Substrate Recognition and Binding by RseP, an Escherichia coli Intramembrane Protease

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I-CLiPs are a class of membrane proteases that are widely distributed from bacteria to higher eukaryotes and play pivotal roles in a variety of biological processes (1, 2). These proteases generally catalyze cleavage of target membrane proteins within or around their transmembrane segments, leading to a release of the soluble domains into the secretory compartments and/or cytoplasm. The soluble domains that are liberated from the membrane act as biologically active molecules such as transcription factors and growth factors. I-CLiPs can be classified into three groups based on their catalytic residues: the S2P (metalloprotease) family, the rhomboid (serine protease) family, and the γ-secretase and signal peptide peptidase (asparyl protease) family (1, 2).

The S2P proteases regulate sterol and lipid metabolism in eukaryotic cells (3–5). They are also involved in the endoplasmic reticulum stress response pathway (6–8). Recent studies revealed that the prokaryotic S2P homologs function in membrane-based events such as stress response (9), sporulation (10), cell division (11), and cell differentiation (12). In Escherichia coli, the S2P homolog RseP is an essential player in the transmembrane signaling involved in the σE pathway of extracytoplasmic stress responses (13–15). σE is a sigma factor dedicated to the transcription of genes that encode proteases, chaperones, and folding catalysts in the cell envelop, and it is activated by a cascade of intracellular proteolysis of its inhibitor (16, 17). Under resting conditions, σE is kept inactive through its tight association with the N-terminal cytoplasmic domain of the membrane-bound anti-σE protein, RseA, which has a type II (NIN-COUT) topology. When cells are exposed to extracytoplasmic stresses, stress signals such as unassembled outer membrane proteins directly activate DegS, a membrane protease with a periplasmic active site (18), to cleave RseA on the periplasmic side (site-1 cleavage). This first proteolysis triggers the second (site-2) cleavage of RseA by RseP within its transmembrane region (RseA TM), leading to the liberation of a complex between the RseA cytoplasmic domain and σE (14, 15, 19). Finally, the RseA cytoplasmic domain is degraded by cytoplasmic proteases, allowing σE to transcribe the target genes (20, 21). RseP can only act on RseA after the site-1 cleavage by the activated DegS. The inability of RseP to act against full-length RseA ensures that the activation of σE is strictly stress-dependent (14, 15). It has been suggested that factors including the PDZ domain of the RseP periplasmic region, the glutamine-rich regions in the RseA periplasmic region, DegS, and periplasmic protein RseB, contribute to the suppression of proteolysis of full-length RseA by RseP (22–24). However, its exact mechanism remains unclear.

Although the essential cellular function of RseP lies in the activation of σE through the cleavage of RseA, model experiments showed that RseP has an unexpectedly wide substrate specificity, by which it can cleave several unrelated transmembrane sequences, including the first and the fifth transmembrane sequences of lactose permease (LacY) (19). Systematic mutational analysis showed that cleavage of a model substrate having the first transmembrane segment of LacY (LacYTM1) is promoted by helix-distabilizing residues in the LacYTM1 sequence (19). The RseP cleavage sites have been determined to lie within the predicted membrane-embedded regions of RseA...
TM and LacYTM1 (19). It has been suggested that I-CLiPs catalyze proteolysis within the lipid bilayer, raising a question of how water molecules required for hydrolysis of a peptide bond are supplied to the proteolytic active site in the membrane. We analyzed the environment of the RseP active site based on the accessibility of various thiol-alkylating reagents to the Cys residues introduced around the catalytic residues of RseP (25). Our results suggested that the active site of RseP is neither totally embedded in the lipid phase nor exposed to the cytoplasm, but rather it is located within a folded protein domain partially embedded in the membrane. This is in sharp contrast to the cases of GlpG, an E. coli rhomboid homolog, and γ-secretase; biochemical, and structural studies have shown that the active sites of these enzymes are open to the extramembrane milieu and freely accessible to water (26–29). It is currently unclear how the substrates and water molecules are presented to the sequestered active site of RseP.

Despite their biological importance, the action mechanisms of I-CLiPs remain largely elusive. In the present study, we biochemically analyzed the interaction between RseP and model substrates. Our results demonstrate the importance of the helix-destabilizing residues of a substrate transmembrane segment in its stable association with RseP and the involvement of the third transmembrane region of RseP in substrate binding. These results provide basic information useful to elucidate the molecular mechanism of proteolysis catalyzed by RseP and the S2P family proteases.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Media**—We used *E. coli* K12 strains that are derivatives of AD16 (Δpro-lac thi F lacZ AM15 Y+ pro+*) (30), KK211 (AD16, rseA::cat, rseP::kan), AD1840 (AD16, rseA::cat, rseP::kan, degS::tet) (14), and KK31 (AD16, rseP::kan, Δ(srl-recA)306::Tn10/pKK6 (Para-rseP)) (13) described previously.

L broth (31) and M9 medium (32) were used as nutrients and minimal media, respectively. Ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), and/or spectinomycin (50 μg/ml) were added for selection of transformants as well as for growing plasmid-bearing strains.

**Plasmids**—The plasmids used in this study are listed in Table 1. Plasmids encoding derivatives of HA-MBP-RseA(LacYTM1)140 having the M1, M6, and M12 mutant forms of the LacYTM1 sequence were constructed by site-directed mutagenesis (33) of pSTD835. pSTD1014 was constructed by cloning a SacI-HindIII fragment of pKK50 (19) into the same sites of pST689 (22). Plasmids for single Cys, H22F, and E23S derivatives of RseP(Cys-less)-His6-Myc were constructed with a site-directed mutagenesis of pSTD892 (25). Plasmids coding for the RseP-His6-Myc derivatives with a replacement of Asn-389 by other residues were also constructed by site-directed mutagenesis of pSTD892 and pKD157. pKD99 (HA-RseA140) was constructed by ligating an EcoRI-HindIII fragment of pKK58 (HA-RseA140) (13) with pSTD689 that had been digested with the same enzymes. For construction of pKD126 encoding a Cys-less derivative of HA-RseA140, the codon for the unique Cys residue in RseA (Cys-109) was changed to the codon for Ala by site-directed mutagenesis of pKD99.

| Plasmid | Encoded protein | References |
|---------|----------------|------------|
| pTVV228 | Vector         | Takara Shuzo |
| pTV807 | Vector         |            |
| pSTD689 | HA-MBP(LacYTM1)-RseA140 | (19) |
| pSTD835 | HA-MBP(LacYTM1/M6)-RseA140 | (19) |
| pSTD840 | HA-MBP(LacYTM1/M1)-RseA140 | (19) |
| pSTD872 | HA-MBP(LacYTM1/M12)-RseA140 | (19) |
| pSTD881 | HA-MBP-RseA140 | (19) |
| pSTD929 | RseP(Cys-less)-His6-Myc | (25) |
| pSTD925 | RseP(P399C)-His6-Myc | This study |
| pSTD1014 | RseP(H22F)-His6-Myc | This study |
| pSTD1017 | HA-MBP(LacYTM1)-RseA140 | (25) |
| pSTD1041 | RseP(N389C/H22F)-His6-Myc | This study |
| pK44 | RseP-His6-Myc | (13) |
| pKD12 | RseP(N389C)-His6-Myc | This study |
| pKD51 | RseP(P399C/H22F)-His6-Myc | This study |
| pKD62 | RseP(N394C)-His6-Myc | This study |
| pKD63 | RseP(P397C)-His6-Myc | This study |
| pKD65 | RseP(N389C/H22F)-His6-Myc | This study |
| pKD67 | RseP(N389G)-His6-Myc | This study |
| pKD68 | RseP(N389L)-His6-Myc | This study |
| pKD70 | RseP(N389Q)-His6-Myc | This study |
| pKD76 | RseP(N389C/H22F)-His6-Myc | This study |
| pKD77 | RseP(N389L/H22F)-His6-Myc | This study |
| pKD79 | RseP(N389Q/H22F)-His6-Myc | This study |
| pKD84 | RseP(Cys-less/E23S)-His6-Myc | This study |
| pKD92 | RseP(N394C/E235S)-His6-Myc | This study |
| pKD96 | RseP(N394C/E235S)-His6-Myc | This study |
| pKD97 | RseP(P397C/E235S)-His6-Myc | This study |
| pKD98 | RseP(P399C/E235S)-His6-Myc | This study |
| pKD104 | HA-RseA140 (having C109) | This study |
| pKD109 | HA-RseA(S111C)-140 | This study |
| pKD114 | HA-RseA(V106C)-140 | This study |
| pKD23 | HA-RseA(G105C)-140 | This study |
| pKD24 | HA-RseA(A108C)-140 | This study |
| pKD26 | HA-RseA(Cys-less)-140 | This study |
| pKD36 | HA-RseA(M104C)-140 | This study |
| pKD137 | HA-RseA(A107C)-140 | This study |
| pKD158 | HA-RseA(V110C)-140 | This study |
| pKD150 | RseP(N389G/P399C/E235S)-His6-Myc | This study |
| pKD151 | RseP(N389L/P399C/E235S)-His6-Myc | This study |
| pKD152 | RseP(N389Q/P399C/E235S)-His6-Myc | This study |
| pKD156 | RseP(P397C/H22F)-His6-Myc | This study |
| pKD157 | RseP(Cys-less/H22F)-His6-Myc | This study |

For single Cys derivatives of HA-RseA140 were constructed by site-directed mutagenesis of pKD126. The DNA sequences of pKD151 and pKD157 were confirmed by sequencing.

**Analysis of the in Vivo Proteolysis of the Model Substrates**—In vivo activity assays (25) of single Cys derivatives of RseP-His6-Myc, cells of KK211 (ΔrseA ΔrseP) carrying pSTD881 (HA-MBP-RseA140) or pSTD1017 (HA-MBP-RseA(LacYTM1)140) were transformed further with a plasmid encoding a derivative of RseP-His6-Myc to be tested. The transformants were grown in M9 medium supplemented with 20 amino acids (20 μg/ml each), thiamine (2 μg/ml), glucose (0.4%), and IPTG (1 mM) at 30 °C for 3 h. For assays of the cleavage of the HA-MBP-RseA(LacYTM1)140 derivatives by chromosomal RseP, cells of AD1811 (ΔrseA) and KK211 (ΔrseA ΔrseP) were transformed with a plasmid encoding a derivative of HA-MBP-RseA(LacYTM1)140. Cells were grown in L broth containing IPTG (1 mM) at 30 °C for 3 h. Proteins were analyzed by SDS-PAGE and immunoblotting using anti-Myc and/or anti-HA antibodies as described previously (19).

**Co-immunoprecipitation Assays**—Cells of KK211 (ΔrseA ΔrseP) carrying an appropriate combination of plasmids encoding derivatives of RseP-His6-Myc and HA-MBP-RseA140 were grown at 30 °C for 3.5 h in L medium containing 1 mM IPTG and...
**RESULTS**

**Helix-destabilizing Residues Are Required for a Substrate Transmembrane Segment to Be Stably Bound by RseP**—A model substrate, HA-MBP-RseA(LacYTM1)140, which is composed of a cytoplasmic, HA-tagged MBP domain, LacYTM1, and a short periplasmic region derived from RseA (Fig. 1), mimics the DegS-cleaved intermediate form of RseA and receives DegS-independent cleavage within the LacYTM1 sequence by RseP (19). When HA-MBP-RseA(LacYTM1)140 was expressed in the ΔrseP strain, it accumulated as the full-length form ( uncleaved, UC), as revealed by anti-ΔHA immunoblotting. In the presence of the chromosomally encoded RseP, it was converted to a cleaved form (CL) (Fig. 2A and B, WT). We previously showed that helix-destabilizing residues in the LacYTM1 sequence promoted its proteolytic cleavage by RseP (19). Residues Leu, Ala, Gly, Tyr, Asn, and Pro have been aligned in this order according to their helix-forming to helix-destabilizing propensities in a membrane-mimicking environment (34). As previously reported (19), replacement of Pro, a strong helix breaker, at position 28 by helix-forming Leu severely impaired the cleavage (Fig. 2, A and B, M1). Also, simultaneous replacement of Gly-13, Gly-25, and Ala-26 by Leu significantly decreased the cleavage efficiency (Fig. 2, A and B, M6), but an additional introduction of Asn at the position of Tyr-19 partially restored the cleavage efficiency (Fig. 2, A and B, M12).

On the basis of these observations, we investigated possible roles of helix-destabilizing residues in the substrate-enzyme interaction. A C-terminally His6-Myc-tagged RseP (RseP-His6-Myc) was co-expressed with the wild-type or mutant forms of the model substrates in the ΔrseP ΔdegS strain. The RseP derivatives used in this experiment and in the following pulldown and cross-linking experiments contained an amino acid alteration (H22F or E23S) in the protease active site motif (HEXXH) to prevent degradation of substrate proteins. Membranes were prepared, treated with or without a membrane-permeable cross-linker, DSP, and solubilized with DDM. Samples were then subjected to immunoprecipitation with anti-ΔHA beads. The solubilized membrane proteins and the anti-ΔHA precipitates were analyzed by immunoblotting with anti-ΔHA and anti-Myc antibodies (Santa Cruz Biotechnology, Inc.) at 4 °C for 3.5 h with rotation. Immunocomplexes were collected, washed three times with buffer C (Buffer B plus 0.1% DDM), and dissolved in SDS sample buffer with or without 2-mercaptoethanol. The samples were analyzed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-Myc and rabbit polyclonal anti-HA.

**Disulfide Cross-linking—**Cells of AD1840 (ΔrseA, ΔrseP, and ΔdegS) containing an appropriate combination of plasmids encoding derivatives of RseP-His6-Myc and HA-RseA140 were grown at 30 °C in L medium containing 1 mM IPTG and 1 mM cAMP for 3.5 h. Then, a portion of the culture was removed and mixed with chloramphenicol (200 μg/ml) and 3 mM 2-phenanthroline. Cells were harvested, washed with 10 mM Tris-HCl (pH 8.1) containing 3 mM 2-phenanthroline, and suspended in 10 mM Tris-HCl (pH 8.1). They were treated with 1 mM Cu2+ (phenanthroline)3 or 3 mM 2-phenanthroline for 5 min. The oxidation reaction was terminated by incubation with 12.5 mM neocuproine for 5 min at 37 °C. Proteins were precipitated with 5% trichloroacetic acid, washed with acetone, and dissolved in buffer containing 1.5% SDS, 100 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 25 mM N-ethylmaleimide at room temperature for 30 min. N-Ethylmaleimide was included to prevent formation of disulfide bonds after protein denaturation. A portion of each sample was mixed with an equal volume of 1 × SDS sample buffer containing no reducing reagent and further incubated at 37 °C for 5 min. For cleavage of disulfide bonds, another portion of each sample was mixed with 1 × SDS sample buffer containing 100 mM DTT and incubated at 37 °C for 5 min. The samples were analyzed by 12.5% SDS-PAGE and anti-Myc and anti-ΔHA immunoblotting.

1 mM cAMP. Cells were harvested, suspended in 50 mM HEPES-KOH (pH 7.5), and disrupted by sonication. Next, total membranes were collected by ultracentrifugation, suspended in buffer A (50 mM HEPES-KOH (pH 7.5)/50 mM KCl/20% glycerol), and treated with 0 or 0.2 mg/ml DSP, a cleavable, homobifunctional cross-linker, at 4 °C for 10 min to quench DSP, diluted 10-fold with buffer B (50 mM HEPES-KOH (pH 7.5)/300 mM KCl/10% glycerol), and solubilized with 1% DDM on ice for 1 h. After clarification by ultracentrifugation, the supernatant was incubated with agarose-conjugated mouse monoclonal anti-HA (F-7) antibodies (Santa Cruz Biotechnology, Inc.) at 4 °C for 3.5 h with rotation. Immunocomplexes were collected, washed three times with buffer C (Buffer B plus 0.1% DDM), and dissolved in SDS sample buffer with or without 2-mercaptoethanol. The samples were analyzed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-Myc and rabbit polyclonal anti-HA.

**Schematic representations of RseP and the model substrates.** A, possible topological arrangement of RseP and the model substrates in the membrane. TM1–4 indicate transmembrane regions 1–4 of RseP, respectively. The approximate positions of the HExxH and LDG motifs that are suggested to constitute the metalloprotease active site of RseP are shown. B, the amino acid sequences of the RseP TM3 and RseA TM regions. The possible transmembrane regions are underlined. The amino acid residues are numbered according to the numbering of the original proteins. The residues that were individually replaced by cysteine in the present study are shown in bold. The residues individually replaced by cysteine in the previous study are marked by dots. The LDG motif is boxed. The arrow indicates the RseP cleavage site.

**FIGURE 1.**
panels lized with 1% DDM and subjected to immunoprecipitation (HA-MBP-RseA(LacYTM1)140 were prepared from cells following sonic disrup-

tion of the substrate transmembrane segment. These results suggest that the helix destabilization of the substrate transmembrane segment is required for the stable formation of the substrate-enzyme complex.

Asn-389 Is Important for the Enzyme-Substrate Interaction—The region of RseP responsible for recognition and binding of a substrate had not been identified. Therefore, we next addressed this issue. We previously constructed a series of RseP-His6-Myc derivatives that contain a single engineered Cys residue in the first (TM1) and third (TM3) transmembrane regions (25). Complementation tests indicated that the N389C substitution in TM3 impaired the functionality along with the mutations at the protease active site motif (HEXXH and LDG) residues (Fig. 1 and supplemental Fig. S1) (25). We then characterized the N389C mutant of RseP in greater details and found that RseP(N389C)-His6-Myc had decreased proteolytic activity in accordance with the loss of complementation activity (Fig. 3). HA-MBP-RseA(LacYTM1)140 accumulated as the full-length protein (UC) when expressed in the ΔrseP strain (Fig. 3A, lanes 2 and 4). When wild-type RseP-His6-Myc or Cys-less RseP-His6-Myc was co-expressed with HA-MBP-RseA(LacYTM1)140, most of the substrate protein was cleaved (Fig. 3A, lanes 1 and 3, band CL). Co-expression of RseP(N389C)-His6-Myc resulted in markedly reduced efficiency of the cleavage (Fig. 3A, lane 5). Similar results were obtained with HA-MBP-RseA140, another model substrate containing RseA TM instead of LacYTM1 (Fig. 3B) (19). The N389C mutant form of RseP-His6-Myc accumulated to a similar level to the parental Cys-less RseP-His6-Myc, excluding the possibility that instability was responsible for the loss of the full function (Fig. 3A and B). These observations, as well as the fact that the N389C mutation has a reduced but significant proteolytic activity, suggest that the N389C mutation does not induce gross conformational changes in RseP.
One possible explanation for the decreased proteolytic activity of RseP(N389C)-His$_6$-Myc would be that the N389C mutation impairs the interaction with the substrate proteins. To test this possibility, we performed co-immunoprecipitation assays (Fig. 4). Membranes prepared from cells expressing a combination of HA-MBP-RseA140 and either RseP(Cys-less/H22F)-His$_6$-Myc or RseP(N389C/H22F)-His$_6$-Myc were solubilized with 1% DDM and subjected to anti-HA immunoprecipitation (IP) with mouse monoclonal anti-HA beads. Solubilized total membrane proteins (upper two panels) and immunoprecipitates (lower two panels) were analyzed by 12.5% SDS-PAGE and anti-HA or anti-Myc Western blotting (WB) as indicated. Filled and open arrowheads indicate the derivatives of HA-MBP-RseA140 and RseP-(Cys-less/H22F)-His$_6$-Myc, respectively.

To address the importance of the Asn-389 residue in RseP, we then mutated it to Gly, Leu, and Gln. Although the RseP(His$_6$-Myc variants with the N389G or the N389Q mutation retained the complementation activity, the N389C mutant was positive in the complementation assay. Taken together, these results demonstrate that Asn-389 plays an important role in the normal interaction of RseP with its substrates.

**Involvement of Other TM3 Residues (Asn-394, Pro-397, and Pro-399) in the RseP-Substrate Interaction**—To investigate the involvement of RseP TM3 in the RseP-substrate interaction, we mutated three additional residues, Asn-394, Pro-397, and Pro-399. Asn-394 and Pro-397 were chosen because they are highly conserved among RseP homologs (35). We included Pro-399, which is less conserved, in our targets, because we supposed that the helix-destabilizing nature of the two prolines in TM3 could have some role in the substrate interaction. These three residues were individually mutated to cysteine to generate RseP-His$_6$-Myc variants having a single Cys at these positions. The resulting mutant proteins had normal complementation activity (supplemental Fig. S1). Like N389C, the N394C and P397C mutant proteins were partially defective in the cleavage of HA-MBP-RseA140 and HA-MBP-RseA(LacYTM1)140 (Fig. 3, A and B, lanes 6 and 7). Although the P399C mutation only marginally affected the ability of RseP to cleave LacY TM1 (Fig. 3B, lane 8), it significantly impaired the cleavage of RseA TM (Fig. 3A, lane 8). The results of the pulldown experiments showed that Cys substitutions for Asn-394, Pro-397, and Pro-399 all impaired the interaction between RseP-His$_6$-Myc and HA-MBP-RseA140 (Fig. 5). These results collectively indicate that the TM3 residues Asn-389, Asn-394, Pro-397, and Pro-399 are required for RseP to normally recognize or bind the substrate protein.

**Disulfide Cross-linking between RseP TM3 and RseA TM**—To examine whether TM3 of RseP directly contacts the substrate TM segments, we carried out disulfide cross-linking experiments, using RseP variants having a single Cys at a position of Asn-389, Asn-394, Pro-397, or Pro-399 and HA-RseA140 variants having a single Cys in its transmembrane region (Fig. 6). HA-RseA140 is a C-terminally truncated version of HA-tagged RseA that is cleaved by RseP in the absence of DegS (19). We used HA-RseA140 as a substrate in this experiment instead of HA-MBP-RseA(LacYTM1)140 or HA-MBP-RseA140, because its size proved more suitable than the latter two in distinguishing between homo- and hetero-cross-linking (see the legend to Fig. 6). We constructed 8 single-Cys variants of HA-RseA140 having a Cys substitution in a segment of position 104 to position 111, in which the site-2 cleavage site is included (Fig. 1). None of these Cys substitutions in HA-RseA140 affected cleavage by RseP (data not shown). Cells expressing a combination of a mono-Cys variant of RseP and a
FIGURE 5. Effects of cysteine substitutions for Asn-394, Pro-397, and Pro-399 in TM3 of RseP on co-immunoprecipitation of RseP-His6-Myc with HA-MBP-RseA140. Plasmids pTYE007 (vector, lane 1), pKD157 (RseP(Cys-less/H22F)-His6-Myc, lane 2), pSTD1041 (RseP(N399C/H22F)-His6-Myc, lane 3), pKD66 (RseP(N389C/H22F)-His6-Myc, lane 4), pKD156 (RseP(N397C/H22F)-His6-Myc, lane 5), and pKD33 (RseP(N399C/H22F)-His6-Myc, lane 6) were introduced into strain KK211 (ΔrseA, ΔrseP1/pSTD881 (HA-MBP-RseA140)). Membrane proteins were solubilized with 1% DDM and subjected to immunoprecipitation (IP) with mouse monoclonal anti-HA beads. Solubilized total membrane proteins (upper two panels) and immunoprecipitates (lower two panels) were analyzed by 12.5% SDS-PAGE and anti-HA or anti-Myc Western blotting (WB) as indicated. Filled and open arrowheads indicate the derivatives of HA-MBP-RseA140 and RseP(Cys-less/H22F)-His6-Myc, respectively.

mono-Cys variant of HA-RseA140 were treated with Cu2+ (phenanthroline), an oxidizing reagent. After quenching the oxidant, proteins were acid-precipitated, solubilized in SDS, and analyzed by SDS-PAGE and immunoblotting with anti-HA and anti-Myc (Fig. 6, A and C). A portion of SDS-solubilized samples was treated with DTT to cleave disulfide bonds prior to SDS-PAGE (Fig. 6, B and D). Oxidation of the sample from cells expressing RseP(P399C/E23S)-His6-Myc and one of the HA-RseA140 derivatives having a Cys at positions 104, 105, 106, 107, or 108 gave rise to a band of ~80 kDa that was detected with either of anti-HA or anti-Myc (Fig. 6A, lanes 3–12). These bands disappeared upon reduction by DTT treatment (Fig. 6B, lanes 3–12) and were not formed when either RseP-His6-Myc (data not shown) or HA-RseA140 (Fig. 6A, lanes 1 and 2) lacked any cysteine residue: Thus, the 80-kDa band represents a disulfide-bonded product between RseP-His6-Myc and HA-RseA140. Additionally, a faint band of approximately the same size was detected with anti-HA and anti-Myc immunoblotting when HA-RseA140 contained Cys at position 109 or 110 (Fig. 6A, lanes 13–16). However, its identity and significance have not been established because of the very faint nature of the signals, especially those detected with anti-Myc. Cross-linked products of ~80 kDa, detectable with anti-Myc and anti-HA, were also generated when RseP(N389C/E23S)-His6-Myc was combined with the M104C, G105C, V106C, A107C, or A108C variants of HA-RseA140 (Fig. 6, C and D). In contrast, neither RseP(N389C/E23S)-His6-Myc nor RseP(N394C/E23S)-His6-Myc gave any detectable cross-linkage with any of the single-Cys variants of HA-RseA140 (data not shown). Use of CuCl2 as an oxidant did not essentially change the results (data not shown), although their oxidation efficiency was much weaker than Cu2+. These results show that the C-terminal region of RsePTM3 is in close contact with the central part of RseA TM. Thus, this region of RseP directly binds the substrate.

We then examined whether the substitutions of Asn-389 affect the disulfide cross-linking between RseP TM3 and RseA TM (Fig. 7). Derivatives of RseP(P399C/E23S)-His6-Myc containing an additional substitution (Gly, Leu, or Gln) for Asn-389 were constructed and examined for disulfide cross-linking with the V106C and A107C variants of HA-RseA140. The Gly and Leu replacements of Asn-389 greatly decreased (by 86 and 90%, respectively, as compared with the control) the formation of the cross-linked product with the V106C variant (Fig. 7, lanes 11–14) and to a much lesser extent (11 and 22% decrease, respectively) with the A107C variant (lanes 19–22). The N389Q mutation did not exhibit such an effect on the cross-linking (lanes 15, 16, 23, and 24). Similar results were obtained repeatedly in several independent experiments (data not shown). These results suggest strongly that Asn-389 is important for the effective contact between RseP TM3 and RseA TM.

DISCUSSION

To delineate the mechanism of specific substrate recognition and cleavage by a protease, it is essential to understand the mode of the substrate-enzyme interaction at the molecular level. However, such information has only very limitedly been available on the S2P family proteases, due mostly to experimental difficulties arising from the membrane-embedded nature of both the enzymes and the substrates. In the present study, we have analyzed the molecular interactions between RseP, an E. coli S2P homolog, and its substrates, and identified elements in the enzyme and the substrate that are important for the recognition and binding.

We previously found that helix-destabilizing residues in LacYTM1 promote its cleavage by RseP (19). Such residues could be relocated to other positions of the transmembrane segment, but their contribution to the lowered overall helix-forming propensities was essential. RseA TM, the physiological substrate of RseP, also contains several helix-destabilizing residues such as Gln-100 and Gln-103, and their substitution to a more helix-prefering Cys residue interfered with the cleavage of RseA TM by RseP (19). We suggest from these observations that disordered structural features of the substrate transmembrane segment is important for efficient cleavage by RseP. Helix-destabilizing residues in the transmembrane sequence of a substrate are also reported to promote cleavage by a signal peptide peptidase (36) and rhomboid proteases (37, 38). An α-helix is generally resistant to proteolytic cleavage, because the conformation makes the amide bonds inaccessible to a protease active site; helix-destabilizing residues may make the polypeptide backbone of a substrate more susceptible to hydrolysis. In the case of GlpG, an E. coli rhomboid homolog, we proposed that helix-destabilizing residues induce a kink to the substrate transmembrane region in a way to enable efficient presentation of the substrate cleavage site located outside the membrane into the enzyme’s active site inside the cavity (38, 39). Our present results show that helix-destabilizing residues
in the case of RseP act to stabilize the substrate-enzyme interaction. Thus, roles played by helix-destabilizing residues in the substrate of I-CLiPs appear to be diverse.

The results of our recent biochemical analyses suggested that the RseP active site is located within a folded, proteinaceous domain that is partly embedded in the membrane and largely sequestered from the surrounding lipidic and aqueous phases (25). Thus, it is conceivable that substrate proteins have to gain access to the recessed proteolytic active site of RseP though a narrow path formed in the enzyme. In this case, efficient access to the active site would require the recognition segment of the substrate protein to lack extensive secondary structure. The use of the proteolytically inactive RseP allowed us to demonstrate that the resulting substrate-RseP interaction is strong enough to withstand the affinity isolation. In contrast to RseP, γ-secretase binds a substrate with a helical conformation (40, 41), indicating that different I-CLiPs use different modes of substrate binding.

After completion of this work, the structures of an S2P homolog from an archaeal species Methanocaldococcus jannaschii were reported (42). The structures revealed that the proteolytic active site in a “closed” state of S2P is positioned within the plane of the membrane and sequestered from the lipidic and aqueous environments, although a water molecule is suggested to reach the active site through a very narrow channel open to the cytoplasm, which is consistent with the results of our thiol-
modifying reagent accessibility assays (25). The authors proposed that, in a “open” state, a substrate TM in an extended conformation makes an access to the recessed catalytic site through the cleft between the transmembrane segments (TM1 and TM6) that act as a gate for substrate entry. It has also been suggested that TM4 of *M. jannaschii* S2P, which corresponds to TM3 of RseP, contributes to the formation of the core active site domain and to substrate binding. Our current results showing that helix destabilization of a substrate TM promotes its stable binding to RseP also fits well with the above model of S2P. It is noteworthy that the proposed gating TMs are not conserved in RseP. Accordingly, RseP might use some other gating mechanism.

It has been suggested that a pair of helix-destabilizing residues (Asn-Pro) in the first transmembrane segment of sterol regulatory element-binding protein, a physiological substrate of the S2P protease, induced a conformational change in the transmembrane domain upon the first cleavage by the S1P protease, making the normally membrane-embedded site-2 cleavage site exposed to the cytoplasm and accessible to the S2P protease (43). However, our previous results show that helix destabilizing residues also promote substrate cleavage by RseP even under a detergent-solubilized condition (19). Additionally, RseP derivatives with a mutated PDZ domain were able to cleave intact RseA without the prior site-1 cleavage (22, 24). These observations suggest that a helix-extending conformational change in the substrate transmembrane region may not be involved in the RseP-catalyzed proteolysis. The role of the helix-destabilizing residues in the promotion of substrate cleavage by RseP is different from the one proposed for the Asn-Pro residues in sterol regulatory element-binding protein.

We have identified the regions of RseP involved in the substrate binding. Pulldown experiments showed that mutational alterations of several amino acid residues (Asn-389, Asn-394, Pro-397, and Pro-399) in RseP TM3 impaired the interaction between the enzyme and the substrate. In addition, disulfide bond formation was observed between some combinations of the Cys residues introduced into RseP TM3 and RseA TM. Some of the Asn-389 alterations that compromised the RseP-substrate interaction significantly interfered with disulfide cross-linking between RseP TM3 and RseA TM, suggesting that the observed cross-linking reflected the direct and functional enzyme-substrate binding. Thus, we conclude that TM3 of RseP has a crucial role in substrate binding.

We showed that Cys at positions 397 and 399 of RseP formed a disulfide bond with a Cys residue introduced into the central part of RseA TM, indicating that they were in a close contact with RseA TM in the membrane-integrated state. However, these substitutions appeared to weaken the RseP-RseA interaction under the solubilized conditions, as revealed by the co-immunoprecipitation assays. Detergent solubilization could have lowered the enzyme-substrate interaction through their increased mobilities or induced a slight structural perturbation to RseP, exaggerating the effects of the Cys substitutions that intrinsically destabilize the interaction. Both of the Cys residues at positions 397 and 399 formed a disulfide bond with Cys at multiple positions (104–108) in RseA TM. This observation could reflect the flexibility in the mode of the substrate recognition/binding by RseP, which appears to be consistent with the fact that RseP can cleave a variety of transmembrane segments with no apparent sequence similarity (19). It has been proposed that γ-secretase possesses the initial substrate-binding site (docking site) distinct from the proteolytic active site and that upon binding to the docking site a substrate is passed over to the nearby active site (44). It is also conceivable that the substrate presentation to the RseP active site follows similar multistep processes with different patterns of residue proximity and, hence, allowing different combinations of cysteines to form a disulfide. The P399C mutation retarded the cleavage of RseA TM more pronouncedly than LacYTM1. Thus, some of the TM3 residues might contribute to the binding preference to different substrates.

In contrast to residues 397 and 399, neither residue 389 nor 394 supported disulfide bond formation with the substrate (data not shown). We also observed that none of cysteines at the periplasmic half of HA-RseA140 TM formed a disulfide bond with RseP(N389C) or RseP(N394C). We reason that Asn-389 and Asn-394 have a role in substrate recognition without directly participating in physical binding. These residues exist in a row of hydrophobic residues (Fig. 1B). Replacement of Asn-389 with Gln had little effect on the interaction of RseP with the model substrate; however, its replacement with Cys, Gly, and Leu significantly compromised the interaction. Asn and Gln are more hydrophilic and helix-destabilizing than the other three residues.
residues, and such properties might be important at this position to establish proper structural arrangement of nearby residues directly involved in the RseP-substrate binding. Asn-394 could have a similar role as well. Asn-394 and Pro-397 are evolutionarily conserved among the S2P family proteins; thus, they might be involved in the substrate binding in this family of proteins.

On the basis of the structure of M. jannaschii S2P, Asn-140, which corresponds to Asn-394 in RseP, was suggested to play a critical role in substrate binding and/or in the formation of the oxyanion hole, which is consistent with our finding that the region of RseP TM3 containing the Asn-394 is important for the substrate binding. The residues of M. jannaschii S2P corresponding to Pro-397 and Pro-399 in RseP are located in a loop that interrupts the TM helix. This loop structure might be important for the recognition and binding of a substrate by conferring flexibility to TM3, which might contribute to the observed flexibility in the relative positioning of the residues in the enzyme and the substrate.

The present study provides insights into the mode of the interaction between RseP and its substrate. The recently solved structure of M. jannaschii S2P serves as a base that is crucial for elucidating the molecular mechanisms of substrate recognition and proteolysis by S2P. However, there are several notable differences between M. jannaschii S2P and RseP; for example, M. jannaschii S2P has no PDZ domain that has been suggested to be essential to understand the function and regulation of this protein.

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REFERENCES

1. Weihofen, A., and Martoglio, B. (2003) Trends Cell Biol. 13, 71–78
2. Wolfe, M. S., and Kopan, R. (2004) Science 305, 1119–1123
3. Sakai, J., Duncan, E. A., Rawson, R. B., Hua, X., Brown, M. S., and Goldstein, J. L. (1996) Cell 85, 1037–1046
4. Dobrosotskaya, I. Y., Seegmiller, A. C., Brown, M. S., Goldstein, J. L., and Rawson, R. B. (2002) Science 296, 879–883
5. Seegmiller, A. C., Dobrosotskaya, I., Goldstein, J. L., Ho, Y. K., Brown, M. S., and Rawson, R. B. (2002) Dev. Cell 2, 229–238
6. Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., Prywes, R., Brown, M. S., and Goldstein, J. L. (2000) Mol. Cell 6, 1355–1364
7. Konno, S., Murakami, T., Tatsumi, K., Ogata, M., Kanemoto, S., Otori, K., Iseki, K., Wanaka, A., and Imaizumi, K. (2005) Nat. Cell. Biol. 7, 186–194
8. Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D. T., Back, S. H., and Kaufman, R. J. (2004) Cell 124, 587–599
9. Schöbel, S., Zellmeier, S., Schumann, W., and Wiegert, T. (2004) Mol. Microbiol. 52, 1091–1105
10. Rudner, D. Z., Fawcett, P., and Losick, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14765–14770
11. Bramkamp, M., Weston, L., Daniel, R. A., and Errington, J. (2006) Mol. Microbiol. 62, 580–591
12. Chen, J. C., Viollier, P. H., and Shapiro, L. (2005) Mol. Microbiol. 55, 1085–1103
13. Kanekura, K., Akiyama, Y., and Ito, K. (2001) Gene (Amst.) 281, 71–79
14. Kanekura, K., Ito, K., and Akiyama, Y. (2002) Genes Dev. 16, 2147–2155
15. Alba, M. B., Leeds, J. A., Onufryk, C., Lu, C. Z., and Gross, C. A. (2002) Genes Dev. 16, 2156–2168
16. Ades, S. E. (2004) Curr. Opin. Microbiol. 7, 157–162
17. Alba, M. B., and Gross, C. A. (2004) Mol. Microbiol. 52, 613–619
18. Walsh, N. P., Alba, B. M., Bose, B., Gross, C. A., and Sauer, R. T. (2003) Cell 113, 61–71
19. Akiyama, Y., Kanekura, K., and Ito, K. (2004) EMBO J. 23, 4434–4442
20. Flynn, J. M., Leveneken, I., Sauer, R. T., and Baker, T. A. (2004) Genes Dev. 18, 2292–2301
21. Chaba, R., Grigorova, I. L., Flynn, J. M., Baker, T. A., and Gross, C. A. (2007) Genes Dev. 21, 124–136
22. Kanekura, K., Ito, K., and Akiyama, Y. (2003) EMBO J. 22, 6389–6398
23. Grigorova, I. L., Chaba, R., Zhong, H. J., Alba, B. M., Rhodius, V., Herman, C., and Gross, C. A. (2004) Genes Dev. 18, 2686–2697
24. Bohn, C., Collier, J., and Boulouc, P. (2004) Mol. Microbiol. 52, 427–435
25. Koide, K., Maegawa, S., Ito, K., and Akiyama, Y. (2007) J. Biol. Chem. 282, 4553–4560
26. Sato, C., Morohashi, Y., Tomita, Y., and Iwatsubo, T. (2006) J. Neurosci. 26, 12081–12088
27. Tolia, A., Chavez-Gutierrez, L., and De Strooper, B. (2006) J. Biol. Chem. 281, 27633–27642
28. Ha, Y. (2007) Curr. Opin. Struct. Biol. 17, 405–411
29. Wang, Y., Maegawa, S., Akiyama, Y., and Ha, Y. (2007) J. Mol. Biol. 374, 1104–1113
30. Akiyama, Y., and Ito, K. (1985) EMBO J. 4, 3351–3356
31. Davis, R. W., Botstein, D., and Roth, J. R. (1980) Advanced Bacterial Genetics: A Manual for Genetic Engineering. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
32. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Sawano, A., and Miyawaki, A. (2000) Nucleic Acids Res. 28, E78
34. Liu, L. P., and Deber, C. M. (1998) J. Biol. Chem. 273, 23645–23648
35. Kinch, L. N., Ginalska, K., and Grishin, N. V. (2006) Protein Sci. 15, 84–93
36. Lemberg, M. K., and Martoglio, B. (2002) Mol. Cell 10, 735–744
37. Urban, S., and Freeman, M. (2003) Mol. Microbiol. 52, 2147–2155
38. Wang, Y., Maegawa, S., Ito, K., and Akiyama, Y. (2007) Mol. Microbiol. 64, 435–447
39. Wolfe, M. S., Xia, W., Moore, C. L., Leatherwood, D. D., Otsatzewski, B., Rahmati, T., Donkor, I. O., and Selkoe, D. J. (1999) Biochemistry 38, 4720–4727
40. Lichtenthaler, S. F., Wang, R., Grimm, H., Uljon, S. N., Masters, C. L., and Breyer, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3055–3058
41. Feng, L., Yan, H., Wu, Z., Yan, N., Wang, Z., Jeffrey, P. D., and Shi, Y. (2007) Science 318, 1608–1612
42. Ye, J., Dave, U. P., Grishin, N. V., Goldstein, J. L., and Brown, M. S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5123–5128
43. Kornilova, A. Y., Bihel, F., Das, C., and Wolfe, M. S. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 3230–3235
44. Akiyama, Y., Yoshihisa, T., and Ito, K. (1995) J. Biol. Chem. 270, 23485–23490