

REVIEW

New insights into S2P signaling cascades: Regulation, variation, and conservation

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Abstract: Regulated intramembrane proteolysis (RIP) is a conserved mechanism that regulates signal transduction across the membrane by recruiting membrane-bound proteases to cleave membrane-spanning regulatory proteins. As the first identified protease that performs RIP, the metalloprotease site-2 protease (S2P) has received extensive study during the past decade, and an increasing number of S2P-like proteases have been identified and studied in different organisms; however, some of their substrates and the related S1Ps remain elusive. Here, we review recent research on S2P cascades, including human S2P, *E. coli* RseP, *B. subtilis* SpoIVFB and the newly identified S2P homologs. We also discuss the variation and conservation of characterized S2P cascades. The conserved catalytic motif of S2P and prevalence of amino acids of low helical propensity in the transmembrane segments of the substrates suggest a conserved catalytic conformation and mechanism within the S2P family. The review also sheds light on future research on S2P cascades.

Keywords: intramembrane; proteolysis; site-1 protease; site-2 protease; substrate

Introduction

Regulated intramembrane proteolysis (RIP) is a conserved mechanism to regulate transmembrane signaling. It is carried out by different types of intramembranously cleaving proteases (I-CLiPs), which can be grouped into three or four families on the basis of their catalytic mechanisms, catalytic properties, and substrates. I-CLiPs have been identified in all kingdoms of life except virus.¹ Through RIP, they activate or inactivate transcription factors or signal-

ing peptides to coordinate the responses between cellular compartments. These enzymes contain multiple membrane-spanning segments, within which the conserved catalytic residues often reside. They specialize in cleaving peptide bonds that are normally embedded in hydrophobic membranes. One family of I-CLiPs consists of serine proteases, designated as Rhomboid proteases, example as GlpG.^{2,3} Another family includes two types of aspartyl proteases: Signal peptide peptidases, example as GXGD and Presenilin1 as the catalytic subunit of γ -secretase.^{4,5} Another includes a large family of metalloproteases, the S2P proteases.⁶

In this review, we focus on the S2P family, starting with the regulation of S2P, SpoIVFB, RseP and the recently identified S2P family members, then discussing the common properties of S2P cascades and finally touching on promising clues for future investigation.

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S2P Cascades in Transmembrane Signaling Processes

S2P in human

As the first member of this family, site-2 protease (S2P) was first identified in humans. It is involved in the feedback regulation of sterol and fatty acid synthesis and uptake by controlling the activity of transcription factors, SREBPs (Sterol regulatory element binding proteins).⁷⁻⁹ Proteins involved in the S2P pathway include site 1 protease (S1P), site 2 protease (S2P), their substrates and other regulatory proteins [Fig. 1(A)]. Before activation, SREBPs reside in the endoplasmic reticulum (ER) membrane while S1P and S2P are located in the Golgi apparatus.^{7,10,11} This spatial separation prevents SREBPs from unregulated degradation by S1P and S2P. SREBPs are membrane-bound transcription factors responsible for sterol and fatty acid biosynthesis and uptake.⁹ They are known to directly promote transcription of more than 30 genes required for uptake and synthesis of cholesterol, triglycerides, fatty acids, and phospholipids.¹² The N-terminal domain of SREBP is a transcription factor of basic helix-loop-helix leucine zipper (bHLH-Zip). The C-terminus forms a tight complex with the WD repeats domain of Scap (SREBP cleavage-activating protein), which functions as the sterol sensor in this system.^{13,14} SREBP contains two transmembrane sequences and a short luminal loop.⁷

In cells with high sterol, Scap sequesters SREBPs and binds to cholesterol in the ER membrane in such a way that it assumes a conformation that is propitious to bind Insig (insulin-induced gene), a small ER membrane-resident protein.¹⁵⁻¹⁷ This binding retains the SREBP-Scap complex in the ER by preventing interaction of Scap with the COPII vesicle-formation proteins at the MELADL motif.¹⁷⁻²¹ As a consequence, SREBPs are protected from activation by S1P and S2P resident in Golgi and the transcription of its target genes declines.

In sterol depleted cells, conformation changes of Scap and Insig disrupt their binding and the SREBP-Scap complex is sorted in COPII-coated transport vesicles to the Golgi.¹⁸ In the Golgi, two sequential proteolytic cleavage events by S1P and S2P finally release the N terminal transcription factor domain of SREBPs from the Golgi membrane.¹⁹ S1P, a serine protease, cleaves after a leucine residue at the consensus sequence RxxL in the luminal loop of SREBPs.^{22,23} Then the zinc metalloprotease S2P cleaves at a Leu-Cys bond predicted to lie within the membrane.⁶ This two-stage proteolysis of SREBPs discharges the mature N-terminal bHLH-Zip domain to the nucleus to bind the sterol regulatory element (SRE) sequences in the promoters of target genes, therefore activating transcription of genes involved in sterol and fatty acid synthesis and

uptake.^{7,9} The resultant increase of cholesterol will later feed back to inhibit activation of SREBP through Scap. Through the S2P cascade, the cholesterol feedback pathway in humans is stringently regulated. Cholesterol content in the ER membrane triggers this regulatory machine by mediating the interaction of Scap with Insigs in a switch-like manner.^{21,24} Thus S2P plays a crucial role in regulating the feedback of cholesterol biosynthesis.

Intriguingly, SREBP is not the only substrate for the S2P pathway. S1P and S2P function *in tandem* to activate the stress response transcription factor ATF6 in response to unfolded protein in a manner similar to SREBP.²⁵ CREBH was identified as a liver-specific transcription factor that is cleaved sequentially by S1P and S2P upon ER stress and is required to activate expression of acute phase response genes.²⁶ OASIS (old astrocyte specifically induced substance), a transcription factor anchored in ER membrane, modulates the unfolded protein response in astrocytes through the cleavage by S1P and S2P.^{27,28} Recent results show that Ichthyosis follicularis with atrichia and photophobia (IFAP syndrome) is caused by functional deficiency of S2P (MBTPS2) through uncertain substrates.²⁹ The diversity of substrates indicates that S2P is a pivotal protease and participates in different signaling transduction pathways.

RseP in *Escherichia coli*

DegS and RseP in *E. coli* are homologs of human S1P and S2P. DegS is a serine protease anchored to the periplasmic face of the inner membrane and RseP is a multiple transmembrane protease with canonical HEXXH and NPDG motifs and located in the inner membrane.³⁰⁻³⁴ They successively process RseA, the membrane-bound anti- σ^E protein. Early studies demonstrated that σ^E mediates extracytoplasmic stress responses by controlling the expression of genes that facilitate refolding or degradation of misfolded periplasmic proteins.³⁵ Under normal growth conditions, σ^E is sequestered by RseA through forming a tight complex with the N-terminal cytoplasmic domain of RseA.³⁶⁻³⁸ When cells are subjected to extracytoplasmic stress which result in unassembled or misfolded proteins signals, RseA is degraded by successive proteolytic events to release σ^E [Fig. 1(B)]. Misfolded outer membrane porins (OMPs such as ompA or ompC) can interact with the PDZ domain of DegS and initiate the first cleavage of RseA by DegS at its periplasmic domain (Val¹⁴⁸-Ser¹⁴⁹).^{33,34,39} This initial, signal-sensing cleavage step is rate-limiting,⁴⁰ and stimulates the second cleavage of RseA₁₋₁₄₈ by RseP. RseP cleaves between Ala¹⁰⁸ and Cys¹⁰⁹, well inside the predicted TM sequence of RseA.⁴¹ Then RseA₁₋₁₀₈, the cytoplasmic fragment of RseA, is further degraded by ClpXP or other ATP-dependent proteases to ultimately release

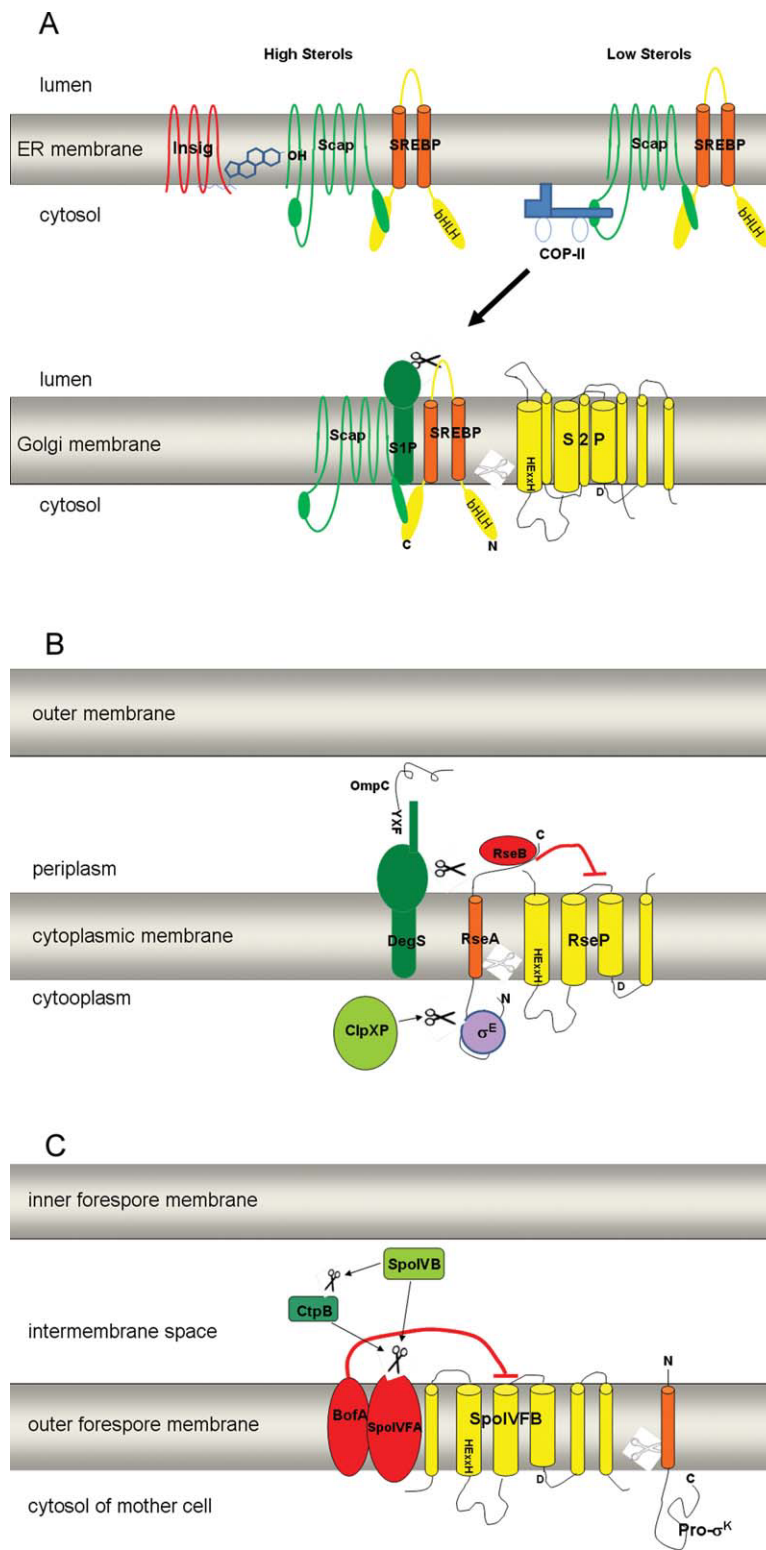


Figure 1. Schematic drawings showing the S2P cascades of human S2P, *E. coli* RseP and *B. subtilis* SpoIVFB. Substrates are shown in orange, Site-2 proteases are shown in yellow. The white scissors represent the cleavage by Site-2 proteases while the black scissors represent the cleavage by Site-1 proteases or other proteases. The metal chelating motif that includes HEXxH and D is shown in black. **A:** Under low levels of sterols, human SREBP is transported from the ER membrane to the Golgi to be sequentially cleaved by S1P and S2P. **B:** In *E. coli*, RseA is cleaved sequentially by DegS and RseP. **C:** After activation by SpoIVB, SpoIVFB cleaves pro- σ^K to release mature σ^K .

σ^E in cytoplasm.⁴⁰ Active σ^E will bind appropriate RNA polymerase complex to modulate expression of target genes.

Many interesting and important studies of the regulation mechanisms have shown complexity and precision of its regulation in nature. As a periplasmic stress sensor, DegS is kept in an inactive state by its PDZ domain under normal conditions.⁴² When protein folding is compromised in the periplasm, the C-terminus of OMPs bind to the PDZ domains of the trimeric DegS protease and activate its cleavage on RseA.^{39,43} Crystallographic and biochemical analysis show that DegS is an allosteric enzyme.⁴⁴ OMPs binding shifts the equilibrium from a nonfunctional state to the functional proteolytic conformation. The ancillary regulatory protein RseB is also involved. Initial studies found that RseP variants with the periplasmic PDZ domain deleted or mutated allowed unregulated cleavage of RseA and consequent σ^E activation.⁴⁵ This indicated that the PDZ domain of RseP might be an inhibitor of proteolytic activity.⁴⁶ A Gln-rich region in the periplasmic domain of RseA was found to be required for the avoidance of the RseP-mediated proteolysis in the absence of site-1 cleavage.⁴⁵ At the same time, it was found that RseP can cleave intact RseA in a Δ RseB strain and RseB binds to the periplasmic region of RseA strongly with 1:1 stoichiometry.⁴⁷ These results suggest RseB plays a vital role in inhibiting RseP cleavage of intact RseA; and RseB, the PDZ domain of RseP and the Gln-rich domain of RseA may together exert an inhibitory function. In the Δ RseB strain, RseP cleavage of RseA is impaired by overexpression DegS, which indicates that RseB and DegS each independently inhibit RseP cleavage of intact RseA.⁴⁷ *In vitro* RseB inhibits proteolysis by DegS by binding tightly to the periplasmic domain of RseA.^{36,48} The inhibitory effect may be due to steric hindrance because RseB prevents the substrate from accessing to the active site of DegS. Though RseB inhibit DegS cleavage of RseA *in vitro*, DegS can cleave RseA *in vivo* under similar conditions. Maybe other factors interact with RseB to relieve its inhibitory effect.

SpoIVFB in *Bacillus subtilis*

SpoIVFB from the gram-positive bacterium *B. subtilis* is another founding member of the S2P metalloprotease family. It is involved in activation of the crucial membrane-associated transcription factors σ^K during sporulation.^{49,50} SpoIVFB processes pro- σ^K to mature and active σ^K by proteolytic removal of an N-terminal extension of 20 amino acids [Fig. 1(C)].^{49,51} Sporulation of *B. subtilis* involves the formation of a polar septum that asymmetrically partitions the cell into a large mother cell and a small forespore. The two cells initially lie side by side, but later the forespore is engulfed by the mother cell to create a cell within a cell.⁵² As a result of engulf-

ment, the forespore is surrounded by two membranes, the inner forespore membrane (IFM) and the outer forespore membrane (OFM). Gene expression is coordinated between these two chambers in part by a series of sigma factors to drive a series of morphological changes that culminate with lysis of the mother cell to release a dormant spore.⁵⁰

As inactive precursor, pro- σ^K associates with the OFM with its N-terminal transmembrane segment (amino acids 1 to 27).⁵³ Proteolytic cleavage by SpoIVFB releases the C-terminal σ^K into the mother cell for directing transcription of genes involved in spore cortex and coat synthesis.⁵⁴ Although σ^K is in charge of the expression of genes in the mother cell, its activation is mediated by signaling proteins from both the mother cell and the forespore cell. Before sporulation, the transmembrane metalloprotease SpoIVFB is held inactive by two other integral-membrane proteins, SpoIVFA and BofA.^{51,55,56} BofA, SpoIVFA and SpoIVFB are all synthesized in the mother cell. They form a complex in OFM. SpoIVFA plays an essential role in the assembly and localization of the complex while BfoA directly inhibits the activity of SpoIVFB on pro- σ^K processing probably by supplying a fourth zinc ligand.^{55,56} The inhibition of SpoIVFB by BofA and SpoIVFA is relieved by two serine proteases, SpoIVB and CtpB.⁵⁷ In the interspace between IFM and OFM, the signaling serine protease SpoIVB from forespore triggers pro- σ^K processing by cleaving the extracellular domain of SpoIVFA at multiple sites.⁵⁸ *In vitro*, these cleavages do not disrupt the interactions between SpoIVFA, SpoIVFB, and BofA, suggesting that SpoIVB-dependent activation of the processing enzyme results from a conformational change in this complex.⁵⁹ CtpB, a second serine protease from the mother cell, also triggers pro- σ^K processing by cleaving SpoIVFA or BofA.^{57,59,60} From the *in vitro* data that CtpB appeared to cleave BofA near its C-terminus upon coexpression in *E. coli* and purified CtpB degraded BofA, a three-step proteolytic cascade was proposed, in which SpoIVB first cleaves SpoIVFA, CtpB then cleaves BofA and finally SpoIVFB cleaves pro- σ^K .⁵⁷ Later Campo and Rudner⁵⁹ proposed another model in which CtpB triggers pro- σ^K processing by cleaving SpoIVFA. They demonstrated that when SpoIVB is unable to cleave SpoIVFA, it can still activate pro- σ^K processing through CtpB. Thus a model was proposed in which SpoIVB regulates intramembrane proteolysis through two proteolytic pathways: directly by cleaving the extracellular domain of SpoIVFA and indirectly by cleaving and activating CtpB to process SpoIVFA, both of which converge on the same regulator SpoIVFA and release SpoIVFB for processing of pro- σ^K .

SpoIVFB is unique among S2P family members in that it does not contain PDZ domain and there is no evidence to suggest that pro- σ^K is cleaved before

SpoIVFB. However, regulation of SpoIVFB activity is achieved by a proteolytic cascade involving serine proteases, which are used as S1P protease in other S2P cascades. SpoIVB and CtpB are not involved in Site-1 cleavage of substrate but are required to relieve the inhibition of SpoIVFB by SpoIVFA and BofA.^{58–60} Recently, Kroos and coworkers⁶¹ constructed a biochemical system to validate the direct relationship between spoIVFB and pro- σ^K . Their results demonstrated that spoIVFB does cleave pro- σ^K in an ATP-dependent manner.⁶¹ This is special in characterized S2P pathways, though more evidence is needed to clarify its regulation mechanism.

RasP in *Bacillus subtilis*

Another S2P homolog in *B. subtilis* is RasP. *B. subtilis* codes for several extracytoplasmic function (ECF) sigma factors including σ^W . As for σ^E in *E. coli*, activity of σ^W is negatively controlled by a membrane-bound anti-sigma factor, RsiW.⁶² The σ^W regulon is induced by different stresses, such as alkaline shock, salt shock, phage infection, and certain antibiotics that damage the cell envelope.^{63–65} RsiW is cleaved at site-1 and site-2 by PrsW (YpdC) and RasP (YluC), respectively,^{66–68} and is then further degraded by cytoplasmic Clp peptidases.⁶⁹ This proteolysis cascade liberates sequestered σ^W into the cytoplasm to exert its function by ultimately binding to RNA polymerase. Though the molecular mechanism of the initialization and regulation remains elusive at present, a simple profile of its proteolysis process can be drawn. Several peptidases are reported to be involved in trimming of RsiW following PrsW and prior to RasP.⁷⁰ In a reconstituted *E. coli* system, PrsW removes 40 amino acids from the C-terminal of RsiW by cleaving between Ala¹⁶⁸ and Ser¹⁶⁹ of the extracytoplasmic domain, generating RsiW-S1. Then the C-terminus of RsiW-S1 is further processed by Tsp (tail-specific protease, a periplasmic carboxyl terminal processing peptidase) before subsequent RasP proteolysis.⁷⁰

Another substrate of RasP in *B. subtilis* has been identified as FtsL, which is involved in division of cells.⁷¹ Alignment of FtsL and RsiW and mutant constructs indicate that the conserved RAS motif before, and the AAA motif inside the transmembrane region of FtsL may be involved in recognition and cleavage, respectively (Fig. 3).⁷¹ However, the so-called S1P seems unnecessary for FtsL processing. A recent investigation suggested that the ABC transporter EcsAB may be involved in the regulation of RasP cleavage of its substrates in an unknown manner.⁷²

MucP in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a gram-negative bacterium, is an important opportunistic human pathogen causing urinary tract, respiratory system and eye infections, and a variety of systemic infections par-

ticularly in individuals with compromised immune defence such as cystic fibrosis (CF).⁷³ During the course of infection in CF, *P. aeruginosa* colonizers convert from low to high producers of the capsular polysaccharide alginate. Thereby they gain higher tolerance against host immune defence and provide a barrier that allows the pathogen to survive treatment with most common antibiotics.⁷³ The overproduction of alginate is often caused by mutations within *mucA*. The antisigma factor MucA is a transmembrane protein located in the inner membrane and contains an N-terminal cytoplasmic domain that binds and represses activity of sigma factor σ^{22} (AlgU/AlgT).^{74–76} MucA also contains a C-terminal periplasmic region for regulation. In response to extracytoplasmic stress, MucA is sequentially cleaved by AlgW and MucP, the homologs of DegS and RseP in *E. coli*, respectively. Then MucA releases the sequestered ECF σ^{22} (AlgU/AlgT) to activate the alginate biosynthesis. Mutations or deletion in *mucA* found in mucoid strains cause truncations of the C-terminal periplasmic domain of MucA. MucB and MucD are negative regulators for alginate synthesis in the periplasm. Without extracytoplasmic stress, MucB binds tightly to the C-terminal of MucA and strongly inhibits the cleavage by AlgW.⁷⁷ This easily explains why truncations of the C-terminal periplasmic domain of *mucA* can induce the mucoid conversion, since it loses the negative regulation by MucB. MucD can eliminate signal proteins that activate AlgW and other proteases to cut MucA.⁷⁸ The defect in MucD caused MucA instability, suggesting wild type MucD degrades overactive cell wall stress signals, for example, the misfolded periplasmic peptides that can activate AlgW protease even in the absence of stress.⁷⁹ MucE is the positive regulator of alginate biosynthesis. Three critical amino acid residues at the C terminus of MucE (WVF) were required for mucoid conversion via proteases AlgW and MucP.⁷⁸ The PDZ domain of AlgW *per se* is a sensor. It binds MucE C-terminal peptide sequences or other stress peptide signals, which represent the folding/assembly status of proteins in the periplasm.⁷⁸ And the PDZ domain plays important roles both in repressing proteolytic activity when appropriate peptide signals are absent and in stimulating cleavage when such peptides are present.⁷⁷ When *P. aeruginosa* suffers from cell wall stress, such as D-cycloserine, a signal peptide from MucE interacts with the PDZ domain of AlgW to induce the site 1 cleavage of MucA by cleaving the C-terminal periplasmic region between Ala¹³⁶-Gly¹³⁷. And then MucP executes its site 2 protease function by cleaving the processed MucA.⁷⁷ In a study of a revertant of mucoid mutant, the ClpXP and ClpP2 proteins were found to degrade the cytoplasmic portion of the truncated MucA N-terminus to release the sequestered AlgU, which finally drives alginate biosynthesis.⁸⁰

MmpA in *Caulobacter crescentus*

Caulobacter crescentus is a dimorphic bacterium that divides asymmetrically and undergoes a characteristic morphological transition as part of its life cycle. During the cell cycle, the polarity determinant PodJ is processed in a S2P cascade. PodJ recruits structural and regulatory proteins required for polar organelle biogenesis to the correct cell pole at a defined time in the cell cycle. The full-length PodJ protein (PodJ_L) contains 974 amino acids, having a cytoplasmic N-terminal part, a single transmembrane domain and a periplasmic C-terminal region.⁸¹ It is synthesized in the early predivisional cell and localizes to the incipient swarmer pole where it recruits PleC histidine kinase/phosphatase and factors required for pili biogenesis.^{81–83} As cells divide, the periplasmic domain of PodJ_L is truncated, resulting in a membrane-anchored short form PodJ_S, which is required for later steps in pole development. Shapiro's group^{84,85} investigated the proteolytic processing of PodJ in *C. crescentus*. They found that truncation of PodJ_L into PodJ_S requires the periplasmic protease PerP, whose expression is controlled by the DivJ–PleC–DivK monitoring system.⁸⁴ PodJ_S is subsequently released from the polar membrane by the S2P homolog MmpA during the swarmer-to-stalked cell transition.⁸⁵ Eventually, the cytoplasmic part of PodJ is removed by an as-yet-unknown protease. The sequential cleavage of PodJ by PerP and MmpA illustrate how the S2P cascade has been adapted to regulate the asymmetric distribution of PodJ isoforms, which lead to the correct succession of polar organelle development during the cell cycle of *C. crescentus*.

HurP in *Bordetella bronchiseptica*

The heme system is one of the three iron retrieval systems in *B. bronchiseptica*, a gram-negative pathogen of humans and animals that colonizes the respiratory tract. HurP, a S2P family member, is essential for heme-dependent induction of *bhuR* and downstream genes.⁸⁶ Expression of the *hurIR bhuR-STUV* heme utilization operons in *B. bronchiseptica* is coordinately controlled by the global iron-dependent regulator Fur and the ECF sigma factor HurI. Activation of HurI requires transduction of a heme-dependent signal via HurI, HurR, and BhuR, a three component heme-dependent regulatory system. HurI is sequestered by its antisigma factor, HurR, when iron is sufficient. BhuR is an outer membrane heme receptor protein. These three components are driven by two promoters, *P_{hurI}* and *P_{bhuR}*. When iron is abundant, expression of both operons is repressed in a Fur-dependent manner. Upon iron depletion, Fur derepression at the *hurI* promoter enables *hurIR* transcription and read-through transcription of *bhuR*, resulting in basal production levels of the

heme transport proteins.⁸⁷ Under this condition, BhuR binds heme and initializes the S2P cascade by activating the unknown S1P cleavage of HurR. Then HurP exerts site 2 cleavage on HurR to release the HruI to bind RNA polymerase.⁸⁶ Finally, this signaling cascade generates the cell response to the iron depletion environment.

S2P homologs in *Arabidopsis*

EGY1 (ethylene-dependent gravitropism-deficient and yellow-green 1) was identified as the first S2P homolog in plants.^{88,89} EGY1 is one of the five S2P homologs in *Arabidopsis* which contain the two canonical motifs HEXXH and NPDG. Located in chloroplast thylakoid membrane, EGY1 is required for development of thylakoid grana and a well-organized lamellae system in chloroplasts. It is also required for the accumulation of chlorophyll and chlorophyll *a/b* binding (CAB) proteins in chloroplast membranes. Another characterized S2P homologs in *Arabidopsis*, AraSP, is a chloroplast inner envelope membrane protease and essential for plant development.⁹⁰ However, the substrates of both EGY1 and AraSP are still under investigation and the relative S1P is elusive.

On the other hand, several membrane-tethered transcription factors (MTF) are reported to be activated by proteolytic release from membrane. Heat stress induces the proteolytic release of the transcription factor domain of MTF AtbZIP28 from ER membrane and results in its redistribution to the nucleus.⁹¹ MTF AtbZIP60 is a proteolysis-activated transcription factor involved in ER stress response.⁹² Processing and relocation to the nucleus of ER MTF AtbZIP17 are important for the up-regulation of salt stress genes.⁹³ AtbZIP17 and AtbZIP28 contain the canonical cleavage site of mammalian S1P as RxxL, and AtS1P responsible for AtZIP17 was identified.⁹³ Genome-scale screening identified at least 85 membrane-bound transcription factors (MTF) in *Arabidopsis*.⁹⁴ They are proposed to be regulated by controlled proteolytic activation in response to various environmental changes.

Other newly identified S2P cascades

In gram-positive bacterium *Enterococcus faecalis*, antibiotic resistance and virulence determinants can be rapidly spread throughout populations by the transport of mobile genetic elements induced by oligopeptide sex pheromone.^{95,96} A S2P family member Eep processes the sex pheromone and inhibitor precursors as they pass through the cell membrane. Eep is involved in the production of pheromone cAD1, cPD1, and cCF10, and inhibitor peptide iAD1 and iCF10.^{97–99} But the substrate recognition and cleavage site remain elusive for Eep. So far no common motif was revealed in the primary sequence of known Eep targets.⁹⁷ It is proposed that possibly the secondary structure provided by

the N terminus of the Eep-recognized polypeptides directs Eep targeting.

Recently, a functionally conserved S2P member Stp1 was characterized in the human fungal pathogen *Cryptococcus neoformans*. The investigators demonstrated that Stp1 cleaves Sre1, a transcription factor similar to SREBP, within its predicted first transmembrane segment.¹⁰⁰ Sre1 pathway is crucial for host adaptation and virulence and is required for both growth and survival in the presence of sterol biosynthesis-inhibiting antifungal drugs,¹⁰¹ thus providing hints for anticryptococcal therapy.

Mycobacterium tuberculosis is an obligate human pathogen threatening global health. In this gram-positive bacteria, knockout mutants of the S2P homolog Rv2869c exhibited altered cell envelope composition and virulence, suggesting S2P mediated RIP as a proximal regulator of cell envelope virulence determinants.¹⁰² Later, penicillin-binding protein 3 (PBP3) was found to be cleaved by Rv2869c (MSMEG_2579) in oxidatively stressed cells. Amino acids A¹⁰² and A¹⁰³ of PBP3 are required for Rv2869c mediated cleavage. While Wag31, by virtue of its interaction with PBP3 through amino acid residues 46NSD48, protects it from oxidative stress-induced cleavage.¹⁰³

Vibrio cholerae is a gram-negative bacterium that causes the severe diarrhoeal disease cholera. Acting in contrast to the common situation of S2P pathway on activating transcription factor, RIP here renders the transcription factors inactive and results in limiting virulence gene expression. TcpP, a membrane-localized virulence activator of *V. cholerae*, bind promoter DNA and activate gene expression under favorable condition. Under adverse conditions, TcpP is degraded by an unknown SIP and a homolog of YaeL, Vc-YaeL.¹⁰⁴

Variation and Conservation of S2P Cascades

As the first known intramembrane protease, mammalian S2Ps have been investigated extensively since Brown and Goldstein discovered their function in regulation of sterol and fatty acid synthesis in 1990s.¹⁰⁵ Thus far, homologous proteases have been identified throughout different organisms from prokaryote, archaea to other eukaryote, but some of the substrates and relative S1Ps still remain elusive (Table I). Here, we try to summarize the variation and conservation of S2P cascades, aiming at providing clues for the future study of newly identified members.

Various site-1 proteases

As summarized above and in Table I, Site-1 proteases differ markedly from one system to another. They will not be discussed further.

Conserved catalytic motif and mechanism of Site-2 proteases

In contrast to the diverse Site-1 proteases, Site-2 proteases are much more closely related to each

other. They all belong to the same family of membrane-embedded, zinc metalloproteases and share the conserved catalytic motif as: HEXXH and NPDG [Fig. 2(A,B)].¹¹³ Most HEXXH motifs lie inside the TM helices, while most NPDG motifs are located at the end of or even outside TM helices. Between these helices containing HEXXH and NPDG, usually there is another TM helix, forming a compact three TM structural core (Fig. 4).^{114,112} Both the HEXXH and NPDG motifs face the cytosolic side, suggesting a conserved active site core domain or conformation to be very close to the membrane surface [Figs. 2(A,B) and 4]. In the studies of substrate recognition and binding by RseP, Koide *et al.* analyzed the environment of the RseP active site based on the accessibility of various thiol-alkylating reagents to Cys residues introduced around the catalytic residues. The results suggested that the active site of RseP is neither totally embedded in the lipid phase nor exposed to the cytoplasm, but rather located within a folded protein domain partially embedded in the membrane.¹¹⁵

Shi and coworkers have determined the crystal structure of the transmembrane core domain of mjS2P, a putative archaea homolog from *Methanocaldococcus jannaschii* (Fig. 4).¹¹² They found that mjS2P has six transmembrane segments (TM1–TM6) and zinc is coordinated by three amino acids, His⁵⁴ and His⁵⁸ in TM2, and Asp¹⁴⁸ in TM4. TM2 and TM4 are stabilized by TM3 and together these three segments constitute a core domain of S2P. These results are consistent with the prior mutational studies. Based on the differences of two mjS2P structures, it was suggested that TM1 and TM6 may function as a lateral gate to allow substrate access to the active site.^{112,116} But since the active sites are physically located very close to membrane surface and proteases appear to cause significant membrane compression around the enzyme, it cannot be ruled out that the active sites may be exposed outside the membrane to interact with substrate during proteolysis.¹¹⁷

In the alignment of identified S2P homologs, other than the known HEXXH motif, an alanine, three amino acids after HEXXH is found conserved among the S2P homologs analyzed [Fig. 2(A)]. Using the alignment of HEXXH and NPDG sequences, an unrooted dendrogram was developed as Figure 2(C). Three main groups appear to represent the eukaryotes homologs, the prokaryote homologs, and the SpoIVFB group. All the Site-2 proteases contain at least four hydrophobic and potentially membrane-spanning segments. S2P, ResP, and SpoIVFB are representatives of the three known structural subclasses within the family.¹¹⁷ The structural classification is consistent with the unrooted dendrogram based on the alignment of the catalytic motif [Fig. 2(C)].

Most of the prokaryote homologs, for example, *E. coli* RseP, *V. cholerae* Vc-RseP, *P. aeruginosa*

Table 1. Currently Characterized S2P Cascades in Different Organisms

	Substrates	S1P	S2P	Location	Inducing signal	Downstream cascade	Ancillary regulators	References
Eukaryotic organisms								
<i>Homo sapiens</i>	SREBPs	S1P/SKL-1	S2P	ER→Golgi	Low levels of membrane cholesterol in ER	Activates cholesterol biosynthesis and uptake	Scap, Insigs, COPII	7–9, 14, 16–18, 21–23
	ATP6	MBTPS1	S2P/MBTPS2	ER→Golgi	Unfolded proteins accumulation as ER stress	Unfolded protein response	BiP/GRP78	25, 106, 107
	OASIS	S1P	S2P	ER→Golgi	ER stress	Unfolded protein response in astrocytes		27
	CREBH	S1P	S2P	ER→Golgi	ER stress	Acute inflammatory response in liver		26
<i>Drosophila</i>	SREBP	S1P	S2P		Low levels of phosphatidylethanolamine	Activating fatty acid biosynthesis		108
<i>Arabidopsis thaliana</i>	bZIP17	AtSIP	Unknown	Unknown				93
	Unknown	Unknown	EGY1 (At2g35220)	Thylakoid membrane				88
	Unknown	Unknown	AraSP (At2g32480)	Chloroplast inner envelope				90
<i>Cryptococcus neoformans</i> (fungi)	Sre1	Unknown	Stp1	Unknown	Hypoxia, nutrient limiting, presence of sterol synthesis inhibitor	Host adaptation and virulence		100, 101
Prokaryotic organisms								
<i>Escherichia coli</i> (G-)	RseA (anti sigma factor of σ^E)	DegS	RseP (YaeL or EcfE)	CM	Extracytoplasmic stress, accumulation of misfolded outer membrane proteins in the periplasm	Extracytoplasmic stress response, cell envelope biogenesis	RseB, RseC, ClpXP	30–34, 40, 41, 43, 47, 48, 109
<i>Pseudomonas aeruginosa</i> (G-)	MucA (anti sigma factor of AlgU)	AlgW	MucP	IM/PM	Cell wall stress (induced by e.g. D-cycloserine)	Extracytoplasmic stress response, alginate biosynthesis	MucB, MucD, MucE, Prc, ClpXP	77–80
<i>Bordetella bronchiseptica</i> (G-)	HurR (anti sigma factor of HurI)	Unknown	HurP	CM/PM	Iron depletion	Activate haem receptor bhurR expression and iron uptake		86
<i>Vibrio cholerae</i> (G-)	TcpP	Unknown	Vc-YaeL	CM/PM	Unfavorable conditions	Limiting virulence gene expression	TcpH	104
<i>Caulobacter crescentus</i>	PodJ (polarity determinant)	PerP	MmpA	CM	In response to the completion of cytokinesis	Polar organelle development	DivJ–PleC–DivK system	84, 85
<i>Bacillus subtilis</i> (G+)	Pro- σ^K	No SIP	SpoIVFB	Outer forespore membrane	Signals for sporulation, e.g. nutrient deprivation	σ^K activates transcription of mother cell genes involved in sporulation	SpoIVFA, BofA, CtpB, SpoIVB	49, 56, 57, 59, 61
	RsiW (anti sigma factor of σ^W)	PrsW (YpdC)	RasP (YluC)	CM/PM	Stress affecting integrity of cell envelope: alkaline stress, salt stress, phage, antimicrobial peptides	Release of σ^W to activate extracytoplasmic stress response	EasAB, Tsp or CptB, ClpXP	64, 66–70, 72

Table I. (Continued)

	Substrates	S1P	S2P	Location	Inducing signal	Downstream cascade	Ancillary regulators	References
<i>Enterococcus faecalis</i> (G+)	FtsL (cell division protein)	Unknown	RasP (YluC)	CM	Induced by DNA damage	Cell division		71
<i>Streptococcus uberis</i> (G+)	cCF10, cAD1, iCF10	Unknown	Eep	CM		Release pheromone, signal for conjugation		96–99, 110
<i>Mycobacterium tuberculosis</i> (G+)	MtuA	Unknown	Eep		Absence of lipoprotein signal peptidase Lsp	Peptidase cleavage		111
Archaea	PBP3 (pbpB)	Unknown	Rv2869c	CM/PM	Oxidatively stress	Regulate cell envelope composition and virulence	Wag31	102, 103
<i>Methanocaldococcus jannaschii</i>	Unknown	Unknown	mjS2P					112

IM, inner membrane; PM, periplasm membrane; CM, cytoplasmic membrane; G+, gram positive; G-, gram negative.

MucP, *B. bronchiseptica* HurP, *C. crescentus* MmpA, *B. subtilis* RasP, *E. faecalis* Eep, and *M. tuberculosis* Rv2869c are cataloged in the RseP subclass. They contain only four TM domains and share additional conserved residues GFG 16 amino acids after HEXXH motif [Fig. 2(A)]. The metal-chelating motif HEXXH and NPDG are located within or near the first and third TM segments and face the cytosolic side of the membrane [Fig. 1(B)]. They cleave type II (N_{in}-C_{out}) TM helices of membrane-spanning regulatory proteins such as antisigma factors in response to extracellular signals and stresses.¹¹⁸ Among the RseP subclass, Chen *et al.* reported that MmpA and RseP are functionally interchangeable in heterologous systems.⁸⁵ Experiments also revealed that the defects in hemin utilization and heme-dependent induction of BhuR were restored when recombinant HurP (or recombinant RseP) was introduced into the mutant. Introduction of HurP into a Vc-yaeL mutant of *V. cholerae* also complemented its S2P defect.⁸⁶ These data provided strong evidence that protease activity and cleavage site recognition is conserved in HurP, RseP, and Vc-YaeL. SpoIVFB has additional TM domains before the first and after the fourth TM domains, bringing the total number to six [Fig. 1(C)]. It removes the membrane associated 20aa pro-sequence of pro-σ^K. S2P from *H. sapiens*, *D. melanogaster*, *C. elegans*, and Stp1 from *C. neoformans* are grouped as the eukaryotic S2P homologs [Fig. 2(C)]. Compared with SpoIVFB, they have either one or several additional TM domain at the N-terminal [Fig 1(A)]. They cleave type II TM helices.

It is intriguing to find that two homologs from *Arabidopsis*, AraSP, and EGY1 are classified in different groups [Fig. 2(C)]. AraSP actually has four TM helices, conserved GFG residues and a PDZ domain,⁹⁰ similar with other homologs in RseP subclass. While EGY1 and its cyanobacterium homologs, sll0862 and slr0643 from *Synechocystis* sp. PCC6803 do not contain PDZ domain and have several additional TM helices. Alignment here suggests they are closer to the SpoIVFB group. Chloroplasts are thought to have derived from a cyanobacterium ancestor by endosymbiosis. The high similarity between EGY1 and sll0862 indicates the nuclear gene of EGY1 may be transferred from the genome of ancestral cyanobacterium. Further investigation of these new members, especially regarding their substrate, relative S1P, and biological function may reveal their relationship clearly.

Substrate recognition

In most S2P cascades, substrates are membrane-bound transcription factors or antisigma factors that sequester sigma factors. These transcription factors or sigma factors are responsible for expression of genes that respond to perturbation of the extracytoplasmic or cytoplasmic environment.¹¹⁸ S2P

A

<i>H. sapiens</i> S2P	141-QVLQVVVPGINLPVNLTYFFFAVLISGVVHEIGHGIAAIREQVRFNFGFIFLFI-195
<i>D. melanogaster</i> S2P	120-VQLEILLPGVNLPLEEIGYITTEVLCVIVHEMGAHALAVMEDVPTVFGFKFIF-174
<i>C. elegans</i> S2P	195-RGLVPIIPGFNLQWGHIPFIMLVIVAVFHELGHPAAWARTSGVTVNGFGFILA-249
<i>C. neoformans</i> Stp1	172-GGLRFLIPGLTVPMWHTPFLILAVVNLIELHGHALSASLDDIQPSKLSFNLHY-226
<i>E. coli</i> RseP	1-----MLSF LMDLASFIVALGVLTIVHEFGHFVARRCGVVRERFSFGFK-46
<i>V. cholerae</i> Vc-RseP	1-----MTDILMNFIAFIALGVLVAVHEFGHFVARRCGVVRERFSFGFK-46
<i>P. aeruginosa</i> MucP	1-----MSALYIMVGTIVALGVLTIVHEFGHFVARRCGVVRERFSFGFK-45
<i>B. bronchiseptica</i> HurP	1-----MLFTLLAFVVALGTLITFHELGHWYARLRGCVRVLRFVSGFGR-43
<i>C. crescentus</i> MmpA	1-----MIGFLIMLVLLFVSVVTVHELGHWYARLRGCVRVLRFVSGFGR-46
<i>A. thaliana</i> AraSP	72--FRSRAISGIDLGSFESVLEAIAVLTITIVVHESGHFLASLQGIHVSKFAFGFP-126
<i>B. subtilis</i> RasP	1-----MEVNTVIAFIIIFGLVFFHELHLLLRAGIILCREFAFGFP-44
<i>E. faecalis</i> Eep	1-----MKTITITIVFGLVLFHEFGHFYAKRAGIILVREFAGMGP-42
<i>M. tuberculosis</i> Rv2869c	1-----MMFVTGIVLFAALISVALHECGHWVARRTKGMKVRVYVFGFP-45
<i>B. subtilis</i> SpoIVFB	13--VHFFLMIIAALGLLTHMHALLCHELLVLELHGHAAALVFFSMRKRVLPLFFGP-67
<i>A. thaliana</i> EGY1	281-AVEPDMELLYPFVDAALPLAVGLVGLLLELHGHFLAARVKKVRLSIPYFIPNI-335
<i>Synechocystis</i> sl10862	242-ALLGFDLVDNWRVGETVPLAIVAGIILLHGHLMWAKKRWGVLRSWPFLLPNW-296
<i>Synechocystis</i> slr0643	230-GLTPEKLGAGIQITLMVGLPYSALITLIGCHELSHYGAARHYKIRTTLPYFIPIP-284
<i>M. jannaschii</i> mjs2P	24--FLVVIIGLSIMNNSIFWAVLFIIEFVSVVLEHGHSHVYAKKYGVKIEKILLPIG-78

B

<i>H. sapiens</i> S2P	435-IDLPVVVEFVKYILISLGGALAVNAVCFALDGGQWILNSFLDA-478
<i>D. melanogaster</i> S2P	421-AAWFDAMLLLLKYNVVSIGLALINAIICFDFDAMHITSTVIHS-464
<i>C. elegans</i> S2P	536-ISWLEHPELTAKVYLFPTLSLALGLFNAMVEVALDGGQIVHTLLKS-579
<i>C. neoformans</i> Stp1	455-SAGTRTGLGLLRYTQIITALSLFLNLLLEPFTDSSQLLEALTEW-498
<i>E. coli</i> RseP	370-MTAEIGVVVYLPFLALISVNLGIINLFLPLVLDGGHLLFLAIEK-413
<i>Vibrio cholerae</i> Vc-RseP	372-TTADYGFVYFLGFLALISINLGIINLFLPLVLDGGHLLFMIEA-415
<i>P. aeruginosa</i> MucP	370-ASAQSGVGDFLNFLAYLSISLGLVNLLELPLVLDGGHLLFYLVEW-413
<i>B. bronchiseptica</i> HurP	362-QTARGLGEAYIAYLALISISLGLVNLLELPLVLDGGHLLYLYVEI-405
<i>C. crescentus</i> MmpA	307-ALAIGLIYQFNLIASLSVSIQFMNLLLELPLVLDGGHLLVMYAYEA-350
<i>A. thaliana</i> AraSP	359-EVARSNIDGLYQFALLNINLAVINLLELPLVLDGGHLLALILEA-402
<i>B. subtilis</i> RasP	340-QVAKTGIVNLFQFAAFLSINLGIINLLELPLVLDGGHLLFLFIEA-383
<i>E. faecalis</i> Eep	340-EASNAQVSTVVFVLMAMLSMNLGIINLLELPLVLDGGHLLVNIIEG-383
<i>M. tuberculosis</i> Rv2869c	308-TVDHGLWAFMFFLAQLNLILAAINLLELPLVLDGGHLLAVFER-351
<i>B. subtilis</i> SpoIVFB	105-REVSVIHQHTPELFTYNLSILFVNLLELPLVLDGGHLLFLFLSK-148
<i>A. thaliana</i> EGY1	418-LHARTVSIHPLVIGWCGLTTFANMLLEVGLDGGHLLVAVQAFGK-461
<i>Synechocystis</i> sl10862	374-LKSSVVISIHLPLVIGWGLVINLALNLELPLVLDGGHLLVQAIYGR-417
<i>Synechocystis</i> slr0643	370-GANTVLDLHPLAIAISYIGLIVTALNLELPLVLDGGHLLIHAMLGQ-413
<i>M. jannaschii</i> mjs2P	116-FDININGYPLLYTSLNMLMGGFNLELPLVLDGGHLLRAILSK-159

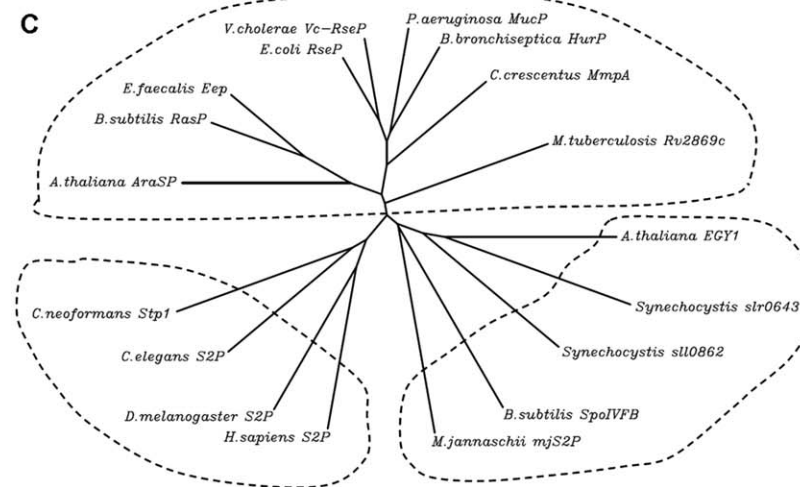


Figure 2. Sequence alignment and relationship among S2P homologs. The sequences of 18 characterized S2P homologs are presented. Only the core TM domains containing the HEXXH and NPDG motifs are shown. **A:** Sequence alignment in the vicinity of the HEXXH motif. The presumed transmembrane helix (TM) is boxed and the consensus amino acids are highlighted in gray. **B:** Sequence alignment for the NPDG motif. **C:** Unrooted dendrogram of S2P homologs based on the alignment of the HEXXH and NPDG motifs. TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and PSIPRED Protein Structure Prediction Server (<http://bioinf.cs.ucl.ac.uk/psipred/>) were used to predict TM helix. The unrooted dendrogram was generated by CLUSTALW (<http://align.genome.jp/>). The aligned sequences are: *H. sapiens* S2P (GI:6016601), *D. melanogaster* S2P (GI:19922044), *C. elegans* S2P (GI:22859116), *C. neoformans* Stp1 (BROAD ID: CNAG_05742), *A. thaliana* EGY1 (GI:15238440), *Synechocystis* sp. PCC6803 sl10862 (GI:16330216), *Synechocystis* sp. PCC6803 slr0643 (GI:16331565), *A. thaliana* AraSP (GI:18402981), *E. coli* RseP (GI:16128169), *V. cholerae* Vc-RseP (GI:20978850), *P. aeruginosa* MucP (GI:146448760), *B. bronchiseptica* HurP (GI:33601589), *C. crescentus* MmpA (GI:20978837), *B. subtilis* RasP (GI:20978800), *E. faecalis* Eep (GI:256853722), *M. tuberculosis* Rv2869c (GI:20978863), *B. subtilis* SpoIVFB (GI:16079849), *M. jannaschii* mjs2P (GI:2499926).

<i>H. sapiens</i> SREBP2	PPVALGMVDRSRILLCVLTFLCLSFNPLTSLIQWGGAHDSQHPHSGSGRSVLSFESG
<i>H. sapiens</i> ATF6	RLKVPSPKRRVVCVMIIVLAFIIINLYGEMSMLEQDSRRMNP SVGPANQRRHLLGFS A
<i>H. sapiens</i> CREBH	VQSTSKSAQTGTCVAVLLLSFALIILEPISSEFGPNKTESPGDFAPVRFVSRTHNDAA
<i>H. sapiens</i> OASIS	KMAATQTGTCLMVAALCFVLVLSLVEFCLPEEFS SSGSQTVKEDP LAADGVYTASQMPSPSLLFYDDG
<i>C. neoformans</i> Sre1	TKITPQSTASAGARVFAAFAMSFVFSASTVFRSASQPQALTDKVLGHATDQQILASVFLIIAEHISRLARS
<i>E. coli</i> RseA	FWQKVRPWAAQLTQMGVAACVSLAVIVGVQHYNGQSETSQQPETPVFNTLPMMGKASPVSLGVPSEATA
<i>P. aeruginosa</i> MucA	-KAEKGPWR-MVGRlavaasvtlavlagvrlYNQNDALPQMAQQGTTPIALPQVKGPAVLAGYSEEQGAP
<i>V. cholerae</i> TcpP	KERIKIEWGKINVVVEYLVFSALYVALLEVIWWSYTGQWYQHELAGITHDLRDLARLPGITIQKLESEQKLT
<i>B. bronchiseptica</i> HurR	AALGRARDRRAMLKSLVAMLAAPPAAWAAALRGARNSGWLADLRTGTGETRTVALGPGTQLRLNTGTAVS
<i>C. crescentus</i> PodJ	GFSTKKSARLGGATVTTALVVFAAAGALGAGVGLLLLNTDDGNNSPRAQAIAGRKADVEVNGPEAD
<i>B. subtilis</i> RsiW	KKRASVKRWFRTHEVIAAAAVFIIIMGGGFFNSWHNDHNF SVSKQPNLVVHNHTVTVPEGETVKGDVTVKNG
<i>B. subtilis</i> FtsL	KKRASITLGERVLLVLFVAAVLSVSLIIVSKAYAAYQTNIIEVQKLEEQISSENKQIGDLEKSVADLSPKQ
<i>M. tuberculosis</i> pbpB	RGASFVFRHRTGNVAVILVLMVAATQLFFLQVSHAAGLRAQAAGQLKVTDVQPAARGSI VDRNNDRLAF
<i>B. subtilis</i> pro- σ^k	-----MVTGVFAALGFVVKELVFLVSYVKNNAFPQPLSSEEKYLLEMAKGDHARNMLIEHN

Figure 3. Sequence and properties of S2P substrates. The sequences of the predicted TM segments of 14 identified S2P substrates are shown. The boxes denote the predicted TM domains. Putative helix-destabilizing residues (N, P, G or repeated amino acids) in the TM regions are highlighted in gray. S1P and S2P cleavage sites, where known, are highlighted between residues in bold (outside TM for S1P and inside or close to TM for S2P, respectively). The consensus sequences of the human S1P site (RxxL or RxxLx) are underlined. The aligned sequences are: *H. sapiens* SREBP2 (GI:27477113), *H. sapiens* ATF6 (GI:2245630), *H. sapiens* CREBH (GI:14211949), *H. sapiens* OASIS (GI:21668502), *C. neoformans* Sre1 (BROAD ID: CNAG_04804), *E. coli* RseA (GI:89109378), *P. aeruginosa* MucA (GI:223702393), *V. cholerae* TcpP (GI:14548354), *B. bronchiseptica* HurR (GI:33603626), *C. crescentus* PodJ (GI:221235062), *B. subtilis* RsiW (GI:62900894), *B. subtilis* FtsL (GI:1122761), *M. tuberculosis* pbpB (GI:15609300), *B. subtilis* pro- σ^k (GI:133478).

cascades liberate the mature form of transcription factors or sigma factors from membrane by sequential cleavages, which enables them to bind RNA

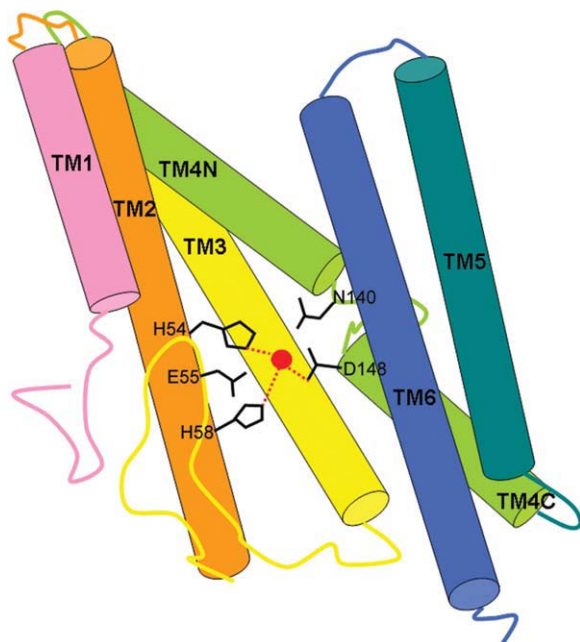


Figure 4. The crystal structure of mjS2P from *Methanocaldococcus jannaschii*.¹¹² Six transmembrane helices (TM) are shown in different colors. The catalytic zinc atom (red) is coordinated by His54 and His58 on TM2 and Asp148 at the N terminal end of helix TM4C. The conserved Glu55 and Asn140 may contribute to catalysis.

polymerase (RNAP). The complete assembly of the RNAP complex will trigger the expression of responsive genes. Generally this cascade is negatively controlled to prevent the cells from constitutively expressing unnecessary genes under normal conditions and it will be initiated immediately once the cells suffer from abnormal environmental stresses.

Interestingly, more than one substrate has been identified for several S2P cascades and some substrates share sequence similarity at the cleavage site. For example, SREBP2, ATF6, CREBH, and OASIS are substrates of human S1P and S2P. They share a similar S1P cleavage site designated as RxxL or RxxLx (Fig. 3).²⁶ RsiW and FtsL are substrates of RasP in *Bacillus subtilis* and they have the same S2P cleavage site with sequence AAAV (Fig. 3).⁷¹ When comparing all the S2P substrates identified so far, it is obvious that there is no consensus sequence at the substrate cleavage site. However, the substrates all contain a transmembrane helix or membrane associated sequence, within which the S2P cleavage site reside inside or near the membrane (Fig. 3). They all contain amino acids of low helical propensity, for example, Pro (P), Gly (G), and Asn (N), in the TM segment (Fig. 3). The resultant helix destabilization may facilitate the cleavage process.

RseP-substrate recognition. Intensive investigations have been conducted on RseP to reveal its substrate recognition. *In vivo* and *in vitro* studies show

that RseP can cleave transmembrane sequences of some model membrane proteins that are unrelated to RseA, provided that the transmembrane region contains residues of low helical propensity. These results show that RseP has proteolytic activity against a broad range of TM sequences and is dependent on residues of low helical propensity in the transmembrane segment of substrates.⁴¹ An α -helix is generally resistant to proteolytic cleavage, because the conformation makes the amide bonds inaccessible to the protease active site; helix-destabilizing residues may make the substrate polypeptide backbone more susceptible to hydrolysis. Helix-destabilizing residues in the TM sequence of a substrate were also reported to promote cleavage by Rhomboid proteases and the signal peptide peptidase.^{119–121}

Through coimmunoprecipitation assays, Koide *et al.* showed that helix-destabilizing residues in a substrate transmembrane segment, which were previously shown to be required for efficient proteolysis of the substrate by RseP, stabilized the substrate-RseP interaction.¹²² The third transmembrane region of RseP is also involved in substrate binding. Asn-394(N) and Pro-397(P) are conserved amino acid in TM3 of RseP and might play a role in the substrate binding.¹²² Inaba *et al.* isolated deregulated RseP mutants which suggested that the proteolytic function is controlled by ligand binding to two tandem PDZ domains in RseP, especially the first one.¹²³

Other than SpoIVFB, most site-2 proteases cut following the site-1 cleavage. Several investigations have explored why Site-1 cleavage must precede Site-2 cleavage. It was found that cleavage by DegS may remove the Gln-rich domain of RseA and eliminate the RseB mediated three-component inhibitory system on RseP (Fig. 1).^{45,46} Recently in the reconstituted sequential cleavages of the *E. coli* RseA by DegS (S1P) and RseP (S2P), Shi and coworkers found that Val¹⁴⁸ of RseA, the newly exposed carboxyl-terminal hydrophobic amino acid is required for the cleavage of RseA by RseP following DegS cleavage. Its mutation to a charged or dissimilar amino acid abolished the Site-2 cleavage. Structural analysis revealed that the putative peptide-binding groove in the second, but not the first, PDZ domain of RseP is poised for binding a single hydrophobic amino acid. Based on the structural evidence, they suggested that after DegS cleavage, the newly exposed carboxyl terminus of RseA Val¹⁴⁸ interacts with the second PDZ domain of RseP to facilitate its cleavage.¹²⁴ This finding parallels observations on the role of ectodomain shedding in γ -secretase.¹²⁵ A common model indicates that ectodomain shedding by S1P frees a single terminal residue of substrates to interact with extracytoplasmic domain of I-CliPs and activate the second cleavage.¹²⁵

SpoIVFB-substrate recognition. Prince *et al.* found a small N-terminal segment (amino acids 1 to

27) was sufficient for membrane localization of a pro- σ^K -GFP chimera.⁵³ Longer segments, however, are required for RIP. It was found that nearly half of pro- σ^K (amino acids 1 to 117) was required for RIP of pro- σ^K -GFP chimeras in sporulating *B. subtilis*. Likewise, pro- σ^K -hexahistidine chimeras demonstrated that the N-terminal 117 amino acids of pro- σ^K are sufficient for RIP, although the N-terminal 126 amino acids allowed much better accumulation of the chimeric protein in sporulating *B. subtilis* and more efficient processing by SpoIVFB in *E. coli*.⁵³

S2P-Substrate recognition. Shen and Prywes found that short luminal domains of ATF6 result in S1P-independent S2P cleavage.¹⁰⁶ The addition of artificial irrelevant domains onto these short ATF6 luminal domains restored the S1P dependence of S2P cleavage, suggesting that dependence of site-2 protease cleavage of ATF6 on prior site-1 protease digestion is determined by the size of the luminal domain of ATF6 but not the specific sequences in the luminal domain.¹⁰⁶

Perspective

As one of the families executing intramembrane cleavage, S2P and its homologs are involved in RIP events that control stress responses,^{33,34} cell division,⁷¹ bacterial mating,⁹⁸ pathogenesis,¹ and polar organelle biogenesis,⁸⁵ among other activities. The nearly universal presence of S2P homologs in different organisms and the diverse functions they play suggest that they perform fundamental functions that convey advantages to cells. Though responsive to different signals and initiated by various S1Ps, the S2P cascades still exhibit common properties. S2P homologs share the conserved HEXXH and NPDG motifs and are easier to identify through a bioinformatics approach.^{113,114} The prevalence of amino acids of low helical propensity in the TM segment of substrates may suggest a conserved catalytic conformation and mechanism in these S2P homologs. Further structural analysis of the S2Ps and their substrates may help to answer this question. The absence of identified substrates is the major bottleneck for protease research because it may hamper further progress in understanding the molecular basis of how these proteases function. Some substrates and relative regulator proteins have been identified through forward genetic approaches that look for suppressors of protease mutant phenotypes. Differential proteomics, protein arrays and yeast two-hybrid screening are useful tools for substrate identification. While the protease cleavage specificity and its subcellular location might help to select candidate substrates. And amino acids of low helical propensity in the TM segment of substrates may help to identify substrates of new S2P homologs.

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