D1-D2 linker that is not on the same protomer, but rather on its neighboring protomer (28). Here, we performed further studies to elucidate the detailed mechanism of how the D1-D2 linker relays the motion transmission from the D2 to the D1 domain. We propose that the dynamic nature of the D1-D2 linker may play a role in the motion-transmission process on the basis of several lines of evidence. First, we observed that there are two adjacent conserved glycine residues located at the C terminus of the D1-D2 linker that have the potential to induce a highly flexible character to the linker. Second, structural studies have previously shown that the D1-D2 linker undergoes striking conformational transitions during the nucleotide hydrolysis cycle (27, 44), as shown in Fig. 5*A*.

To directly test the involvement of the dynamic movement of the D1-D2 linker in the motion-transmission process, we abrogated the linker flexibility either by mutating of the two consecutive glycine residues to proline residues or by introducing a disulfide bond formed between the D1-D2 linker and the D2 domain to covalently lock the linker to the D2 domain. Our results demonstrated that the presence of the dynamically impaired D1-D2 linker of p97 caused a dramatic defect in ERAD, in agreement with the proposal that its dynamic nature is crucial for the D1-D2 linker to function as a motion relay.

These results, along with those of our previous study (28), led us to propose a mechanism delineating how the nucleotide-induced motion is mediated by the D1-D2 linker of its neighboring protomer, as shown in Fig. 5*B*. In this model, before ATP hydrolysis in the D2 domain, the D1 and D2 domains are tightly held by the D1-D2 linker of its neighboring protomer (Fig. 5*B*, 1). Upon ATP hydrolysis, the D2 domain undergoes a conformational change, which leads to an overall rotation, as previously observed in the crystal structural studies (44) (Fig. 5*B*, 2). This rotation changes the distance between the interacting region on the D2 domain (the region near F563) and that on the D1-D2 linker of the neighboring protomer (residue L464). The increased distance causes a weakening of the interaction between these two regions. Meanwhile, the rotation of the D2 domain also drives the movement the D1-D2 linker, and therefore changes the spatial location of the region (the linker residue L464) that interacts with the D1 and D2 domains of its neighboring protomer (Fig. 5*B*, 3). In this manner, the nucleotide-induced motion is sensed and thereby transferred from the D2 domain of one protomer to the D1-D2 linker of its neighboring protomer. Similarly, the movement of the D1-D2 linker leads to an increased distance between residue L464 and its interacting region (the region near residues 358–360) in the D1 domain of its neighboring protomer. This process results in a weakened tension force between these two regions, and this triggers the conformational change in the D1 domain. In this way, the nucleotide state information is transmitted to the D1 domain (Fig. 5*B*, 4), and further to the N domain. Indeed, in support of this model, during the course of ATP hydrolysis, the distances among the interacting regions along the interprotomer motion-transmission pathway dramatically change. For example, after ATP hydrolysis, the distance between F563 and L464 of the neighboring protomer increases about 3 Å, and the distance between L464 of the neighboring protomer and F360 increases 1.4 Å. In this scenario, the highly flexible D1-D2 linker functions as a bridge connecting the communication between the D2 to D1 domains of its neighboring protomer, and the two consecutive C-terminal glycine residues function as pivoting points that allow the dynamic movement of the D1-D2 linker.

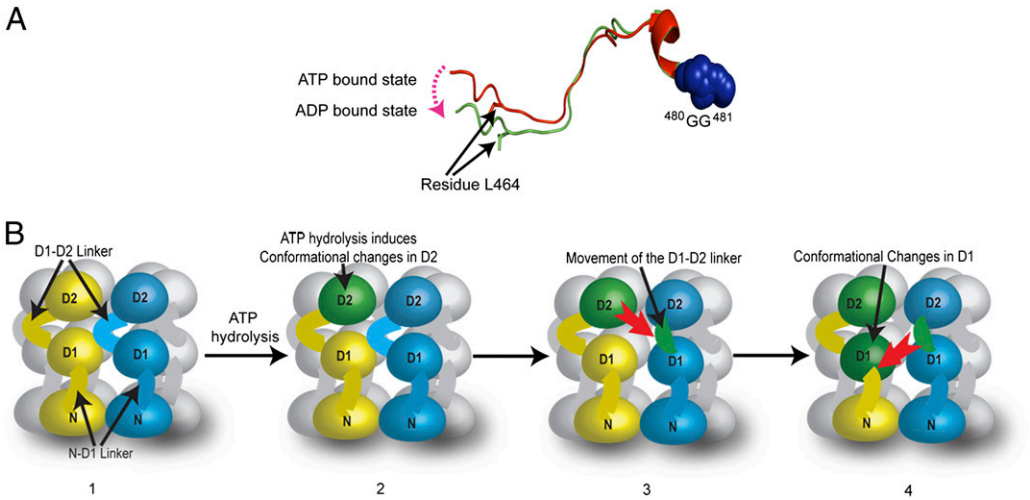

Fig. 5. Proposed model of how dynamic movement of the D1-D2 linker relays motion transmission from the D2 to the D1 domain of its neighboring protomer. (*A*) Structure of the D1-D2 linker at two nucleotide bound states. Red: ATP bound state. Green: ADP bound state. The Gly-Gly sequence residing at the C terminus of the D1-D2 linker (shown as blue sphere) functions as a pivoting point that allow the dynamic movement of the D1-D2 linker. The D1-D2 linker residue L464 that mediates the motion transmission is shown as sticks. (*B*) Proposed model of stepwise inter protomer motion transmission between D2 and D1 domains. AAA ATPase p97 is shown as a hexamer. The interprotomer motion transmission pathway is shown for two protomers, which are highlighted in yellow and blue with domains/regions labeled. Red arrows indicate the direction of interprotomer motion transmission. Numbers below depict different motion transmission states of p97. The conformational changes in domains resulted from the motion transmission are depicted as color changes to green. 1: Before ATP hydrolysis, the D2 and D1 domains are held tightly by the D1-D2 linker its neighboring protomer. 2: ATP hydrolysis induces conformational changes in the D2 domain. 3: Conformational changes in the D2 domain drive movement of the D1-D2 linker of its neighboring protomer. In this manner the motion is transmitted from the D2 domain to the D1-D2 linker of its neighboring protomer, as shown by a red arrow. 4: Movement of the D1-D2 linker triggers conformational changes in the D1 domain of its neighboring protomer. Therefore, the nucleotide-induced motion is transmitted to the D1 domain, as shown by a red arrow.

Materials and Methods

Construction of p97 Mutants. All mammalian p97 mutants were derived from the wild-type mouse p97 (mp97) fusion constructs with pmcherry-p97, as described previously (28, 45).

In Vivo ERAD Activity Assay. The ERAD activity assay was carried out as previously described (28).

Spotting Assay for Growth. The spotting assay experiments were performed as previously described (28).

Purification of His-Tagged p97 Variants. Full-length wild-type and p97 mutants were expressed in *E. coli* BL21(DE3)-Codon Plus RIL cells using pET28a vector (Invitrogen) at 15 °C after induction with 0.4 mM isopropyl β-D-thiogalactopyranoside at an A_{600} of 0.6–0.8. Purification of N-terminal His6-tagged p97 variants was performed using the ÄKTAprime plus protein purification system (GE Healthcare Life Sciences) with HisTrap FF crude Column (GE Healthcare Life Sciences).

ATPase Activity Assay. ATPase assays were carried out in assay buffer containing 50 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 0.1 mM EDTA, 80 mM NaCl, 0.5 mM ATP, and 0.1 μM p97, as previously described (24). The release of inorganic phosphate by ATP hydrolysis was measured using a colorimetric ATPase assay kit (Innova Biosciences). Briefly, 100 μL of p97 was added to 100 μL of assay substrate/buffer mix. After shaking at 37 °C in a Thermomixer (Eppendorf) for 15 min, the reaction was stopped according to the kit manual. After 30-min incubation at room temperature, 250 μL from each reaction was transferred to a 96-well plate, and the absorbance at 620 nm was measured using a Synergy 2 Multi-Mode Microplate Reader (Bio-Tek). All assays were repeated at least three times, and the average activities with SEs of measurement are presented.

Disulfide Bond Identification by Mass Spectrometry. Mass spectometry was performed by Optimum Biotech. Briefly, wild-type p97 and R465C/G610C double-cysteine mutant were digested by sequence grade trypsin (Promega); 10 mM DTT was used for reduction for one hour at 56 °C after enzyme digestion. A Bruker Autoflex MALDI-TOF mass spectometrer was used in data collection. Saturated α-Cyano-4-hydroxycinnamic acid solution in 50% (vol/vol) acetonitrile and 0.05% trifluoroacetic acid was used for matrix. The instrument was set at positive reflectron mode covering 0.4–6 kDa, and calibrated with a mixture of peptides with different lengths. A total of 1,000 laser shots were collected from each sample spot. Disulfide bond mapping was performed manually by matching peak values ($M+H^+$) of the mass spectometry spectrum to theoretical peptide values of enzyme digestions based on protein sequence.

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