Cleavage of RseA by RseP requires a carboxyl-terminal hydrophobic amino acid following DegS cleavage

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Regulated intramembrane proteolysis (RIP) by the Site-2 protease (S2P) results in the release of a transmembrane signaling protein. Curiously, however, S2P cleavage must be preceded by the action of the Site-1 protease (S1P). To decipher the underlying mechanism, we reconstituted sequential, in vitro cleavages of the *Escherichia coli* transmembrane protein RseA by DegS (S1P) and RseP (S2P). After DegS cleavage, the newly exposed carboxyl-terminal residue Val-148 of RseA plays an essential role for RseP cleavage, and its mutation to charged or dissimilar amino acids crippled the Site-2 cleavage. By contrast, the identity of residues 146 and 147 of RseA has no impact on Site-2 cleavage. These results explain why Site-1 cleavage must precede Site-2 cleavage. Structural analysis reveals that the putative peptide-binding groove in the second, but not the first, PDZ domain of RseP is poised for binding to a single hydrophobic amino acid. These observations suggest that after DegS cleavage, the newly exposed carboxyl terminus of RseA may facilitate Site-2 cleavage through direct interaction with the PDZ domain.

membrane protein | PDZ domain | regulated intramembrane proteolysis | S2P

Regulated intramembrane proteolysis (RIP), ubiquitously conserved from bacteria to humans, plays an essential role in numerous aspects of biology (1–8). RIP signaling requires a site-specific cleavage of a transmembrane signaling protein by a specific membrane-embedded protease within the lipid bilayer. One of the founding examples of RIP signaling is cleavage of the membrane-bound transcriptional factor SREBP by the intramembrane metalloprotease Site-2 protease (S2P) (9–11). In response to low cellular levels of cholesterol, SREBP is cleaved at an intramembrane site proximal to the cytosol (12). Consequently, its amino-terminal transactivation domain is released from the Golgi membrane and translocated into the nucleus, where it induces expression of genes that control synthesis and uptake of sterols and fatty acids. Cleavage by S2P is essential in this signaling process and, curiously, must be preceded by a prior cleavage mediated by the Site-1 protease (S1P) (13). S1P cleaves SREBP in the lumen of Golgi, at loop sequences between two transmembrane helices (14).

RseP (also known as YaeL) is an *Escherichia coli* homolog of S2P. In response to accumulation of unfolded outer membrane proteins (OMPs) in the envelope, RseP cleaves a membrane-anchored protein RseA at an intramembrane site close to the cytoplasm (Fig. 1*A*) (15–18). Because the amino-terminal sequence of RseA is bound to the transcription factor $\acute{o}^E$, this cleavage results in the release of the amino-terminal RseA–$\acute{o}^E$ complex into the cytoplasm, where RseA is selectively degraded by proteases, and the freed $\acute{o}^E$ activates transcription of genes that cope with envelope stress (15). Similarly to all characterized S2Ps, RseP cleaves RseA only after a prior cleavage mediated by DegS, the *E. coli* S1P in envelope stress response (16, 17). DegS is a serine protease with a carboxyl-terminal PDZ domain and an amino-terminal transmembrane segment (Fig. 1*A*). In the absence of envelope stress, DegS exists in an autoinhibited state (19). Binding of the carboxyl-terminal residues of unfolded OMPs to the PDZ domain of DegS triggers its allosteric activation (20, 21), which subsequently cleaves the periplasmic domain of RseA between amino acids Val-148 and Ser-149 (Fig. 1*A*) (22).

Despite intense investigation, several important aspects remain to be elucidated in S2P-mediated RIP signaling. A central unanswered question is: Why must the action of S2P be preceded by S1P cleavage? In this manuscript, we provide strong evidence that after DegS cleavage, the newly exposed carboxyl-terminal residue of RseA 1–148 is required for the subsequent RseP cleavage.

Results and Discussion

In Vitro Reconstitution of Site-2 Cleavage. An important tool for deciphering the underlying mechanisms of intramembrane signaling is in vitro reconstitution of the sequential cleavages using recombinant proteins. After many attempts, we succeeded in purification of the full-length, recombinant proteins DegS, RseA, and RseP (Fig. 1*B*). These proteins exhibited excellent solution behavior under detergent micelles. First, we examined cleavage of RseA by DegS. The carboxyl-terminal PDZ domain of DegS was shown to inhibit its serine protease activity, and this inhibition was relieved by binding of the OMP peptides to the PDZ domain (19–21, 23). Consistent with this conclusion, free DegS exhibited little activity for the RseA substrate (Fig. 1*C*, lane 2); addition of the OMP peptide RDGNVYYF resulted in complete cleavage of RseA (Fig. 1*C*, lane 3). Analysis by amino-terminal peptide sequencing and MS identified the two cleavage fragments of RseA as residues 1–148 and 149–216 (Fig. S1), confirming the scissile peptide bond to be between Val-148 and Ser-149 (22).

Next, we investigated RseP cleavage. The full-length RseP failed to cleave RseA in the absence of Site-1 cleavage by DegS (Fig. 1*C*, lane 4); incubation of the OMP peptide failed to activate RseP in the absence of DegS (Fig. 1*C*, lane 6). Under conditions where RseA was cleaved by DegS in the presence of OMP peptide, the RseA fragment 1–148 was further processed by the RseP protease into two fragments of smaller molecular weight (Fig. 1*C*, lane 5). Analysis by amino-terminal peptide sequencing and MS revealed that the Site-2 cleavage occurred between residues 108 and 109 (Fig. S1), confirming previous observations (18). These results match the in vivo observations and show that the cleavage of RseA by RseP must be preceded by a prior cleavage mediated by DegS. Thus, the sequential cleavages of RseA by DegS and by RseP have been recapitulated in vitro through biochemical reconstitution. Protease

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Data deposition: The atomic coordinates of the RseP PDZ1, WT PDZ2, PDZ-GKASPV fusion, and PDZ2-I304A have been deposited in the Protein Data Bank (ID codes 3ID1, 3ID2, 3ID4, and 3ID3).

See Commentary on page 14737.

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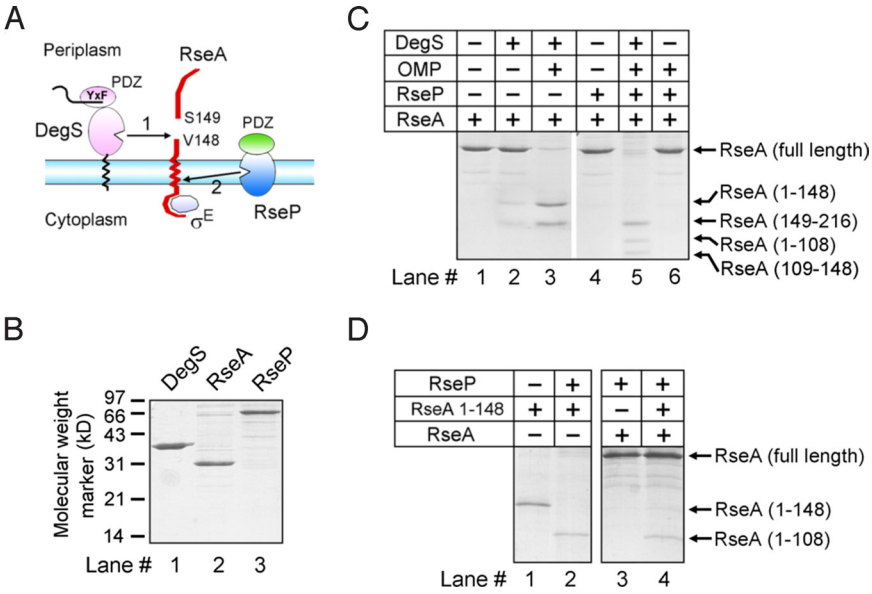

Fig. 1. In vitro reconstitution of sequential cleavages of RseA by DegS and RseP. (*A*) A schematic diagram of the proteolytic cascade across the inner membrane of *E. coli*. After OMP peptide binding to the PDZ domain, DegS is activated and cleaves RseA between Val-148 and Ser-149. Then, the membrane-embedded protease RseP makes the second cleavage after residue 108, at a site that is close to the cytoplasm. (*B*) A representative SDS/PAGE gel showing the full-length, recombinant proteins DegS, RseA, and RseP. All gels shown in this study were stained by Coomassie blue. (*C*) Reconstitution of sequential cleavages of RseA by DegS and RseP. Note that RseP only cleaved RseA after it was first cleaved by DegS in the presence of OMP peptides. (*D*) RseA 1–148 contains the essential factor for Site-2 cleavage. RseP efficiently cleaved the isolated RseA 1–148 (lane 2) but exhibited no activity for the full-length RseA (lane 3). RseP failed to cleave the full-length RseA in the presence of RseA 1–148 but completely cleaved RseA 1–148 (lane 4).

activity of the S2P family is thought to rely on a catalytic zinc ion. Consistent with this notion, RseP was inactivated by the zinc chelator 1,10-phenanthroline in a concentration-dependent manner (Fig. S2).

The observation that the protease activity of RseP was unleashed by the first cleavage event suggested that the RseP activity might be induced by the RseA fragment 1–148, by RseA 149–216, or by both. To differentiate between these distinct scenarios, we biochemically purified the RseA fragment 1–148 after DegS cleavage of the full-length RseA (Fig. 1*D*, lane 1). RseP efficiently cleaved this RseA fragment (Fig. 1*D*, lane 2) but exhibited no detectable activity toward the full-length RseA (Fig. 1*D*, lane 3). This experiment shows that the essential factor for RseP cleavage of the RseA fragment 1–148 is contained within this fragment itself. To examine whether RseA 1–148 can unleash RseP activity *in trans*, we incubated RseP with the full-length RseA in the presence of RseA 1–148. Although RseA 1–148 was completely cleaved by RseP, the full-length RseA remained intact (Fig. 1*D*, lane 4), indicating that RseA 1–148 can only induce the protease activity of RseP *in cis*.

Critical Role of RseA Residue 148 in RseP Cleavage. Because the RseA fragment 1–148 is sufficient for unleashing the protease activity of RseP, we hypothesized that the newly exposed carboxyl-terminal amino acid following RseA cleavage might play an important role. To investigate this possibility, we generated four missense mutants of the full-length RseA: two involving conserved mutations V148T and V148I, and the other two involving nonconserved mutations V148H and V148K. We purified these four mutant proteins and examined their cleavages by DegS and RseP. Similarly to the WT RseA (Fig. 2*A*, lanes 1–3), the mutants V148T and V148I were cleaved by DegS in the presence of OMP peptides, and the RseA fragment 1–148 was further cleaved by RseP to generate the fragment 1–108 (Fig. 2*A*, lanes 4–9). In contrast, although V148H and V148K were also cleaved by DegS, the fragment 1–148 resisted cleavage by RseP (Fig. 2*A*, lanes 10–15). These results suggest that the identity of residue 148 plays a critical role in Site-2 cleavage.

To corroborate the in vitro finding, we sought to examine the consequence of these mutations in *E. coli*. To accomplish this task, we first generated RseA-null cells and put the ORF of β-galactosidase under the control of a $\acute{o}^E$-driven promoter. Then, we transformed the cells with the WT as well as mutant RseA plasmids, challenged the cells with envelope stress signals, and monitored the β-galactosidase activity as a measurement of the envelope stress response. We recognized that results from such in vivo assays could be difficult to interpret because many steps, both before and after Site-2 cleavage, were involved. Nonetheless, the fact that only one amino acid was mutated in each RseA construct suggested a reasonable chance for us to see a differential effect. Cells transformed by the WT RseA plasmid gave rise to the highest level of β-galactosidase activity (Fig. 2*B*), suggesting robust envelope stress response. Cells transformed by plasmids containing conserved mutations V148T and V148I exhibited a modest reduction of 24% and 18%, respectively, of the β-galactosidase activity (Fig. 2*B*). In contrast, cells transformed by plasmids carrying RseA mutations V148H and V148K displayed a marked reduction of β-galactosidase activity by 69% and 73%, respectively (Fig. 2*B*). These in vivo results are consistent with the in vitro observations.

Conserved Mutation of Val-148 Is Tolerated. Characterization of the four RseA mutants suggests that the identity of residue 148 may

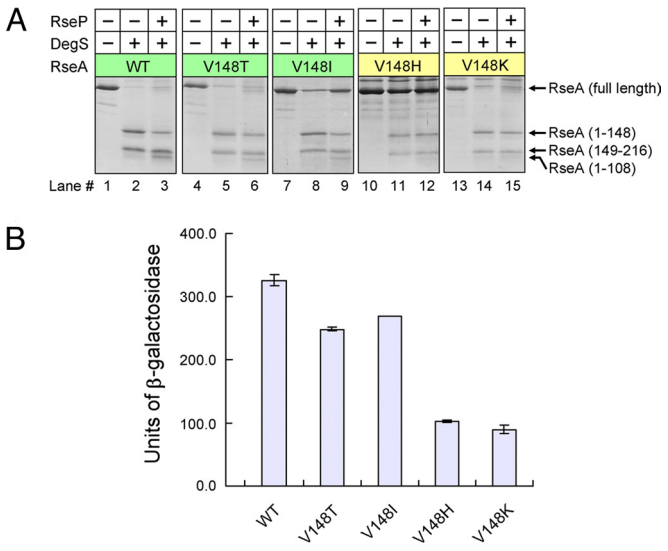

Fig. 2. Residue 148 of RseA plays an important role for Site-2 cleavage. (*A*) Mutation of Val-148 to Thr or Ile, but not His or Lys, allowed Site-2 cleavage. DegS and OMP peptide were added together to the reactions where DegS is indicated. (*B*) Mutation of Val-148 to Thr or Ile in RseA allowed a more robust envelope stress response than mutation of Val-148 to His or Lys.

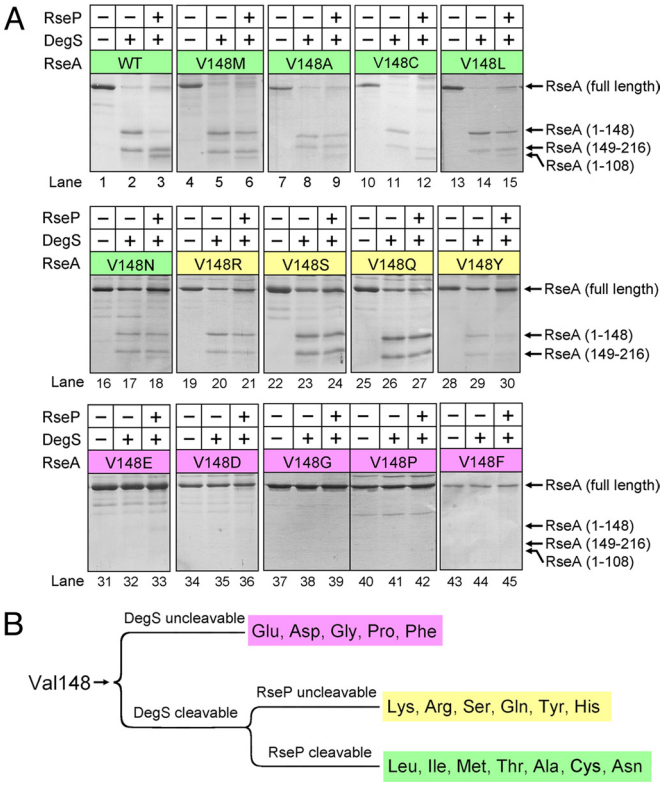

Fig. 3. Conserved mutation of Val-148 in RseA allowed retention of Site-2 cleavage. (*A*) Mutation of Val-148 to conserved, but not dissimilar or charged, amino acids in RseA allowed retention of Site-2 cleavage. DegS and OMP peptide were added together to the reactions where DegS is indicated. (*B*) Classification of three categories of amino acids at position 148 of RseA based on their impact on Site-1 and Site-2 cleavages. Mutation of Val-148 to any of the five amino acids—Glu, Asp, Gly, Pro, and Phe—crippled Site-1 cleavage of RseA by DegS. Among the mutations that allow Site-1 cleavage, six (mutation of Val-148 to Lys, His, Arg, Ser, Gln, and Tyr) do not allow Site-2 cleavage.

play an essential role in the Site-2 cleavage of RseA by RseP. To systematically investigate this scenario, we sought to mutate Val-148 to all other 15 amino acids and succeeded in all except the mutation V148W. These 14 RseA mutant proteins were individually purified and subjected to DegS and RseP cleavage assays (Fig. 3*A*).

Compared with the WT RseA (Fig. 3*A*, lanes 1–3), mutants V148M, V148A, V148C, and V148L, each involving replacement of Val-148 by a conserved amino acid, exhibited a reduced but detectable level of RseP cleavage (Fig. 3*A*, lanes 4–15). RseA-V148N was also cleaved by RseP (Fig. 3*A*, lanes 16–18), although the cleavage efficiency was markedly reduced compared with the WT RseA (Fig. S3). By contrast, V148R, V148S, V148Q, and V148Y allowed the Site-1 cleavage by DegS but crippled the Site-2 cleavage by RseP to an undetectable level (Fig. 3*A*, lanes 19–30). The remaining mutations, including V148E, V148D, V148G, V148P, and V148F, did not even allow Site-1 cleavage (Fig. 3*A*, lanes 31–45).

These in vitro results give rise to a pattern (Fig. 3*B*). Val appears to be the optimal amino acid at position 148, allowing efficient cleavage by both DegS and RseP. Uncharged amino acids, which are similar to Val by side-chain length (Thr and Asn) or hydrophobicity (Ala, Cys, Ile, Leu, and Met), appear to be tolerated at position 148 of RseA and allowed the Site-2 cleavage to occur. Positively charged or dissimilar polar amino acids at position 148 (Lys, Arg, His, Gln, and Tyr) allowed DegS cleavage but failed to allow RseP cleavage. Finally, negatively charged amino acids (Asp and Glu), Gly, Pro, and Phe did not allow Site-1 cleavage by DegS. Therefore, the identity of the carboxyl-terminal residue of the RseA fragment 1–148 governs whether Site-2 cleavage of RseA occurs and, if so, to what extent. This finding suggests an explanation to the requirement of Site-1 cleavage before Site-2 cleavage.

Unique Importance of Residue 148 for Site-2 Cleavage. To further determine the sequence requirement for Site-2 cleavage, we investigated the importance of residue 147 by generating 19 mutant RseA proteins, each involving a single missense mutation of Pro-147 to a distinct amino acid. These 19 RseA mutant proteins were individually purified and subjected to DegS and RseP cleavage assays (Fig. S4). The results indicate that none of the mutations crippled the ability of the mutant RseA to be cleaved by DegS or by RseP. Next, we took the same exhaustive approach to examine the role of residue 146 in RseA. Again, the results show that all mutant RseA proteins retained the ability to be cleaved by DegS and RseP (Fig. S5). These results are in sharp contrast to those on residue 148, and they unambiguously demonstrate that the identity of residue 148, but not residue 147 or 146, governs the Site-2 cleavege of RseA by RseP.

Essential Role of RseP PDZ Domains in Site-2 Cleavage. But how does the carboxyl terminus of RseA 1–148 bring about RseP-mediated Site-2 cleavage? There are two possibilities: the carboxyl terminus of RseA 1–148 may directly activate the RseP protease, or it may help recruit the RseA fragment to RseP for cleavage. Both scenarios require interactions between RseP and the carboxyl terminus of RseA 1–148. Because PDZ domains are known to specifically recognize carboxyl-terminal residues (24), the carboxyl terminus of RseA 1–148 is likely to transiently interact with the PDZ domains of RseP. In this case, mutation of the peptide-binding groove on the surface of the PDZ domains might abolish interactions with the carboxyl terminus of RseA 1–148, resulting in loss of Site-2 cleavage. To examine this scenario, we generated two full-length RseP mutants, G214A/I215A and G303A/I304A, each of which contained two missense mutations that were designed to disrupt the putative peptide-binding groove. We purified these two RseP mutants and examined their activity on the WT RseA substrate (Fig. 4*A*). Strikingly, both RseP mutant proteases abrogated their proteolytic activity and were unable to further process RseA 1–148 (Fig. 4*A*, lanes 5–8). To pinpoint the essential residues, we generated and purified two missense RseP mutants, I215A and I304A, each of which contained a single missense mutation in the putative peptide-binding groove. Compared with the WT RseP, these mutants also exhibited crippled protease activity toward RseA 1–148 (Fig. 4*B*). These observations indicate that the integrity of the putative peptide-binding grooves in the PDZ domains of RseP is required for Site-2 cleavage.

Binding of Val-148 to the Second PDZ Domain of RseP. Our analysis suggests a direct interaction between the PDZ domains of RseP and the carboxyl-terminal peptide derived from RseA 1–148. However, despite repeated trials, we failed to detect such interactions by using isothermal titration calorimetry. We reasoned that this could be due to two possibilities: the relatively weak interactions between the isolated PDZ domain and the peptide, and/or the inappropriate conformation of the PDZ domains for binding to the RseA peptide.

To investigate these possibilities, we individually crystallized the first (PDZ1; residues 127–220) and the second (PDZ2; residues 222–309) PDZ domains of RseA and determined their X-ray structures at 1.7- and 3.0-Å resolution, respectively (Table S1). The overall structures are similar to each other and are both classified as a circular permutation of normal PDZ domains (24), where the carboxyl-terminal strand (β5) forms one side of the putative peptide-binding groove (Figs. 4 *C* and *D*, and 5*A*). Despite overall similarity, there are important structural differences between the two PDZ domains. Although PDZ1 was crystallized as a monomer (Fig. 4*C*), PDZ2 existed as an artificial homodimer in the crystals (Fig. 4*D*). Whereas the anticipated peptide-binding pocket is well-

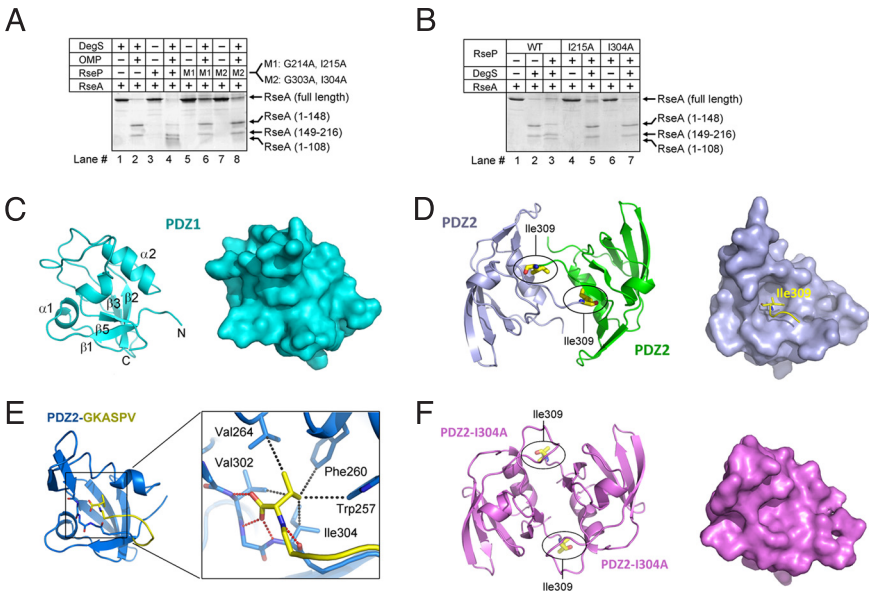

Fig. 4. Integrity of the peptide-binding grooves in RseP PDZ domains is essential for Site-2 cleavage. (*A*) Mutation of residues in the putative peptide-binding groove of RseP PDZ domains led to loss of Site-2 cleavage. Gly-214/Ile-215 and Gly-303/Ile-304 are predicted to be located in the putative peptide-binding grooves of PDZ domains 1 and 2, respectively. (*B*) A single missense mutation in the putative peptide-binding groove of RseP PDZ domains led to loss of Site-2 cleavage. (*C*) Structure of the first PDZ domain (PDZ1; cyan) is shown in ribbon diagram (*Left*) and surface representation (*Right*). Note the absence of the putative peptide-binding groove. (*D*) Structure of the second PDZ domain (PDZ2). (*Left*) The homodimer of PDZ2 in an asymmetric unit. (*Right*) The surface representation of one PDZ2 domain, with its peptide-binding pocket accommodating Ile-309 from the adjacent PDZ domain. (*E*) Structure of PDZ2-GKASPV, which has the RseA peptide $G_{143}KASPV_{148}$ fused to its carboxyl terminus. Val-148 binds to the surface pocket of PDZ2 through four specific hydrogen bonds (red dashed lines) and a number of van der Waals contacts (black dashed lines). (*F*) Structure of PDZ2-I304A. (*Left*) The two molecules of PDZ2-I304A in an asymmetric unit. (*Right*) The surface representation of PDZ2-I304. The mutation I304A deforms the putative peptide-binding pocket.

formed on PDZ2, it is blocked by a short α-helix in PDZ1. Interestingly, within the homodimer of PDZ2, the putative peptide-binding pocket of one domain was occupied by the carboxyl-terminal residue Ile-309 of the adjacent domain (Fig. 4*D*). In contrast to PDZ2, the putative peptide-binding pocket of PDZ1 was occupied by Val-210 from the short α-helix (Fig. S6*A*). Thus, PDZ1 is unable to accommodate peptide ligand in the present conformation. These observations strongly suggest that PDZ2, but not PDZ1, may directly bind to the carboxyl terminus of RseA 1–148.

To obtain direct evidence, we fused the RseA peptide $G_{143}KASPV_{148}$ to the carboxyl terminus of PDZ2, crystallized the fusion protein, and solved its structure at 1.6-Å resolution (Table S1). In contrast to the WT domain (Fig. 4*D*), PDZ2-GKASPV existed as a monomer in the crystals (Fig. 4*E*). The carboxyl-terminal residue Val-148 of the peptide $G_{143}KASPV_{148}$ bound to the hydrophobic pocket of PDZ2 (Fig. 4*E*). The carboxylate oxygen atoms of Val-148 accept three hydrogen bonds from the backbone amid groups of residues 302–304, whereas the amide nitrogen atom

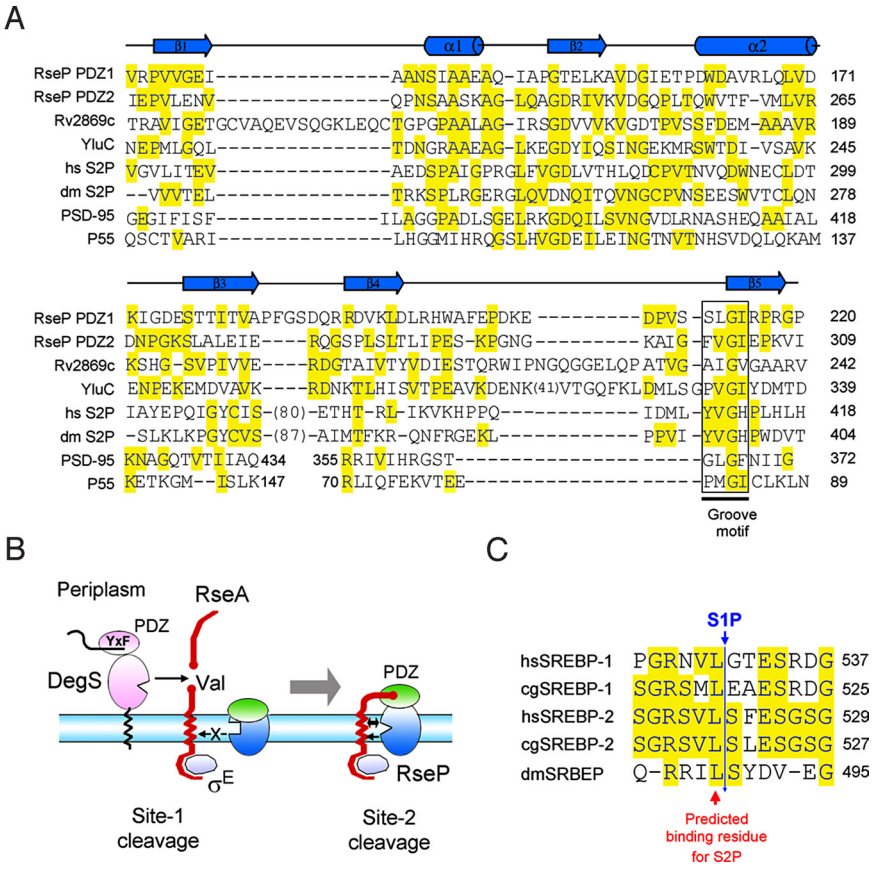

Fig. 5. A proposed model on the mechanism of Site-2 cleavage. (*A*) Sequence alignment of PDZ domains from representative S2Ps and two non-S2P proteins (PSD-95 and P55). Conserved amino acids are colored yellow. Shared secondary structural elements of RseP PDZ1 and PDZ2 are shown above the sequences. Note the presence of extra amino acids preceding strand β5 of S2P proteins. (*B*) A proposed model on the mechanism of Site-2 cleavage. After DegS cleavage, the newly exposed carboxyl terminus of RseA directly binds to the PDZ domains of RseP, and this binding is essential for the Site-2 cleavage. This binding may activate RseP or help recruit RseA 1–148 to the protease activity of RseP. (*C*) Sequence alignment of representative SREBPs reveals a hydrophobic amino acid at the newly exposed carboxyl terminus after the Site-1 cleavage.

of Val-148 donates a hydrogen bond to the carbonyl group of Ile-304. In addition, the hydrophobic side chain of Val-148 is nestled in a greasy pocket formed by Trp-257, Phe-260, Val-264, Val-302, and Ile-304. Consistent with our biochemical analysis, Pro-147 and Ser-146 of the peptide $G_{143}KASPV_{148}$ do not interact with PDZ2. The size of the PDZ2 peptide-binding pocket can only accommodate one carboxyl-terminal amino acid, as opposed to that of a canonical PDZ domain, which accommodates three residues (24).

These structural features suggest a scenario in which Val-148 of RseA, after DegS cleavage, directly binds to PDZ2. In this case, mutation in the peptide-binding pocket of PDZ2, such as I304A, may change the conformation of the pocket and negatively affect Val-148 binding, consequently crippling Site-2 cleavage. To examine this hypothesis, we crystallized and determined the structure of the PDZ2 mutant I304A (Table S1). Compared with the WT PDZ2 (Fig. 4*D*), the size of the peptide-binding pocket in the I304 mutant was substantially reduced (Fig. 4*F* and Fig. S6*B*). The reduced size of the surface pocket in PDZ2-I304A was no longer capable of binding to Val-148 or a carboxyl-terminal hydrophobic residue. Indeed, the carboxyl-terminal residue Ile-309 of PDZ2-I304A was not accommodated in the pocket (Fig. 4*F*). These observed structural features of PDZ2-I304A are fully consistent with the loss of protease activity of RseP-I304A (Fig. 4*B*).

Discussion

In this study, we demonstrated that the identity of the newly exposed carboxyl-terminal residue 148 of RseA, as a result of Site-1 cleavage by DegS, determines whether Site-2 cleavage can occur and, if so, the extent. We also show that the identity of residues 146 and 147 has no impact on Site-2 cleavage. These findings provide a plausible explanation to the puzzle of why Site-2 cleavage must be preceded by Site-1 cleavage. Conserved mutation of Val-148 (to Ala, Cys, Ile, Leu, Met, Thr, and Asn) allowed both Site-1 and Site-2 cleavages. Then, we showed that mutation of residues in the putative peptide-binding groove of RseP PDZ domains abolished the protease activity of RseP. Subsequent structural analysis revealed that the second, but not the first, PDZ domain of RseP exists in an open conformation and directly binds to Val-148 through a combination of hydrogen bonds and van der Waals contacts.

If the carboxyl terminus of RseA 1–148 binds to PDZ2, why does mutation in PDZ1 also lead to loss of Site-2 cleavage, then? We speculate that in the context of full-length RseP, peptide binding to PDZ2 may require an appropriate conformation of PDZ1, and perturbation of PDZ1 conformation may negatively affect the ability of PDZ2 to interact with the carboxyl terminus of RseA 1–148. This speculation is based on sequence and experimental considerations. First, the linker sequence between the core structural domains of PDZ1 and PDZ2 only comprises two amino acids (Fig. 5*A*), suggesting tight coupling and potential interaction between PDZ1 and PDZ2. Second, a number of other mutations in PDZ1 and PDZ2, which do not map to the peptide-binding groove, also led to abrogation of Site-2 cleavage. These findings suggest that the native conformations of both PDZ1 and PDZ2 are required for binding of the carboxyl terminus of RseA 1–148 to the PDZ2 domain of RseP.

The interactions between the RseP PDZ domains and the isolated RseA peptide are transient in nature. In contrast to the canonical peptide-binding PDZ domains (24), the PDZ2 domain of RseP contains a pocket, not a groove. Consequently, PDZ2 is ideally suited to accommodate a single hydrophobic amino acid, as opposed to a three-residue peptide (24). The interactions between Val-148 of the RseA peptide GKASPV and the surface pocket of PDZ2, involving four specific hydrogen bonds and a number of van der Waals contacts, were nicely captured by the crystal structure (Fig. 4*E*). The binding of Val by PDZ2 (Fig. 4*E*) involves appreciably more specific interactions than the accommodation of Ile, which has only two hydrogen bonds (Fig. S6*C*). This is consistent with our observation that the WT RseA, where Val-148 is exposed after DegS cleavage, is most efficiently cleaved by RseP (Fig. 3). In the context of lipid membrane, the transient interactions between the carboxyl-terminal residue of RseA 1–148 and RseP may be greatly facilitated by two-dimensional diffusion and by additional interactions between the transmembrane helices of RseA and RseP (25).

What is the biochemical consequence of PDZ2 binding by the carboxyl-terminal residue of RseA 1–148? One possibility is that this binding may directly activate the RseP protease (Fig. 5*B*), either by inducing some conformational changes in the RseP protease or by relieving the inhibitory role of the PDZ domains. Consistent with the latter scenario, deletion of the PDZ domains in RseP led to constitutive, S1P-independent Site-2 cleavage (26, 27). Another distinct possibility is that the PDZ domains of RseP may simply serve as a receptor for the carboxyl-terminal residue of RseA 1–148, and the binding results in the recruitment of RseA 1–148 to the protease active site of RseP (Fig. 5*B*). This scenario would be similar to the case of γ-secretase, in which the ectodomain of nicastrin directly binds to the newly exposed amino terminus of a substrate protein, such as amyloid precursor protein (APP) or Notch, after it is cleaved by a prior protease (28). Differentiation between these two possibilities may require additional biochemical and structural analysis.

The PDZ domains of known S2Ps are predicted to contain a surface peptide-binding site, with the sequence Ö[I/V/L/M]G[I/V/F/H] (where Ö denotes an uncharged amino acid) as one side of the putative binding pocket (Fig. 5*A*). Except RseP, most S2Ps contain only one PDZ domain, which likely corresponds to PDZ2 of RseP and is responsible for binding to the newly exposed carboxyl-terminal residue after Site-1 cleavage of the substrate protein. This notion is supported by analysis of the S1P cleavage sites in characterized SREBP sequences (Fig. 5*C*). For example, cleavage of human SREBP-2 by S1P exposes the carboxyl-terminal, hydrophobic amino acid Leu-522 (Fig. 5*C*).

One caveat is that our experiments were mostly performed in vitro under conditions of detergent micelles, rather than lipid membrane. As such, how relevant are the conclusions to living cells? We note that membrane proteins are thought to adopt similar conformations in detergent micelles compared with those in lipid membrane. In fact, structures of most integral membrane proteins were obtained under detergent micelles, and they reveal important functional insights. In vitro characterization represents an indispensable approach for elucidating the detailed molecular mechanisms and has been applied successfully to the study of RIP signaling and yielded important insights (3, 8). Last, but not least, the sequential cleavages of RseA by DegS and RseP were recapitulated in vitro (Fig. 1), arguing that the essential elements of S2P cleavage have been faithfully retained in the in vitro system.

While this manuscript was under preparation, Inaba et al. (29) reported the biochemical characterization of the two PDZ domains of RseP. In their study, a majority of the loss-of-function mutations were mapped to the putative peptide-binding region of PDZ1, suggesting a potential role in ligand binding (29). We note that PDZ1 exists in a closed conformation, where Val-210 occupies the putative peptide-binding pocket (Fig. 4*C* and Fig. S6*A*). Displacing Val-210 may be energetically unfavorable for the putative ligand and would be even less likely in cases of transient ligand binding. Hence, we favor the idea that the putative ligand, which we identified as the carboxyl terminus of RseA 1–148, binds to the unobstructed pocket on PDZ2. A RseP mutant, A115V/G214E, was reported to cleave RseA independently of Site-1 cleavage by DegS (29). We purified this mutant RseP but were unable to detect any protease activity under conditions in which the WT RseP was active. Finally, the PDZ structures in our study, but not in the previously published study (29), represent those of the WT proteins.

Our study answers the question of why Site-2 cleavage must be preceded by Site-1 cleavage in the case of bacterial envelope stress

response. The answer is surprisingly simple—the newly exposed carboxyll terminus of RseA after DegS cleavage plays an essential role in the activation of RseP. Yet, how this is accomplished remains to be investigated. It also remains to be seen whether the conclusions derived from RseP are generally applicable to other PDZ-containing S2Ps (30).

Materials and Methods

Protein Preparation, Crystallization, and Structure Determination. A detailed description of protein preparation, crystallization, data collection, and structure determination of PDZ domains is available in the *SI Materials and Methods*. Full data collection and processing statistics are shown in Table S1.

In Vitro DegS and RseP Cleavage Assays. Protein preparation is described in *SI Materials and Methods*. The WT and mutant RseA proteins were used as the substrate. The assay was performed at 37 °C for 90 min in a buffer containing 50 mM $NaHPO_4$, pH 8.4; 400 mM NaCl; 0.02% n-dodecyl-β-D-maltoside (wt/vol); and 3.3% glycerol. The final concentrations of the substrate proteins were $\approx$0.1 μM. The final concentrations of DegS and OMP peptide (DNRDGNVYYF) were 0.05 and 10 μM, respectively. The final RseP concentration was 0.05 μM. The reaction was stopped by SDS sample buffer, and the cleavage products were analyzed by SDS/PAGE and Coomassie staining.

In Vitro Cleavage of RseA 1–148 by RseP. The WT RseA 1–148 protein was used as the substrate. The assay was performed at 37 °C for 90 min in a buffer containing 10 mM Tris·HCl, pH 8.0; 150 mM KCl; 0.02% DDM (wt/vol); and 4 mM DTT. The final concentrations of the substrate protein were $\approx$6 μM. The final RseP concentration was 1.5 μM. The reaction was stopped by SDS sample buffer, and the cleavage products were analyzed by SDS/PAGE and Coomassie staining.

β-Galactosidase Assays. The σ^E activity was assayed by monitoring β-galactosidase activity expressed from a σ^E-dependent lacZ reporter gene. After growing for 4 h at 37 °C in M9 medium, the transformed cells were stressed by being transferred to 42 °C for 3 h. The final OD_{600} of the cells was 1.0–1.5. All assays were performed at least twice reproducibly. Data from all samples with error were shown. Assays were performed as described previously (31).

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