Regulation of the periplasmic stress responses in *E. coli* and *P. aeruginosa*

by

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ABSTRACT

The ability to adapt to changing environments is essential to survival. Bacteria have developed sophisticated means by which they sense and respond to stresses imposed by changes in the environment. I have undertaken the study of elements of the σ^E stress response pathway in the bacterium *Escherichia coli* and the orthologous pathway in the bacterium *Pseudomonas aeruginosa*. These pathways sense stress in the periplasm and relay the signal into the cytoplasm by a series of proteolytic cleavages of a transmembrane regulatory protein.

In *E. coli*, I have undertaken the study of the regulation of the cleavage of transmembrane regulator RseA by the first protease, DegS. I discovered that RseB, an RseA-binding protein, inhibits cleavage of RseA by DegS. The interaction between RseA and RseB is strong and specific, and the inhibition of cleavage is independent of the autoinhibition of DegS by its PDZ domain.

In *P. aeruginosa*, I have demonstrated that AlgW, the homolog of DegS, cleaves the transmembrane regulator MucA. I have shown similar inhibitory effects of the ortholog of RseB on the ortholog of RseA. Interestingly, the PDZ domain of AlgW appears to function differently from that of DegS. In addition, I observed that a regulatory loop in the AlgW protease plays an inhibitory role in the binding of substrate.

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CHAPTER ONE

Introduction to the periplasmic stress response

The selective advantage of adaptive changes to physiology in response to changes in environment

The fitness of a replicating entity depends on the abilities of its constituents within a population to survive changes in the environment. In order to survive and reproduce effectively under diverse conditions a population of replicators must rely on heterogeneity of behavior, either among individuals or temporally within individuals. Some heterogeneity is manifested in differences among individuals. This can be due to heritable differences in behavior or to stochastic responses to environmental stimuli among individuals. Such strategies allow for a range of responses to be realized in a population, thus increasing the likelihood that at least one individual will survive a stress and reproduce.

It is apparent, however, that organisms are not static or purely stochastic in response to changing environments. When environmental conditions change rapidly with respect to the generation time of the organism, the toll on a stagnant population can be large even if the range of strategies employed by individuals is diverse. Furthermore, changes in environment that are detrimental to survival may also slow the generation time of those that do survive, exacerbating the problem of reproduction for a non-responsive strategy. Hence, selection has favored temporal adaptations at the level of the individual, fundamentally the cell, to cope with such environmental changes. In such systems, the individual possesses sensory systems to detect and respond to different aspects of or changes in the environment or perturbations of its own physiology.

What are the physiological changes consequent to environmental changes and how are they detected? We focus on orthologous stress responses of two Gram-negative bacterial species intimately connected with human life that serve as model systems: *Escherichia coli* and *Pseudomonas aeruginosa*. *E. coli* is a mutualistic symbiotic resident of the gut, although some strains are pathogenic. The study of *E. coli* is facilitated by tools developed over a long history of genetic, biochemical, and cell biological experimentation on the organism. *P. aeruginosa* is an opportunistic pathogen in humans, particularly in people who suffer from cystic fibrosis (CF), whose lungs are often infected by this bacterium in a process that involves the stress response of the bacterium (Govan and Deretic 1996). This has afforded the advantage of population-genetic type studies to observe the evolution of stress response during the course of infections (Mathee et al. 2008).

Our research emphasis has been on the σ^E pathway, one of the stress responses in the bacterial envelope. We will discuss the structure of the Gram-negative cell envelope, followed by a summary of the σ^E stress response system in *E. coli* and the orthologous AlgU system in *P. aeruginosa*, looking in detail at each of the proteins involved in the signalling cascade. We then turn to discuss the HtrA class of molecules that play a role in both the detection of and response to envelope stress. Finally, we discuss the importance of the mammalian homologs of this class of molecule.

Structure of the Gram-negative cell envelope

The *E. coli* envelope comprises the outer membrane, the inner membrane, and the periplasmic space between them. The inner membrane is a phospholipid bilayer. The inner layer of the outer membrane is composed primarily of phospholipid and the outer layer is composed chiefly of lipopolysaccharide (LPS). The periplasmic space includes the peptidoglycan, which is anchored to the outer membrane by covalent attachments to the major outer membrane lipoprotein (Bos and Tommassen 2004). Under some conditions, *P. aeruginosa* (but not *E. coli*) produce the polysaccharide alginate, which becomes the outermost surface of the cell (Govan and Deretic 1996).

Proteins are present in all regions of the envelope. Integral inner membrane proteins are recognized and inserted into the membrane by virtue of their hydrophobic transmembrane domains, with orientation governed by the charges of the extramembrane domains (Luirink et al. 2005). Soluble periplasmic proteins as well as outer membrane proteins and nascent lipoproteins are transported to the periplasm by virtue of their N-terminal sequences, which serve as secretion signals. After transport into the periplasm, secretion signals are either retained, keeping the protein attached to the inner membrane, or they are cleaved by signal peptidase, which allows the protein to become soluble in the periplasm.

Lipoproteins are present in both the inner and outer membranes. Their localization requires a secretion signal whose sequence determines whether the lipoprotein will remain in the inner membrane or be shuttled to the outer membrane. Lipoproteins contain a cysteine residue after the

signal sequence to which a diacylglycerol group is attached via the sulfhydryl group. A third acyl group is added to the amino terminus of the protein after removal of the signal sequence. Depending on the identity of residues near the cysteine, lipoproteins either remain in the inner membrane or are transported to the outer membrane by the LolA/LolB proteins (Narita et al. 2004).

Porin molecules provide channels in the outer membrane through which ions and small molecules can pass between the extracellular space and the periplasm. Porins contain secretion signals which are cleaved after transit through the inner membrane. They are then chaperoned to the outer membrane by soluble periplasmic chaperones and are inserted into the membrane by the Omp85 (YaeT) complex (Gentle et al. 2005, Mogensen and Otzen 2005), possibly by recognition of porin C termini (Robert et al. 2006).

Stress responses in the envelope

Environmental conditions can affect the structure and function of proteins in the envelope, and one way the cell deals with this is to change the profile of proteins that are produced under different conditions. Protein production can be controlled in a variety of ways: the levels of production of messenger RNAs that encode the proteins, secondary processing of messenger RNAs, stability of messenger RNAs, levels of protein expression, post-translational modification, protein stability, and activity (e.g. allosteric effectors).

Transcriptional control is a common way in which large changes in the proteome can be effected rapidly. Whereas individual proteins have different sensitivities to environmental conditions, stabilities, and activities, transcriptional control is based on patterns of promoters that are controlled by different transcription factors. Thus, numerous proteins can be controlled at the level of transcription by being under the control of a transcription factor whose activity is a function of that environmental condition.

Control of transcription factors can be accomplished in a number of ways. They, too, can be controlled at the level of their own transcription. Post-translational modifications such as phosphorylation can control the activity of transcription factors, as they do in the Cpx stress response in *E. coli* (Raivio and Silhavy 2001). In the σ^E response, the transcription factor is activated by proteolysis of its inhibitor in the presence of environmental stress (Alba and Gross 2004, Hasenbein et al. 2007).

The σ^E system in *E. coli*

σ^E was initially identified as a subunit of RNA polymerase involved in expression of genes at high temperatures (Erickson and Gross 1989). The gene corresponding to σ^E activity was identified and named *rpoE* (Raina et al. 1995, Rouviere et al. 1995). *rpoE* is an essential gene in *E. coli* because the cell requires a basal level of σ^E activity even in the absence of stress (De Las Penas et al. 1997). σ^E directs transcription of a variety of genes, including those encoding proteins that act as chaperones for proteins in the periplasm or the outer membrane (Rhodius et al. 2006).

rpoE is the first gene in an operon that contains three other genes: *rseA*, *rseB*, and *rseC*. RseA is an inner membrane spanning protein whose cytoplasmic domain binds and inhibits the activity of σ^E (De Las Penas et al. 1997, Missiakas et al. 1997, Campbell et al. 2003). The inhibitory effect of RseA is broken in the presence of stress by its cleavage in succession by three proteases. It is cleaved first in the periplasm by the membrane-tethered serine protease DegS (Ades et al. 1999, Alba et al. 2001), and subsequently in its transmembrane region by the integral membrane metalloprotease YaeL (also known as EcfE or RseP; Alba et al. 2002, Kanehara et al. 2002) (Figure 1). This cleavage releases the cytoplasmic portion of RseA from the membrane and reveals a C terminal recognition motif for the cytoplasmic protease ClpXP, which degrades the cytoplasmic domain of RseA and frees σ^E (Flynn et al. 2004). This type of signal transduction, which involves cleavage of a transmembrane protein within the membrane, has been termed 'regulated intramembrane proteolysis (RIP)', and occurs in diverse signal transduction systems including those involved in sporulation in *Bacillus subtilis*, sterol regulation in eukaryotes, the mammalian unfolded protein response, and intercellular communication through Notch and EGF-signalling (Brown et al. 2000, Urban and Freeman 2002, Wolfe and Kopan 2004).

How does heat stress activate the proteolytic cascade that results in the activation of σ^E? The first clue came from a genetic screen for genes that act as activators of σ^E activity when present on multicopy plasmids. This experiment yielded a number of outer membrane porins, and it was shown that overexpression of porins activates σ^E (Mecsas et al. 1993). It was later discovered that inactivation of the DegS protease inhibited the σ^E response (Ades et al. 1999). In addition, it was discovered that the proteolytic activity of DegS could be stimulated by the carboxyl terminal

sequences of porins (Walsh et al. 2003). It is believed that folded porins do not have exposed C termini, and thus that porin C termini can act as a proxy for detecting protein folding defects in the periplasm or disruptions in the outer membrane. Whether this is a consequence of porins that have come out of the membrane or it is due to nascent membrane proteins that are unable to be inserted in the membrane is not clear.

The AlgU system in *P. aeruginosa*

One of the environmental changes that is of great human relevance is that of the infection by the opportunistic pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* has emerged as a significant problem especially for those who suffer from cystic fibrosis. The lungs of CF patients are often infected with *P. aeruginosa*, and although the lungs may be cleared of infections with treatment, infections are often recurrent, and result in a reduction of lung function with each episode. A noticeable and important phenotypic change is the mucoid phenotype of many *P. aeruginosa* isolates from CF patients' lungs. The mucoid phenotype is the consequence of the production of the external polysaccharide alginate, which is generally concomitant with the induction of the extracytoplasmic stress response. These phenotypic changes in the bacterium allow it to resist clearance by the immune system via protection from oxidative stress imposed by macrophages as well as by the protection from killing by many antibiotic compounds. As a result, *P. aeruginosa* infections in the CF lung are difficult to treat, and also make the lung susceptible to infections by other bacterial species (Govan and Deretic 1996).

Work on determining the genetic basis for mucoidy resulted in the identification of a gene named *mucA*, which when deleted conferred the mucoid phenotype (Martin et al. 1993). In addition, numerous mucoid CF isolates were sequenced and a large fraction of them were found to have mutations in this gene that resulted in nonsense codons in the periplasmic domain (Boucher et al. 1997, Bragonzi et al. 2006). MucA is homologous to *E. coli* RseA, and binds and inactivates the transcription factor AlgU (AlgT), which controls both the stress response and the production of alginate (Erickson and Gross 1989, DeVries and Ohman 1994). It was evident that mutant MucA from CF isolates was less stable than wild-type, though it was not clear how or by what protease MucA was being cleaved (Rowen and Deretic 2000). Work in *E. coli* gave hints, and it has been suggested since (and we have verified independently *in vitro*) that the *P. aeruginosa* ortholog of DegS, AlgW, is responsible for cleaving MucA (Wood et al. 2006, Qiu et al. 2007).

The role of PPP proteins in stress sensing and response

Many of the proteins involved in stress tolerance in gram negative bacteria are periplasmically localized PDZ-domain containing proteases (PPP proteins) (Figure 2). In *E. coli* these proteins include DegS, DegP, as well as DegQ, which is similar in domain structure to DegP and is located adjacent to *degS* on the chromosome. The YaeL protease is a member of this class, as is Prc (Tsp) which has been identified as a protease that recognizes substrates with nonpolar C termini (Silber et al. 1992, Keiler and Sauer 1996).

PDZ domains

PDZ domains are compact, small (about 90 amino acid) protein domains that are rich in beta sheets. They are present in prokaryotes and eukaryotes in proteins of diverse function, and often mediate interactions with other proteins. They are prevalent in proteins that are linked directly or indirectly to a membrane, especially in neuronal synapses where they serve to scaffold proteins along the synaptic membrane (Kim and Sheng 2004). The most common method by which PDZ domains bind proteins is by motifs at the carboxyl terminus (Songyang et al. 1997, Harris and Lim 2001). Several classes of PDZ domains have been discovered that recognize different carboxyl terminal motifs. PDZ domains have also been shown to bind internal peptide sequences. Some PDZ domains are capable of binding both C-terminal motifs and internal sequences as well, though not necessarily the same motif. PDZ domains have also been shown to form homo- and hetero-oligomers (Hillier et al. 1999, Im et al. 2003) and to interact with lipids (Mortier et al. 2005). In eukaryotes PDZ domains are often present in multiple copies in tandem within a single protein, with each PDZ domain mediating a different function. In other cases, a tandem of PDZ domains is required for a single function which each PDZ domain is unable to perform on its own (Grootjans et al. 2000, Kang et al. 2003 and references 5, 13 therein, Cierpicki et al. 2005, Long et al. 2003, Grembecka et al. 2006).

PDZ domains have been grouped into different classes based on the polypeptide sequences to which they bind (Songyang et al. 1997, Harris and Lim 2001). Class I PDZ domains bind the C terminal motif S/T-X-Φ, where Φ represents a hydrophobic amino acid and X represents a position with no strong preference. Class II PDZ domains bind the C terminal motif Φ-X-Φ.

Other classes of PDZ domains bind the C terminal motif E/D-X-Φ or X-X-C (Harris and Lim 2001, Nourry et al. 2003). Some PDZ domains bind proteins of one class, while others are capable of binding to proteins of multiple classes (Nourry et al. 2003). Furthermore, a PDZ domain that binds a peptide in a given class will not necessarily tolerate all possible mutations in its binding partner that still remains within that class.

The tandem arrangement of PDZ domains has been shown to be important for their function. It has been demonstrated structurally with the PDZ tandom of X11/Mint scaffold proteins, wherein one PDZ domain inhibits binding of low-affinity peptides by blocking the binding site on the adjacent PDZ domain (Long et al. 2005). In the glutamate receptor-interacting protein (GRIP), it has been shown that PDZ4 and PDZ5 are both required for binding to the glutamate receptor, and biochemical studies have demonstrated that neither PDZ4 or PDZ5 is stably structured alone unless they are in tandem (Zhang et al. 2001, Feng et al. 2003).

DegS

DegS is a trimeric, inner membrane-tethered periplasmic protein (Walsh et al. 2003). It contains a trypsin-like protease domain and a PDZ domain. The structure of DegS has been determined by x-ray crystallography (Wilken et al. 2004, Zeth 2004) (Figure 3). DegS was identified as the potential protease acting on RseA because deletion of it among multiple periplasmic proteases was the only one that significantly and substantially reduced σ^E activity (Ades et al. 1999). *degS* is an essential gene in *E. coli* because it is required for σ^E activity (Alba et al. 2001).

The PDZ domain of DegS is believed to play an inhibitory role with respect to proteolysis of RseA. $DegS^{\Delta PDZ}$ suppresses the lethality of a *ΔdegS* strain. Furthermore, strains containing $DegS^{\Delta PDZ}$ have higher basal activity of sigE than those with full-length DegS (Walsh et al. 2003). In vitro cleavage of RseA by DegS is very slow in the absence of activator, but cleavage by $DegS^{\Delta PDZ}$ is rapid in the absence of activator (Cezairliyan and Sauer 2007, Sohn et al. 2007). Binding of the PDZ domain of DegS to the C termini of porins is believed to be the activating signal in the initiation of the stress response (Walsh et al. 2003).

YaeL

YaeL (RseP) is a member of the membrane metalloprotease class. It traverses the inner membrane four times and has a long periplasmic region between the third and fourth transmembrane domains. It contains two PDZ domains in tandem (Kinch et al. 2006), which are located in the periplasm. The the proteolytic function of YaeL was initially inferred by sequence homology to the human site-2-protease (Kanehara et al. 2001). *yaeL* is an essential gene in *E. coli* (Kanehara et al. 2001) because it is required for σ^E activity (Alba et al. 2002, Kanehara et al. 2002).

YaeL has been shown to cleave RseA *in vivo* after cleavage by DegS (Alba et al. 2002, Kanehara et al. 2002). While *ΔyaeL* strains are inviable, *ΔyaeLΔrseA* strains are viable (Kanehara et al. 2002). Furthermore, *rpoE* was identified as a multicopy suppressor of the *ΔyaeL* lethal phenotype, suggesting that its sole essential function is to activate σ^E (Kanehara et al. 2002). A gene encoding a small RNA was also isolated as a suppressor of the *ΔyaeL* phenotype, and is

believed to act by downregulating porins (Douchin et al. 2006). When two porins (OmpA and OmpC) are deleted together, a *yaeL* deletion strain is viable (Douchin et al. 2006).

One of the PDZ domains of YaeL (the closest to the amino terminus) has been implicated as a repressor of its proteolytic acitivity (Kanehara et al. 2003, Bohn et al. 2004). Not only is a $YaeL^{\Delta PDZ1}$ construct capable of complementing the *ΔyaeL* lethal phenotype, but cleavage of RseA occurred in a DegS-independent manner (Kanehara et al. 2003, Bohn et al. 2004). Point mutations in critical positions of PDZ1 show similar phenotypes. Furthermore, it appears that there are regulatory elements of the periplasmic region of RseA that prevent it from being cleaved by *yaeL* prior to cleavage by DegS. Specifically, there is a glutamine-rich region of RseA that has been shown to be important for the inhibition of proteolysis by YaeL prior to cleavage by DegS. The proteolytic activity of YaeL has been reconstituted *in vitro*, however it is not evident that such a system recapitulates the complex regulation by the PDZ domain and by aspects of the sequence of RseA (Akiyama et al. 2004).

RseB

rseB, the gene immediately downstream of *rseA* in the *rpoE* operon, encodes a 34 kilodalton soluble periplasmic protein. RseB was found to interact with the periplasmic domain of RseA. The absence of RseB correlates with decreased stability of RseA as well as an increase in the level of σ^E activity (De Las Penas et al. 1997, Missiakas et al. 1997, Ades et al. 1999), suggesting that binding of RseB to RseA inhibits the ability of DegS or YaeL to cleave RseA. Biochemical evidence suggests that RseB binding to RseA inhibits cleavage of RseA by DegS (Cezairliyan

and Sauer 2007). Genetic evidence has implicated RseB as an inhibitor of YaeL proteolysis of full-length RseA, since YaeL can cleave full-length RseA in a *ΔdegSΔrseB* strain (Grigorova et al. 2004). This inhibitory role may be dependent on the PDZ domain of YaeL because RseB does not inhibit cleavage of full-length RseA very well against $YaeL^{\Delta PDZ1}$ (Grigorova et al. 2004).

The structure of RseB has been determined by x-ray crystallography (Kim et al. 2007, Wollmann and Zeth 2007), which shows that it is a beta-sheet rich dimeric protein with two domains (Figure 4). One domain, colored blue, resembles that of fatty-acid binding proteins, suggesting that it may serve as a sensor of unincorporated membrane lipid components in the periplasm.

DegP and DegQ

DegP (HtrA) was identified as a protein that cleaves unfolded periplasmic proteins (Strauch and Beckwith 1988). It was also shown to be an essential gene in *E. coli* at high temperatures. Transcription of *degP* is highly induced upon heat stress and other stresses that activate the σ^E or Cpx stress responses (Raivio and Silhavy 2001, Alba and Gross 2004, Rhodius et al. 2006).
In *E. coli degQ* is adjacent to *degS* in the chromosome, although their transcription is regulated by different promoters and *degQ* expression is not induced by heat shock (Waller and Sauer 1996). Neither *degQ* nor *degP* is essential for the transduction of the σ^E stress response (Ades et al. 1999). The function of *degQ* is unclear, but it likely overlaps partially with that of *degP* as multicopy *degQ* was found to suppress the high-temperature lethality of a *degP*$^-$ strain (Waller and Sauer 1996).

DegP and DegQ are soluble hexameric periplasmic proteins (Waller and Sauer, Krojer et al. 2002). Each monomeric subunit of DegP and DegQ has a trypsin-like serine protease domain followed by two PDZ domains. The PDZ domains of DegP play an important role in the proteolytic function of DegP, although in a manner different from that of DegS. In one study, DegP lacking either PDZ domain was shown to have approximately 5% of the proteolytic activity of wild-type against the substrate beta-casein, whereas a mutant lacking both PDZ domains showed less than 0.1% activity (Spiess et al. 1999). In another study, deletion of the first PDZ domain of DegP severely disrupted its proteolytic activity in vitro, whereas deletion of the second PDZ domain had little effect on proteolytic activity (Jomaa et al. 2007). Deletion of both PDZ domains also severely diminished proteolytic activity (Iwanczyk et al. 2007). DegP constructs with one or both PDZ domains deleted were trimeric rather than hexameric (Jomaa et al. 2007, Iwanczyk et al. 2007).

A non-proteolytic role for the DegP class of proteins was postulated when a *Rickettsia* homolog was discovered in which all of the catalytic triad residues are absent (Bass et al. 1996). It was later shown that overexpression of a *degP* variant in *E. coli* that has the catalytic serine mutated to alanine can rescue the high-temperature lethal phenotype of a *degP*$^-$ strain (Spiess et al. 1999). It is clear that the protease domain of DegP is sufficient to mediate chaperone activity, although the roles of the PDZ domains in this function are not clear. DegP mutants lacking either of the PDZ domains retain chaperone activity *in vitro* (Spiess et al. 1999, Jomaa et al. 2007). Mutants lacking both PDZ domains had unaffected chaperone activity in one study (Iwanczyk et al. 2007), whereas in another study, up to fourfold higher concentrations were required for chaperone activity equivalent to wild-type (Spiess et al.1999). Interestingly, complementation of

temperature sensitivity of a *degP*⁻ strain was not possible with DegPΔPDZ1 or DegPΔPDZ1+2, but high levels of DegPΔPDZ2 could complement (Spiess et al. 1999). The ability of DegPΔPDZ2 to complement may also be assisted its ability to act as a protease to some extent.

MucD, the DegP ortholog in *P. aeruginosa*, has been shown to be important for virulence (Yorgey et al. 2001). In *Burkholderia cenocepacia*, another opportunistic pathogen in the lungs of cystic fibrosis patients, *degP* mutants were also sensitive to thermal and osmotic stresses, and had impaired virulence (Flannagan et al. 2007). It was shown that the active site serine and the PDZ domains were important for resistance to stress. The effect of *degP* mutation on virulence and survival under stress has been tested in a number of pathogenic bacteria (Mo et al. 2006). *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Salmonella typhimurium*, *Streptococcus pyogenes*, *Yersinia enterocolitica*, and *Yersinia pestis* all showed decrease of virulence in the absence of functional DegP.

HtrA proteins in mammals

There are four human homologs of HtrA, although only one of them, HtrA2, has been well-studied. The nuclear-encoded mitochondrial serine protease HtrA2 (Omi) is the mammalian homolog of DegS. It possesses a trypsin-like serine protease domain and a PDZ domain (Li et al. 2002). HtrA2 has been implicated in programmed cell death because it was found to interact with inhibitors of apoptosis (IAPs) (Suzuki et al. 2001, Hegde et al. 2002, Verhagen et al. 2002, van Loo et al. 2002). It was later discovered that IAPs are substrates of Htra2 (Yang et al. 2003, Srinivasula et al. 2003). Recently, HtrA2 has been linked to a rhomboid-like protease in

mitochondria, where it appears that the inner mitochondrial membrane rhomboid protease PARL, which regulates release of mitochondrial proteins during apoptosis, acts on HtrA2 (Chao et al. 2008).

Mutations in human HtrA2 have been implicated as a susceptibility factor in Parkinson's disease (Strauss et al. 2005). It has been shown that Htra2 interacts with another Parkinson's deasease-associated protein, PINK1, which is a mitochondrial protein kinase that phosphorylates HtrA2 (Plun-Favreau et al. 2007). Mutation of HtrA2 has also been implicated as the causative factor in the Parkinson's-like neuromuscular disorder mnd2 in mice (Jones et al. 2003, Martins et al. 2004), and this phenotype has been attributed to the deficiency in proteolytic activity of HtrA2. These studies have shown that while cytosolic HtrA2 plays a pro-apoptotic role, the role of HtrA2 in the mitochondrion is anti-apoptotic.

Two studies have also provided evidence that HtrA2 can cleave amyloid precursor protein (APP), although they disagree on the mechanism. One suggests that some APP is localized to the mitochondria, where it is cleaved (Park et al. 2006), while another suggests that some HtrA2 is present in the endoplasmic reticulum and that APP is cleaved by HtrA2 there (Huttunen et al. 2007).

The PDZ domain of HtrA2 was found to be a negative regulator as constructs lacking it were more proteolytically active toward a non-native substrate (Li et al. 2002). Like DegS, HtrA2 proteolytic activity can be induced by binding of hydrophobic C-terminal peptides to its PDZ domain (Martins et al. 2003), although it is not clear that the rate enhancement is as great as it is

for DegS (Sohn et al. 2007). The hydrophobic C-terminal tail of the presenilin protein was identified as a potential activator of HtrA2 proteolysis (Gupta et al. 2004). Although the last three residues of presenilin were critical for activation, it was shown that residues as far as 10-15 from the C terminus were important for potentiation of the activating effect. Additional studies of the binding specificity of the HtrA2 PDZ domain also found that it could bind internal hydrophobic motifs (Zhang et al. 2007).

These observations adduce the relevance to human health of studying proteins involved in the bacterial envelope stress response and homologous proteins in eukaryotes. These proteins are key factors in innate human disease as well as diseases caused by pathogenic bacteria. The research detailed in the following chapters centers on two aspects of control in such signalling pathways. First, the role of structural features of the DegS and AlgW proteases in their activation and inhibition. Second, the nature of the inhibitory effect of RseB and the *P. aeruginosa* ortholog MucB on the action of DegS and AlgW on their substrates.

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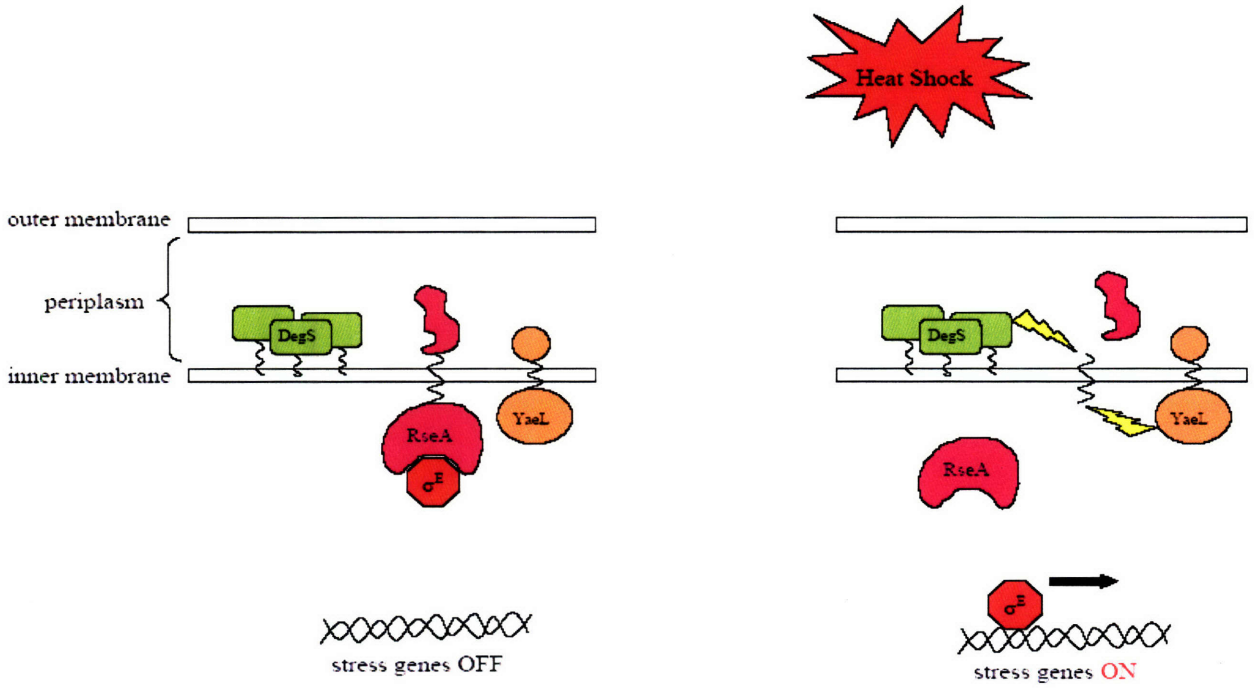

Figure 1: Proteolytic cleavages regulate activity of σ^E

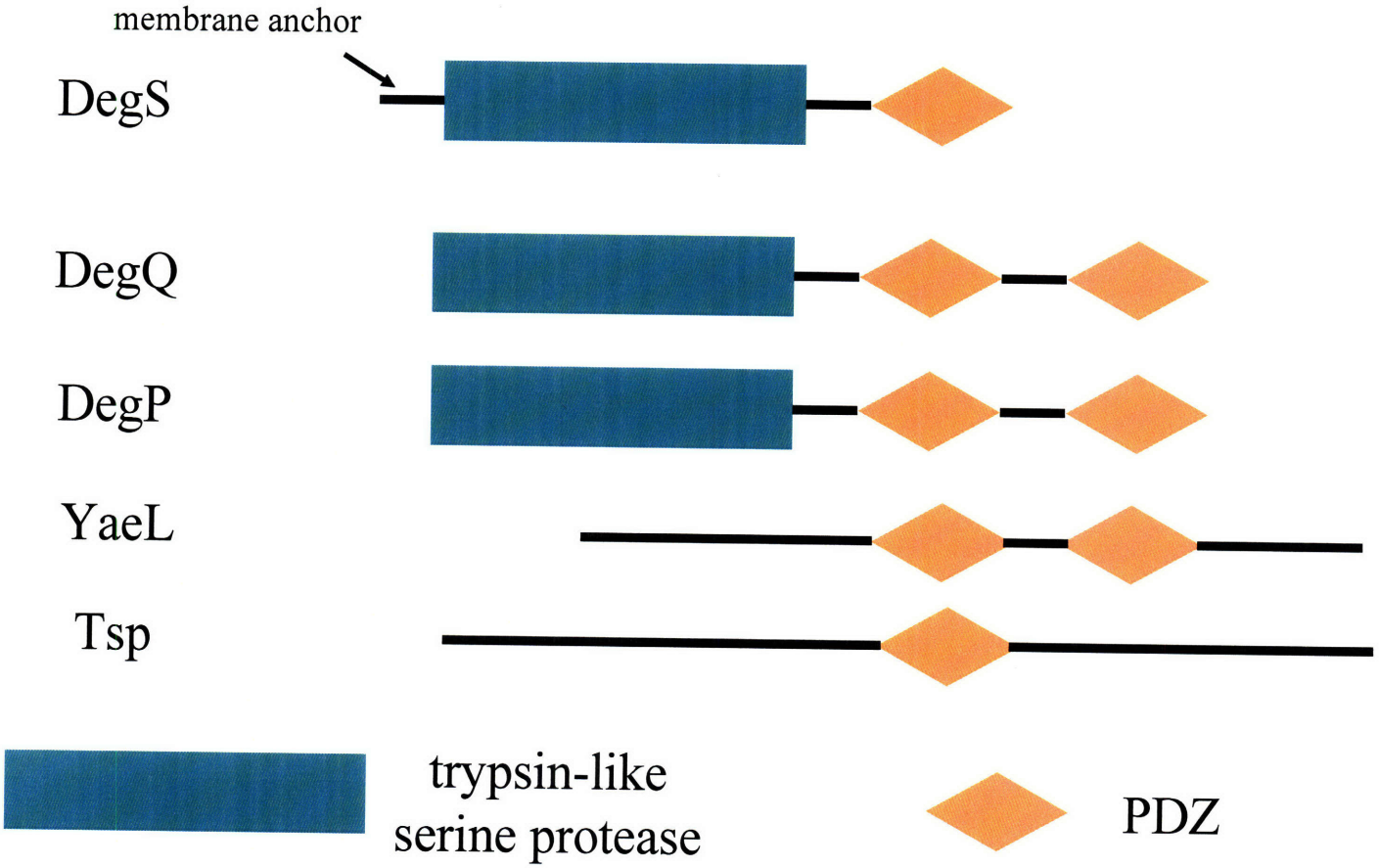

Figure 2: Periplasmic PDZ domain-containing Proteases (PPP) of *E. coli*

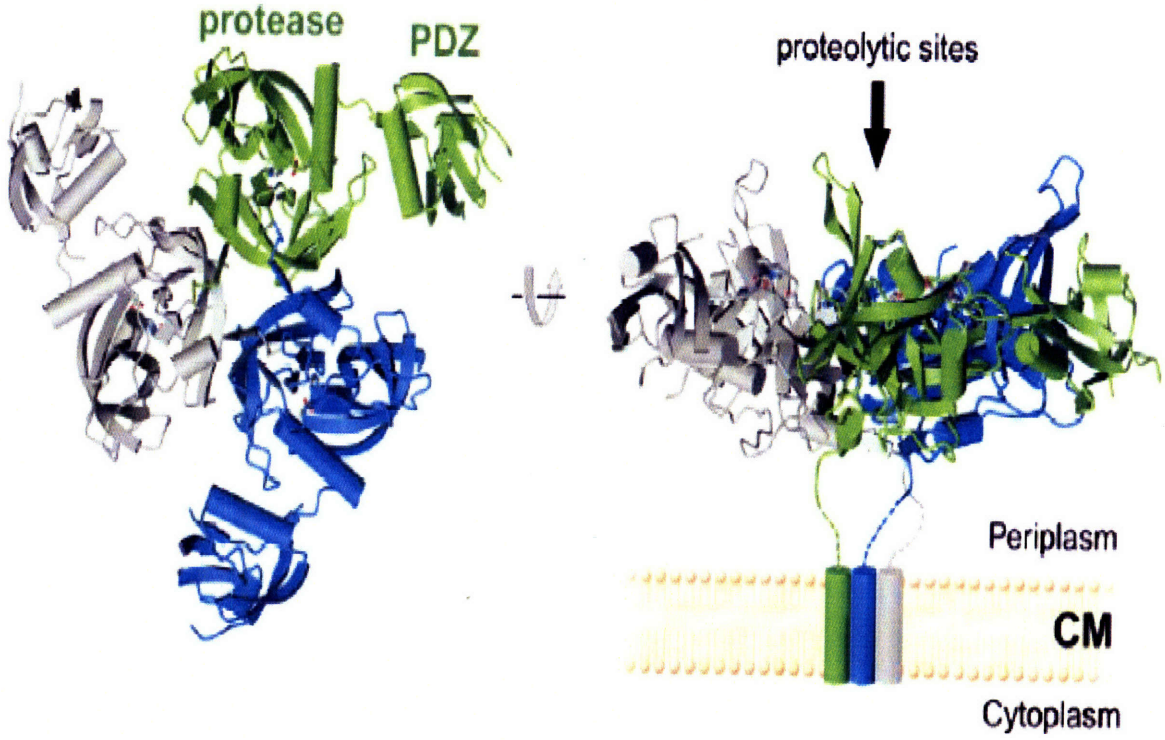

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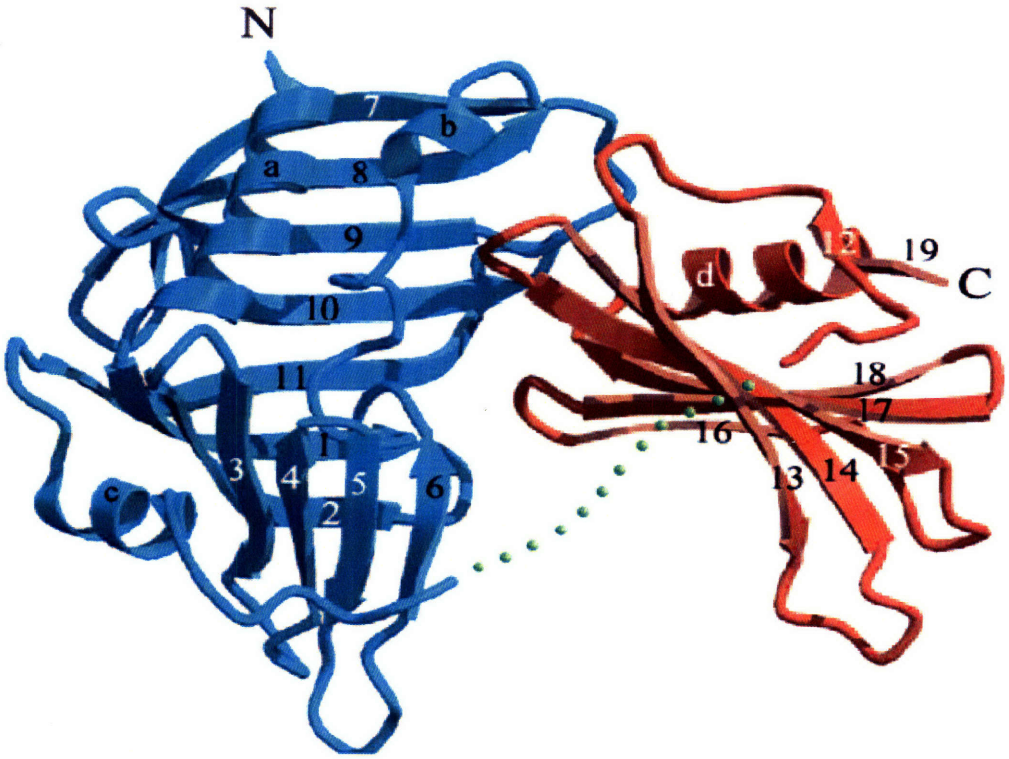
Figure 3: Structure of DegS

A



B

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Figure 4: Structure of RseB.
(A) monomer only shown, with colors corresponding to the N- and C- domains
(B) one of two observed dimer conformations

CHAPTER TWO

Inhibition of Regulated Proteolysis by RseB

Abstract

The envelope-stress response of *E. coli* is a sensor system that increases transcription of stress genes in the cytoplasm when misfolded porins are detected in the periplasm. This response is initiated by DegS cleavage of the periplasmic domain of RseA, a transmembrane protein. Additional proteolysis of transmembrane and cytoplasmic portions of RseA then frees the σ^E transcription factor, which directs the transcriptional response. We show that RseB protein, a known negative regulator, inhibits proteolysis by DegS *in vitro* by binding tightly to the periplasmic domain of RseA. Inhibition of DegS cleavage requires RseB binding to a conserved region near the C terminus of the poorly structured RseA domain, but the RseA sequences that mediate DegS recognition and RseB binding do not overlap directly. Although DegS cleavage of RseA is normally activated by binding of the C termini of porins to the PDZ domain of DegS, RseB inhibition is independent of this activation mechanism.

Introduction

A variety of physiological sensor systems use proteolytic cleavage of a membrane-spanning regulatory protein as a key early step in initiating rapid changes in gene expression (Urban and Freeman, 2002). This method of signal transduction has been named "regulated intramembrane proteolysis" (Brown et al., 2000). Although these systems permit information to be transmitted across membranes in diverse pathways and organisms, the biochemical mechanisms by which regulated intramembrane proteolysis is modulated remain largely undetermined.

The envelope-stress response pathway of *Escherichia coli* is a regulated intramembrane proteolysis system that includes the σ^E transcription factor, the RseA and RseB regulators, and the DegS and RseP (YaeL) proteases (Ades, 2004; Alba and Gross, 2004; Duguay and Silhavy, 2004; Ehrmann and Clausen, 2004; Ruiz and Silhavy, 2005). σ^E controls expression of gene products that facilitate the refolding or degradation of misfolded proteins in the periplasm (Dartigalongue et al., 2001; Kabir et al., 2005; Rhodius et al., 2006). The association of σ^E with RNA polymerase is normally inhibited by formation of a tight complex between σ^E and the cytoplasmic domain of RseA, a transmembrane protein (Campbell et al., 2003). At high temperature or under other conditions that result in misfolding of periplasmic proteins, a series of proteolytic cleavages destroy RseA and liberate σ^E to activate gene expression (Alba et al., 2002). The periplasmic domain of RseA is initially cleaved by DegS, a protease which is anchored to the periplasmic face of the inner membrane (Ades et al., 1999; Alba et al., 2001). This periplasmic cleavage event activates RseP cleavage within the transmembrane region of RseA (Akiyama et al., 2004), releasing the complex of σ^E and the cytoplasmic domain of RseA

from the membrane. The final step in σ^E activation involves degradation of the RseA cytoplasmic domain by ClpXP or other intracellular proteases (Flynn et al., 2004).

The envelope-stress signaling cascade that activates σ^E can be initiated by misfolded outer membrane porins (OMPs) that have a Tyr-Xxx-Phe motif at their C terminus. This tripeptide is buried in native membrane-embedded OMPs but is likely to be accessible to other proteins when OMPs are in an unassembled or denatured state. Peptides containing the C-terminal Tyr-Xxx-Phe motif (OMP peptides) activate DegS cleavage of the periplasmic domain of RseA *in vitro*, and secretion of proteins bearing these C-terminal OMP sequences activates σ^E-mediated gene expression *in vivo* (Walsh et al., 2003). DegS is a trimer, with each subunit consisting of a membrane anchor, a serine-protease domain, and a PDZ domain (Walsh et al., 2003; Wilken et al., 2004). OMP peptides bind to the DegS PDZ domain (Walsh et al., 2003), and crystallographic studies suggest that the bound peptide plays a direct role in activating the protease (Wilken et al., 2004). Another model, in which OMP-peptide binding to the DegS PDZ domain relieves an inhibitory interaction with the DegS protease domain, has also been proposed because DegS lacking the PDZ domain has OMP-independent activity *in vivo* (Walsh et al., 2003). Regardless of uncertainty about the detailed mechanism, however, it is clear that misfolded OMPs or OMP peptides are required to activate cleavage of RseA by full-length DegS.

RseB is a periplasmic protein that negatively regulates the envelope-stress response. Mutational inactivation of RseB results in faster degradation of RseA and increased activity of σ^E in the absence of stress (De Las Penas et al., 1997; Missiakas et al., 1997; Ades et al., 1999; Ades et al.,

2003; Grigorova et al., 2004). RseB also appears to inhibit RseP proteolysis of full-length RseA (Grigorova et al., 2004). RseA and RseB, which interact with each other and are encoded in the same operon with σ^E, have orthologs in numerous bacterial species. In *Pseudomonas aeruginosa*, for example, MucA and MucB (the RseA and RseB orthologs) regulate both the heat-stress response and alginate production by modulating the activity of the AlgU transcription factor (Schurr et al., 1996; Schurr and Deretic, 1997; Rowen and Deretic, 2000). Interestingly, inactivation of either MucA or MucB can result in comparably large increases in AlgU activity (Schurr et al., 1996; Rowen and Deretic, 2000), supporting major regulatory roles for both proteins.

To understand the regulatory role of RseB in greater detail, we have carried out biochemical studies using purified components. In this paper, we show that RseB binds to the periplasmic region of RseA strongly and with one-to-one stoichiometry. The association of RseA with RseB directly inhibits RseA degradation by DegS. This inhibition is independent of OMP peptides and the PDZ domain of DegS. We find that RseB recognizes a small C-terminal region of RseA, which would be released from the membrane following DegS cleavage. Our results suggest that RseB needs to be inactivated by a cellular signal that is distinct from C-terminal OMP peptides to allow RseA release and subsequent cleavage by the DegS and RseP proteases during the envelope-stress response.

Results

RseB inhibition of RseA degradation by DegS

His_6-tagged RseB lacking its N-terminal signal sequence was cloned, overexpressed, and purified (see Methods). This variant contains residues 24-318 of the RseB precursor protein and should be similar to mature periplasmic RseB after cleavage of its signal sequence. Soluble His_6-tagged variants of DegS and the periplasmic region of RseA ($RseA^{peri}$) were also purified (Walsh et al., 2003). We tested the effect of RseB on DegS proteolysis of $RseA^{peri}$ monitored by SDS-PAGE and Coomassie staining (Figure 1). As expected, DegS degraded $RseA^{peri}$ in the presence OMP peptide when RseB was absent. When RseB and OMP peptide were both present, however, degradation of $RseA^{peri}$ by DegS was strongly inhibited (Figure 1).

A DegS variant lacking the PDZ domain ($DegS^{\Delta PDZ}$) was used to test if RseB inhibition of RseA degradation was mediated via interactions with the OMP peptide or the PDZ domain. We found that $DegS^{\Delta PDZ}$ degraded $RseA^{peri}$ at comparable rates both in the presence and absence of OMP peptide (Figure 1). Moreover, cleavage of $RseA^{peri}$ by $DegS^{\Delta PDZ}$ under both conditions was at least as fast as cleavage observed using full-length DegS and OMP peptide. This result demonstrates that the serine-protease domain of DegS is sufficient for recognition and cleavage of RseA; neither the DegS PDZ domain nor the OMP peptide is required for this reaction. RseB inhibited $RseA^{peri}$ degradation by $DegS^{\Delta PDZ}$ in the absence or presence of OMP peptide. We conclude that RseB inhibits degradation of RseA by a mechanism independent of the regulation of DegS activity by the PDZ domain and OMP peptide.

It has been suggested that unfolded or misfolded proteins in the periplasm bind RseB, causing it to release RseA and providing a second physiological signal for initiation of the envelope-stress response (De Las Penas et al., 1997; Missiakas et al., 1997; Collinet et al., 2000). To test this model, we added excess quantities of four different largely unstructured proteins (α-casein, β-casein, an unfolded variant of the titin I27 domain, and an unfolded variant of RNase H) to reaction mixtures containing DegS, OMP peptide, $RseA^{peri}$, and RseB. None of these non-native proteins prevented DegS cleavage of $RseA^{peri}$ in the absence of RseB, and none of them allowed cleavage in the presence of RseB (data not shown).

RseB•RseA binding

We covalently modified $RseA^{peri}$ by attaching a fluorescein dye and assayed RseB binding by changes in fluorescence anisotropy (Figure 2A). Fitting of the resulting binding curve gave an equilibrium dissociation constant (K_D) of 20 nM (25 °C, pH 7.4, 200 mM KCl). Unlabeled $RseA^{peri}$ competed efficiently for fluorescein-$RseA^{peri}$ binding to RseB (Fig. 2A, inset). RseB•RseA binding was roughly 3-fold stronger at 5 and 15 °C than at 25 °C and was about 3- to 4-fold weaker at 35 and 45 °C (data not shown). Although RseB•RseA binding was weaker at higher temperatures, RseB had a native structure at 50 °C (see Fig. 3B) and still inhibited DegS cleavage of $RseA^{peri}$ at this temperature (data not shown).

To determine the binding stoichiometry, we performed non-denaturing gel electrophoresis of mixtures of $RseA^{peri}$ and RseB at concentrations high enough to ensure complex formation. In this assay, maximum formation of the RseB•$RseA^{peri}$ complex was observed when the concentration of $RseA^{peri}$ was equal to that of RseB (Figure 2B). Hence, the binding

stoichiometry appears to be 1:1. A similar result was obtained when increasing concentrations of RseB were titrated against a fixed concentration of $RseA^{peri}$ and binding was assayed by changes in anisotropy of trace amounts of fluorescein-$RseA^{peri}$ (Figure 2C).

The kinetics of dissociation of the fluorescein-$RseA^{peri}$•RseB complex at 25 °C (pH 7.4, 200 mM KCl) were determined following addition of excess unmodified RseA (Figure 2D). Fitting of these kinetic data gave a dissociation rate constant (k_{diss}) of 0.055 s^{-1}, corresponding to a half-life of approximately 13 s. The association rate constant (k_{assn}) calculated as k_{diss}/K_D was $2.8 \bullet 10^6$ $M^{-1}s^{-1}$. Addition of excess α-casein did not cause dissociation of the fluorescein-$RseA^{peri}$•RseB complex (Fig. 2D). This result and those discussed above indicate that RseB is still able to bind RseA and to inhibit its cleavage by DegS in the presence of significant quantities of non-native proteins.

RseB secondary and quaternary structure

The circular-dichroism (CD) spectrum of RseB was consistent with a predominately β-sheet structure (Figure 3A). In thermal-denaturation experiments monitored by CD, RseB showed a cooperative melt but the unfolding transition appeared biphasic (Figure 3B), suggesting that RseB may contain multiple domains. Interestingly, PSI-BLAST searches revealed statistically significant sequence homology (E value < 10^{-20}) between LolA, a periplasmic protein of known structure which transports lipoproteins to the outer membrane, and the N-terminal 180 or so RseB residues in our construct. This observation supports the idea that RseB contains more than one structural domain.

Two forms of RseB were observed in gel-filtration experiments. One of these forms (peak II) eluted at a position expected for globular dimer (Figure 3C); this form inhibited DegS cleavage of RseA (data not shown). Another form (peak I) eluted at a position corresponding to a species with a molecular weight 2 to 3-fold larger (Figure 3C). Peak-I RseB did not inhibit DegS (data not shown). $RseA^{peri}$ is monomeric in solution (Walsh et al., 2003). When a mixture of RseB and fluorescein-$RseA^{peri}$ was chromatographed on the gel-filtration column, the $RseA^{peri}$ eluted at a position expected for a globular complex containing two RseB molecules and two $RseA^{peri}$ molecules (Figure 3D).

In freshly purified RseB samples, the peak-I species typically represented 40-50% of the total protein. However, peak I increased and peak II decreased during storage of purified RseB. Furthermore, peak I and peak II exhibited identical CD spectra (data not shown). These observations suggest that peak I is formed from peak II by an oligomerization reaction. In some aged samples, peak I represented >90% of the total RseB. However, urea denaturation followed by renaturation of inactive peak-I material produced protein that largely eluted as peak II and was active in inhibition of DegS cleavage of RseA. Based on these observations, we believe that peak-II RseB represents the protein species that is biologically active in RseA binding and inhibition of DegS cleavage.

RseA residues required for interaction with RseB

In sequence comparisons with orthologs, residues 165-189 of *E. coli* RseA displayed higher conservation than most parts of the periplasmic region (Figure 4A). To determine if this RseA region bound RseB, we synthesized a peptide corresponding to RseA residues 160-189. A

fluorescein-labeled variant of this peptide bound RseB with a K_D of roughly 6 μM (Fig. 4B), and the unlabeled peptide competed for RseB binding to fluorescein-RseA^{peri} (Figure 4C). Thus, RseA residues 160-189 comprise a major site of interaction with RseB. We also created C-terminally truncated variants of RseA^{peri} (residues 121-161 and 121-175) in which most or part of the 160-189 sequence was removed. Neither truncated variant competed substantially with fluorescein-RseA^{peri} for RseB binding (Figure 4B). Taken together, these results demonstrate that RseA residues 160-189 are sufficient for the RseB•RseA interaction and suggest that residues between 176-191 play a key role in binding. Because the RseB affinity of the 160-189 peptide was lower than the affinity of full-length RseA^{peri}, regions of RseA outside of the 160-189 sequence probably also contribute to the binding interaction.

Effect of RseB binding on RseA degradation by DegS

Several experiments were performed to determine whether RseB binding to RseA is the mechanism of inhibition of DegS cleavage. First, we assayed RseB inhibition of DegS degradation of the C-terminally truncated RseA^{peri} variants. DegS degraded both truncated variants, albeit somewhat less efficiently than it degraded full-length RseA^{peri}, but RseB did not inhibit DegS cleavage of either substrate (Figure 5A). Thus, RseA mutations that severely impair RseB binding prevent RseB inhibition of DegS cleavage. Second, we tested whether the 160-189 peptide could relieve RseB-mediated inhibition of DegS degradation of RseA^{peri}. This peptide did not affect DegS cleavage of RseA^{peri} in the absence of RseB but largely reversed the inhibitory effect of RseB (Figure 5B). These experiments show that RseB does not inhibit DegS cleavage of RseA when binding is impaired by mutations in RseA or by peptide competition. We

conclude that RseB exerts its inhibitory effect by binding to RseA and making RseA a poor substrate for DegS.

DegS cleaves RseA between Val^{148} and Ser^{149} (Walsh et al., 2003), whereas our results indicate that residues farther towards the C terminus of RseA participate in RseB binding. To test the importance of the spacing between the scissile peptide bond and the RseB binding determinants, we created RseA variants in which 8, 16, or 24 residues were inserted between the DegS cleavage site and the known site of contact with RseB. Each insertion mutant was degraded by DegS (Figure 5C). Moreover, RseB inhibited DegS degradation of each of these insertion variants. Thus, moving the cleavage site and the primary site of RseB contact farther apart in RseA does not prevent RseB binding from inhibiting DegS cleavage of RseA.

Discussion

Our results demonstrate that RseB binds directly to the periplasmic region of RseA and that this binding prevents or severely slows RseA degradation by DegS. The precise mechanism by which RseB binding prevents cleavage of RseA by DegS remains to be determined, but results presented here and previously constrain potential models. Free $RseA^{peri}$ is molten-globule-like, with little stable tertiary structure (Walsh et al., 2003). Thus, the Val^{148}-Ser^{149} cleavage site and any other parts of the periplasmic domain of RseA that are required for DegS recognition should be freely available to the enzyme in the absence of RseB. The primary RseB-binding site and the DegS-cleavage/recognition sites in RseA do not overlap to any substantial degree. For example, RseB binds the $RseA^{160-189}$ peptide, whereas DegS still degrades the $RseA^{121-161}$ fragment. Moreover, inserting 8-24 residues between the Val^{148}-Ser^{149} cleavage site and the primary RseB-

binding site in RseA did not prevent bound RseB from inhibiting DegS cleavage. This result and the low degree of homology between periplasmic-domain sequences of RseA orthologues from closely related organisms make it unlikely that RseB binding causes the entire periplasmic region of RseA to fold into a compact structure that is resistant to DegS cleavage.

Nevertheless, RseB binding must in some fashion shield RseA sequences that are required for proteolysis by DegS. This could occur by the model depicted in Fig. 6, in which RseB bound to the primary RseA site also interacts with and blocks DegS access to more distant, secondary RseA sequences. This model does not require the intervening RseA sequences to be folded. In fact, these intervening sequences would need to be sufficiently flexible to allow formation of both sets of RseB•RseA interactions without significant strain. A model of this type would also explain why full-length $RseA^{peri}$ binds RseB more tightly than the $RseA^{160-189}$ peptide. The secondary contacts could contribute to overall binding affinity, because of effective-concentration considerations, but not be sufficiently strong to allow RseB binding in the absence of the primary RseA binding site. We note that DegS cleavage of RseA would release a C-terminal fragment that retains affinity for RseB. If active RseB were limiting in the cell, then increasing quantities of the C-terminal RseA fragment could compete with intact RseA for RseB binding, potentially acting to enhance the rate of DegS cleavage.

Independently of its effects on DegS cleavage of RseA, RseB also appears to inhibit RseA cleavage by the second-site protease RseP *in vivo* (Grigorova et al., 2004). RseA variants that lack the primary RseB-binding site or have mutations in or near this site are degraded by RseP in the absence of DegS (Kanehara et al., 2003). Moreover, RseP posesses two predicted PDZ

domains (Kinch et al., 2006), one of which has been shown to regulate its activity. RseP variants with mutations in the C-terminal PDZ domain cleave full-length RseA in the absence of DegS (Kanehara et al., 2003; Bohn et al., 2004; Grigorova et al., 2004), but RseB does not inhibit this degradation efficiently (Grigorova et al., 2004). Intriguingly, the region of RseA to which RseB has a strong affinity is between two glutamine-rich regions that play some role in conferring resistance to RseP proteolysis (Kanehara et al., 2003). One model, consistent with these results, is that RseB binding to RseA allows binding of this complex to the PDZ domain(s) of RseP, which in turn negatively regulates RseA cleavage by RseP. Alternatively, the added bulk of bound RseB might block RseA recognition by RseP, potentially by steric clashes between RseB and the PDZ domains of RseP. In a different regulated intramembrane proteolysis system, addition of bulky structured domains to the eukaryotic ER stress sensor ATF6 results in inhibition of proteolysis by a second-site protease (Shen and Prywes, 2004).

In our studies *in vitro*, little DegS cleavage of RseA occurred when sufficient RseB was present, even when DegS activity was fully induced by OMP peptide. Moreover, the affinity of RseB for RseA should ensure efficient complex formation in the cell. For example, there are approximately 5000 molecules of σ^E in an *E. coli* cell (Grigorova et al., 2006) and RseA is likely to be present in comparable quantities, which would correspond to a concentration of roughly 80 μM in the periplasm. Because the K_D for the RseB-RseA interaction is less than 1 μM, most molecules of RseB in the periplasm should be bound to RseA in the absence of competing interactions. However, *E. coli* mutants lacking RseB show only modest increases in σ^E-mediated gene expression compared to mutants lacking RseA (Ades et al., 2003; Grigorova et al., 2004). These results suggest that the negative regulatory role of RseB *in vivo* is smaller than might be

expected from our biochemical results. Several factors could explain these differences. (*i*) The steady-state intracellular RseB level is not known, and there may be less RseB than RseA. (*ii*) Other cellular signals may reduce RseB binding to RseA, diminishing its inhibitory ability. (*iii*) Another regulator of envelope stress in *E. coli* may slow DegS cleavage of RseA in the absence of RseB.

In *Pseudomonas aeruginosa*, elimination of the RseB or the RseA orthologue causes a comparable increase in AlgU-mediated gene expression under some conditions (Schurr et al., 1996; Rowen and Deretic, 2000). This result shows that RseB orthologues can have major regulatory roles and suggests that cellular mechanisms must be present to allow relief of RseB inhibition and efficient induction of the envelope-stress response. Our experiments show that temperature increases within the physiological range for *E. coli* growth (up to 50 °C) do not prevent RseB inhibition of DegS cleavage of RseA. Thus, temperature *per se* is unlikely to represent an inducing signal for relief of RseB inhibition. Nevertheless, high temperatures and other environmental conditions that cause envelope stress might result in denatured macromolecules, fragments, or the accumulation of other molecular species that prevent RseB binding to RseA. In principle, stress-induced molecular signals could drive formation of the inactive peak-I RseB species observed in our studies or could compete reversibly for RseA binding to RseB. Because dissociation of RseB from RseA is realtively fast (half-life $\approx$ 15 s), a competition mechanism could rapidly inactivate RseB on a timescale consistent with the transcriptional response to envelope stress, which is detected within minutes of the initial stimulus (Ades et al., 2003).

Competition between RseA and other non-native proteins for RseB binding has been proposed, because RseB is recovered with the unstable MalE31 mutant protein in inclusion bodies (Collinet et al., 2000). However, Grigorova *et al.* (2004) found that MalE31 overproduction relieved RseB inhibition only modestly. Moreover, denatured OMPs are unlikely to be the stress signal that affects RseB activity. We observe RseB inhibition of DegS cleavage of RseA in the presence of high concentrations of C-terminal OMP peptides *in vitro*, and overproduction of OmpC did not affect RseB inhibition of RseP cleavage of RseA *in vivo* (Grigorova et al., 2004). In addition, our studies show that RseB binds and inhibits DegS cleavage of RseA even in the presence of significant concentrations of several different non-native and unfolded proteins. Thus, bulk unfolded protein in the periplasm is unlikely to prevent RseB binding to RseA. RseB shares homology with LolA, which transports lipoproteins to the outer membrane (Matsuyama et al., 1995; Narita et al., 2004), and antibiotic-induced changes in the structure of the lipopolysaccharide (LPS) in the outer membrane of *E. coli* are sufficent to induce the σ^E-stress response (Tam and Missiakis, 2005). Hence, unassembled lipoproteins, periplasmic lipids, or modified LPS molecules or fragments might provide inputs into the envelope-stress response by regulating RseB activity. The biochemical assays described here should provide a useful tool for future studies of the control of RseB activity.

Materials and methods

Proteins and peptides

DNA encoding an RseB variant lacking the periplasmic localization sequence (residues 2-23) was cloned between the NdeI and XhoI sites of pET21b, appending an LEHHHHHH tag to the C terminus of the protein. Transformants of *E. coli* strain X90(DE3) were grown at 37 °C in LB

medium with ampicillin (100 μg/mL) to an OD_{600} of approximately 0.6, and protein expression was induced by addition of IPTG (100 μg/mL). Cells were harvested after 2 h, resuspended in 1/50 volume of lysis buffer (50 mM sodium phosphate (pH 8), 500 mM KCl, 20 mM imidazole), and lysed by sonication. The cell lysate was spun for 30 min at 23,000 X g, and the supernatant was applied to a Ni-NTA column pre-equilibrated in lysis buffer. The column was washed with 60 volumes of lysis buffer, prior to addition of elution buffer (50 mM sodium phosphate (pH 8), 500 mM KCl, 500 mM imidazole). Fractions containing the most concentrated RseB, on the Bradford-stain assay, were combined and dialysed overnight against 1000 volumes of buffer A (50 mM sodium phosphate (pH 6), 100 mM NaCl). The dialysate was loaded onto a MonoS cation exchange column equilibrated in buffer A, and RseB was eluted with a linear gradient from buffer A to buffer B (50 mM sodium phosphate (pH 6), 1 M NaCl). Fractions contained purified RseB were visualized by SDS-PAGE, pooled, dialysed against 1000 volumes of degradation buffer (50 mM sodium phosphate (pH 7.4), 200 mM KCl, 10% glycerol) and stored frozen at –80 °C.

$DegS^{\Delta PDZ}$, which contains an N-terminal His_6 tag but lacks the DegS membrane anchor (residues 2-26) and PDZ domain (residues 257-355), was cloned and expressed in strain X90(DE3). Purification and storage were similar to that for RseB, except that after the Ni-NTA column, appropriate fractions were pooled and dialysed overnight into degradation buffer containing 5 mM EDTA and the ion-exchange step was not performed. $RseA^{peri}$, DegS, and OMP peptide (NH_2-DNRDGNVYYF-COOH) were purified as described (Walsh et al., 2003). For labeling, Ser^{154} in $RseA^{peri}$ was mutated to cysteine; the mutation did not affect purification. Purified $RseA^{peri\text{-}C154}$ was reduced with TCEP, mixed with a 10-fold molar excess of fluorescein-5-

maleimide, and incubated overnight at 4 °C. Products were separated by reverse-phase HPLC. The fluorescein-modified protein ran at the same position as $RseA^{peri}$ in SDS-PAGE but fluoresced when illuminated with UV light. The labeled protein was lyophilized and resuspended in degradation buffer. Solid-phase peptide synthesis was performed by the MIT Biopolymers lab. Peptides were purified by reverse-phase HPLC using a Vydac C18 column. α- and β-casein were purchased from Sigma. Unfolded carboxymethylated titin and unfolded L78D/L112D RNase H* were prepared as described (Kenniston et al., 2003; Kenniston et al., 2004) and were a gift from Jon Kenniston.

$RseA^{peri}$ insertion mutants were created by first mutating the codon for glycine151 from GGG to GGA, which introduces a unique KpnI site without altering the protein sequence. Oligonucleotides (5'-CTT CTG AAG CGA CCG CAA AGG TAC-3', 5'-CTT TGC GGT CGC TTC AGA AGG TAC-3') were phosphorylated, annealed, and ligated into the vector cut with KpnI. Transformants were screened by PCR for insertions of one, two, or three cassettes.

Degradation assays

DegS cleavage was performed in degradation buffer at 37 °C for 16 h. Reactions were quenched by addition of SDS-PAGE loading buffer, boiled, electrophoresed on 12% or 15% Tris-tricine gels, and stained with Coomassie brilliant blue.

RseB characterization

Circular-dichroism spectra were taken with an Aviv 60DS instrument. RseB protein (145 μM in degradation buffer) was diluted with water to the desired concentration and placed in a 1 cm

path-length cuvette. Spectra were taken at 25 °C with 1 s integration time. Temperature melts were performed in increments of 1 °C with 30 s equilibration and 10 s integration.

Gel filtration was performed on a SMART system (Amersham Biosciences) at 4 °C using an Superdex 200 column. Elution of RseB was monitored by absorbance at 280 nm, whereas elution of fluorescein-labeled $RseA^{peri}$ and fluorescein-labeled $RseA^{peri}$•RseB was monitored by absorbance at 490 nm. For some experiments, RseB or $RseA^{peri}$•RseB mixtures were added to an equal volume of 9 M urea, diluted with an equal volume of degradation buffer, filtered, and then loaded onto the column. Molecular-weight standards were from BioRad (#151-1901).

Binding and kinetic assays

Binding of fl-$RseA^{peri}$ to RseB was monitored by changes in fluorescence anisotropy at 25 °C using a PTI QM-2000-4SE spectrofluorometer. The fl-$RseA^{peri}$ protein was diluted in degradation buffer to a final concentration of 43 nM in a 60 μL volume cuvette. RseB protein was serially diluted into degradation buffer. Increasingly concentrated RseB dilutions were titrated into the cuvette in 1 μL increments. The sample was excited at 467 nm and emission was monitored at 520 nm. Anisotropy was calculated based on the scattering correction of a sample containing an identical amount of RseB but no fl-$RseA^{peri}$.

To determine binding stoichiometry, different amounts of RseB and $RseA^{peri}$ were allowed to mix in 4.65 M urea and were then electrophoresised on a native 10% Tris•glycine polyacrylamide gel. This procedure eliminated most inactive peak-I RseB material and resulted in reproducible binding, presumably because the urea was diluted during electrophoresis. The

native gel was stained with Coomassie brilliant blue. In a second stoichiometry experiment, a mixture of 43 nM fl-RseA^{peri} and 1.54 μM unlabeled RseA^{peri} was added to a cuvette and fluorescence anisotropy was measured (excitation, 467 nm; emission, 520 nm). Another mixture with 43 nM fl-RseA^{peri}, 1.54 μM RseA^{peri}, and 10 μM RseB was prepared. The RseB protein used for this experiment was urea-denatured, diluted, and buffer exchanged, resulting in >95% of active peak-II RseB as judged by native gel and gel filtration. A fraction of the cuvette mixture was withdrawn and replaced with an equal volume of the mixture containing RseB, and fluorescence anisotropy was measured again. This procedure was repeated to obtain data for a range of RseA:RseB ratios.

For measurement of dissociation kinetics, fl-RseA^{peri} (50 nM) and RseB (164 nM) were mixed and the fluorescence anisotropy was measured. Unlabeled RseA^{peri} or α-casein were then added and mixed thoroughly, and fluorescence anisotropy was measured as a function of time. For equilibirum competition assays, RseA^{peri}, RseA 121-161, RseA 121-175, or the RseA 160-189 peptide were added in a volume of 1 μL to the fl-RseA^{peri}•RseB sample (61 μL) and fluorescence anisotropy was measured after 5 min.

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Figure legends

Figure 1 RseB inhibition of DegS cleavage of the periplasmic domain of RseA. SDS-PAGE analysis of samples following incubation of full-length $RseA^{peri}$ (20 μM) and DegS (32 μM) with or without OMP peptide and RseA at the concentrations indicated. The DegS and RseB bands, which have similar electrophoretic mobilities, are not shown.

Figure 2 RseB interactions with the periplasmic domain of RseA. (**A**) Increasing concentrations of RseB were added to fluorescein-$RseA^{peri}$ (43 nM) and binding at 25 °C (50 mM sodium phosphate (pH 7.4), 200 mM KCl, 10% glycerol) was assayed by changes in fluorescence anisotropy. The solid line is a nonlinear least squares fit of the data with a K_D of 20 nM. **Inset.** Unlabeled $RseA^{peri}$ competed efficiently for binding of fl-$RseA^{peri}$ to RseB. (**B**) RseB binding to RseA assayed by native-gel electrophoresis saturated at a stoichiometry of approximately 1:1 (subunit equivalents). (**C**) Stoichiometry of RseB•RseA binding formation assayed by change in fluorescence anisotropy. Increasing concentrations of RseB were added to a mixture of fl-$RseA^{peri}$ (43 nM) and unlabeled $RseA^{peri}$ (1.54 μM). The dotted line is a least squares linear fit to the first four data points. The solid line represents the anisotropy associated with maximal binding. (**D**) Dissociation kinetics. RseB (60 nM) and fl-$RseA^{peri}$ (43 nM) were preincubated at 25 °C and excess $RseA^{peri}$ (5.3 μM) or α-casein (32 μM) was added approximately 10 s before complex dissociation began to be monitored by changes in fluorscence anisotropy.

Figure 3 Characterization of RseB and its complex with RseA. (**A**) Circular-dichroism spectrum of RseB (1 μM) at 25 °C in 0.34 mM sodium phosphate (pH 7.4), 1.4 mM KCl, 0.07% glycerol.

(**B**) Thermal unfolding of RseB (3 μM) in 1 mM sodium phosphate (pH 7.4), 0.47 mM KCl, 0.21% glycerol was assayed by changes in CD ellipticy. The solid line is a fit for a three-state denaturation model. (**C**) Gel filtration of RseB on a Superdex 200 column (Pharmacia) at 4 °C in 50 mM sodium phosphate (pH 7.4), 200 mM KCl, 10% glycerol. The open diamonds mark the elution positions of molecular-weight standards. The dotted line is an exponential fit of molecular mass versus elution volume. The relative proportions of peak-I RseB and peak-II RseB varied in different preparations. In the chromatogram shown, RseB was urea denatured and allowed to refold immediately before chromatography. (**D**) Gel filtration (same column and conditions as panel C) of fl-RseA^{peri} (circles) or fl-RseA^{peri} with excess RseB (triangles).

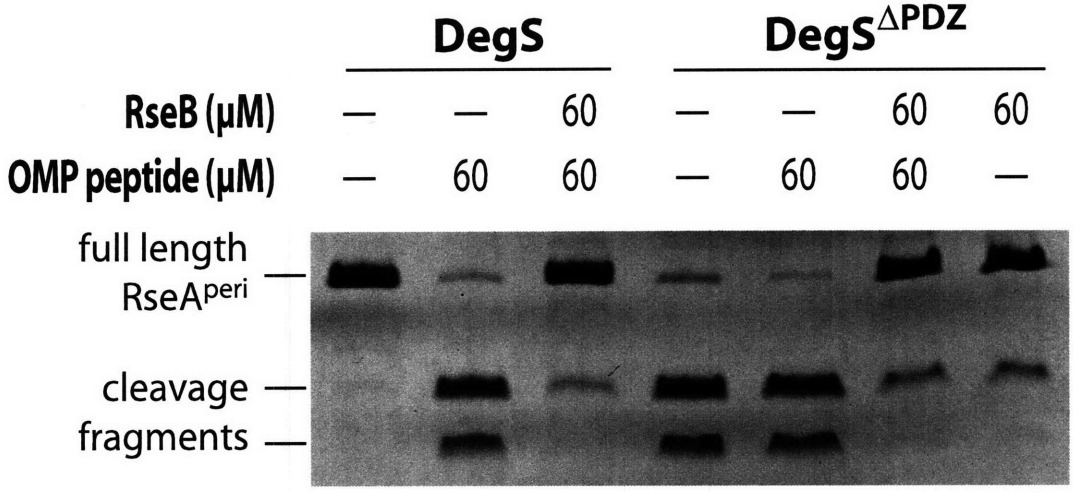
Figure 4 Some fragments of the periplasmic domain of RseA bind RseB. (**A**) Sequence alignment of the periplasmic domains of RseA from *Escherichia coli* (Ec; GI 1173288), *Salmonella typhimurium* (St; GI 16765959), *Yersinia pestis* (Yp; GI 16122916), *Haemophilus influenzae* (Hi; GI 1173289), *Pasteurella multocida* (Pm, GI 15603653), and *Vibrio cholerae* (Vc; GI 15642462). The positions of fragments of the *E. coli* protein are shown below the alignment. (**B**) RseB binding to fluorescein-labelled RseA 160-189 (50 nM) assayed by fluorescence anisotropy. The solid line is a nonlinear least squares fit with a K_D of 6.5 μM. (**C**) Competition experiments. RseB (160 nM) and fl-RseA^{peri} (43 nM) were mixed and fluorescence anistropy was assayed 5 min after the addition of competitor proteins.

Figure 5 Effects of mutations in RseA or the presence of competitor on RseB inhibition of DegS cleavage of RseA. (**A**) DegS cleavage of 20 μM RseA^{peri} or truncated variants was assayed by SDS-PAGE in the presence or absence of OMP peptide and RseB. (**B**) Relief of RseB-mediated

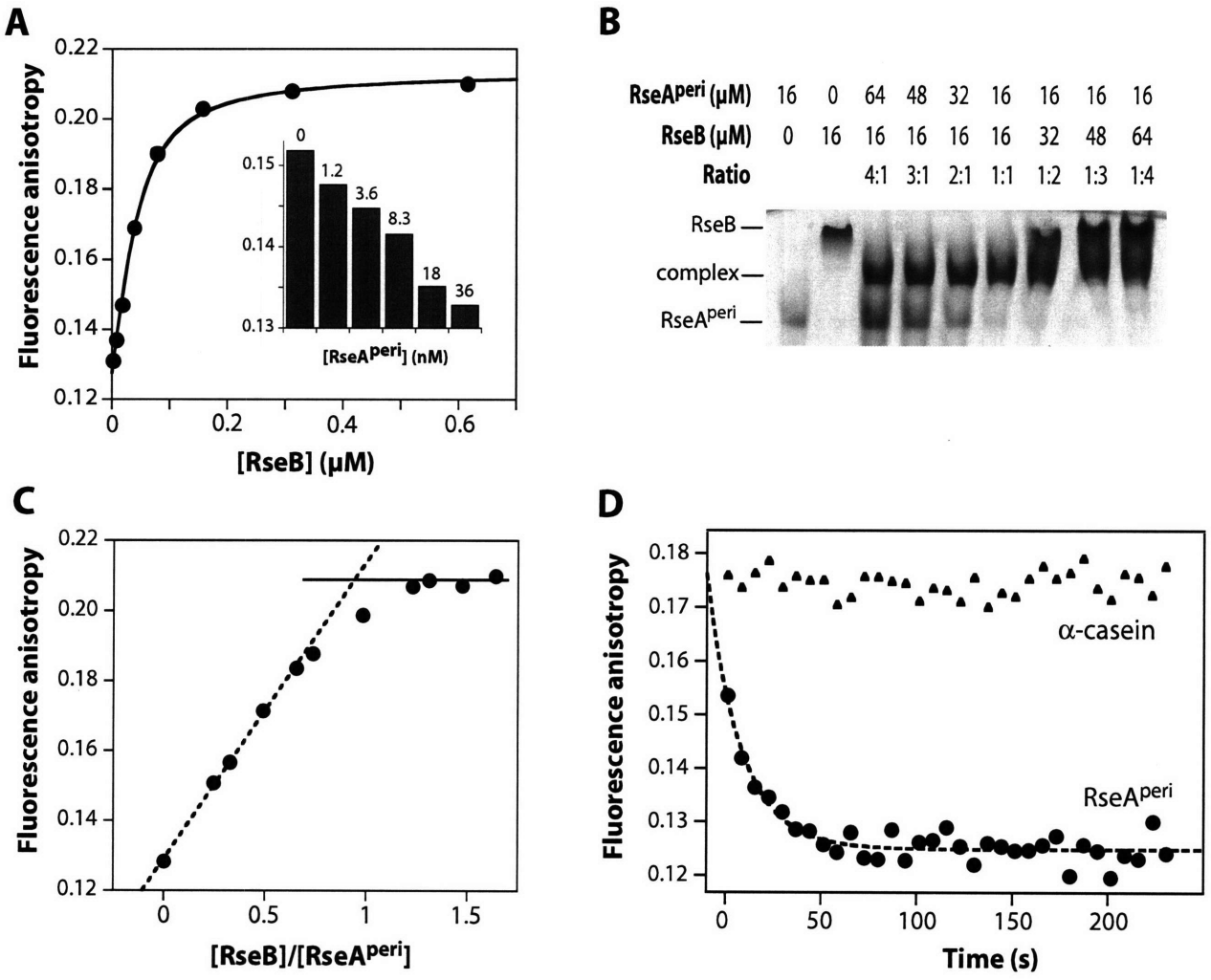
degradation inhibition of $RseA^{peri}$ by the RseA 160-189 peptide. (**C**) $RseA^{peri}$ variants with insertions between the DegS-cleavage site and RseB-binding site were incubated with DegS in the presence or absence of OMP peptide and RseB. The DegS concentration in all lanes in this figure was 32 μM.

Figure 6 Model for RseB binding to the periplasmic domain of RseA. A primary site in RseA (including of residues between 160 and 189) mediates binding to RseB. A secondary RseA site (overlapping determinants required for DegS recognition) strengthens binding to RseB.

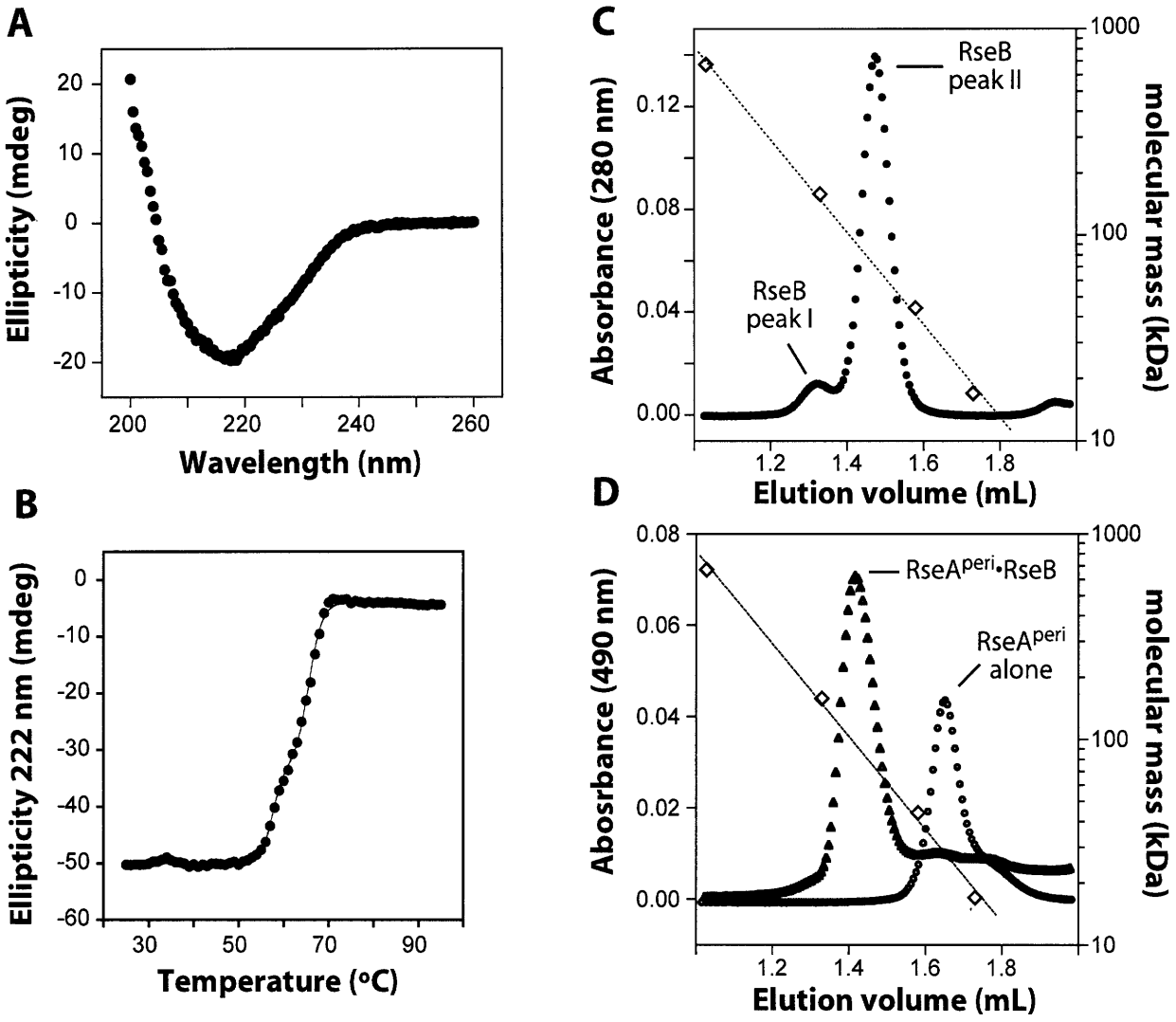
RseB inhibition of DegS cleavage of the periplasmic domain of RseA



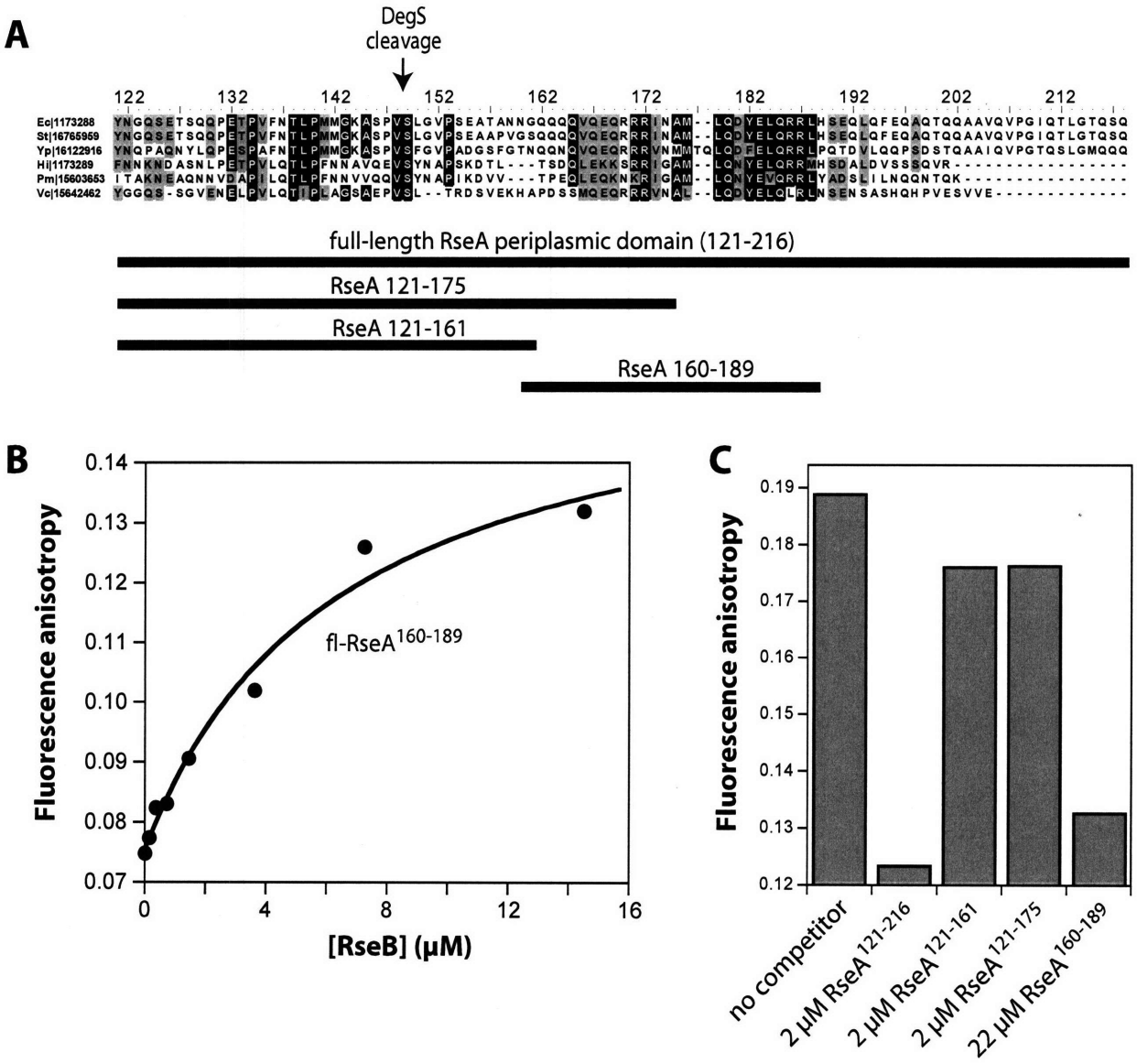
RseB interactions with the periplasmic domain of RseA



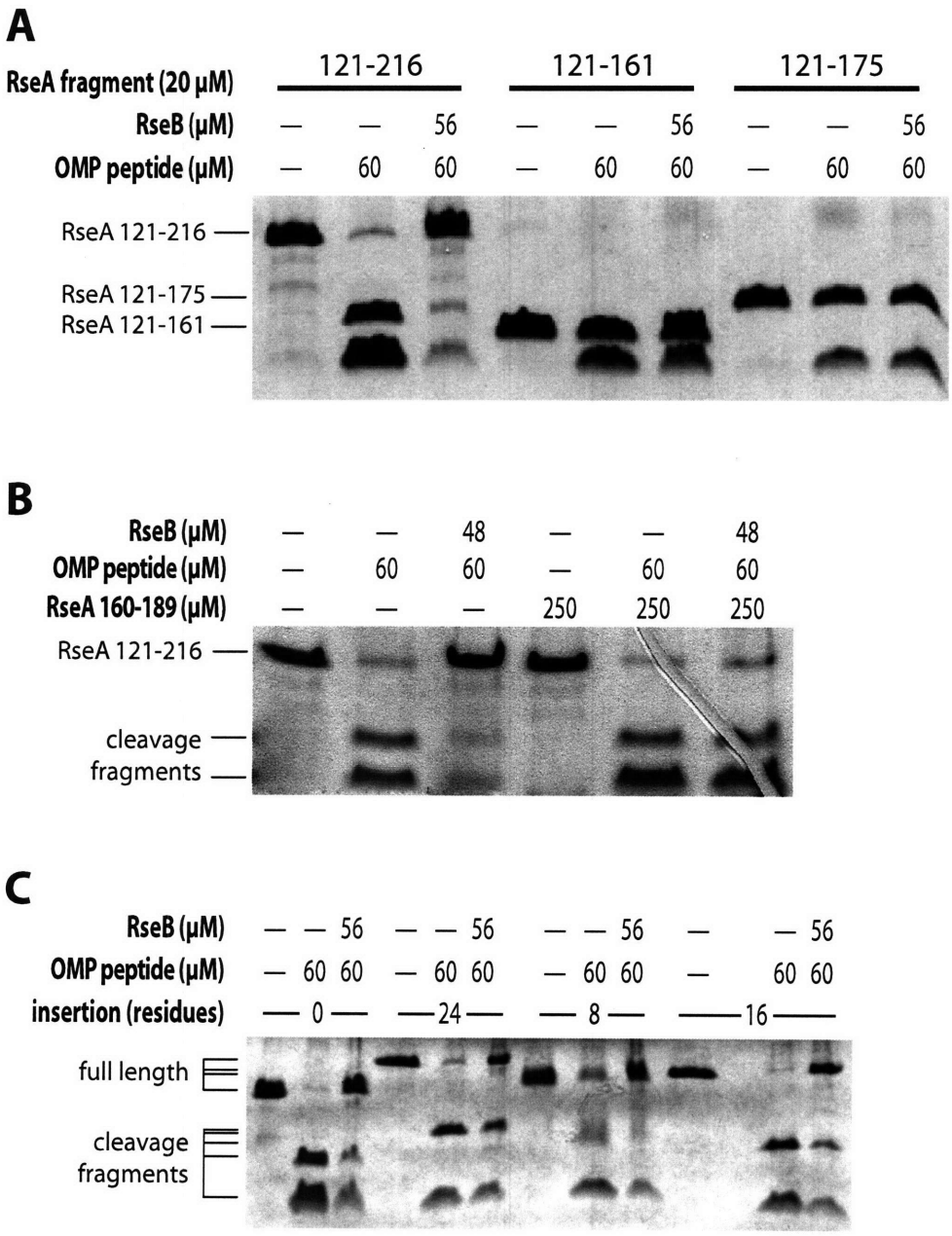
Characterization of RseB and its complex with RseA



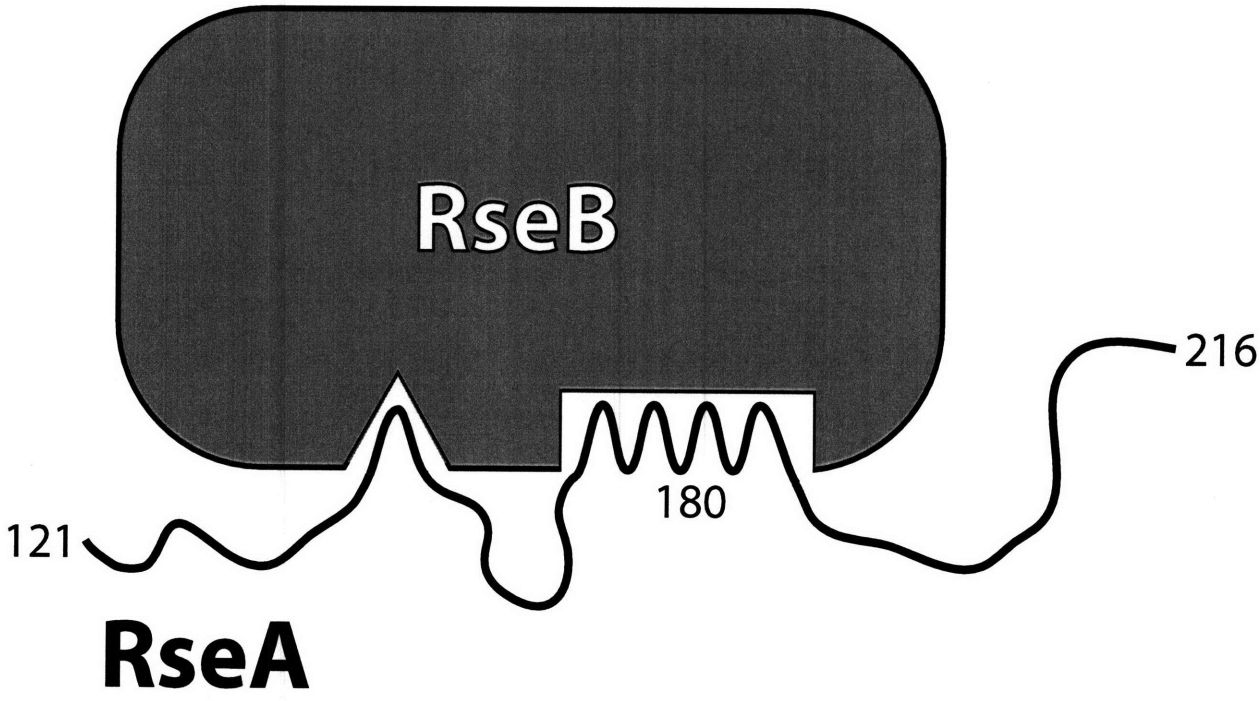
Some fragments of the periplasmic domain of RseA bind RseB



Effects of mutations in RseA or the presence of competitor on RseB inhibition of DegS cleavage of RseA



Model for RseB binding to the periplasmic domain of RseA



CHAPTER THREE

Mechanisms of positive and negative regulation of the extracytoplasmic stress response and alginate production in *Pseudomonas aeruginosa*

Abstract

The ability of a pathogen to survive the defensive responses of its host requires the detection of and response to perturbations in its own physiology. Activation of the extracytoplasmic stress response in the pathogen *Pseudomonas aeruginosa* results in higher tolerance against immune defenses as well as the production of alginate, a surface polysaccharide that confers resistance to many antibiotic treatments. The response is regulated by the transcription factor AlgU, which is controlled by the transmembrane repressor protein MucA. AlgU activity is also repressed by the the periplasmic protein MucB. Here, we show that specific peptides bind to the periplasmic AlgW protease and activate its cleavage of MucA and demonstrate that tight binding of MucB to MucA strongly inhibits this cleavage. We also probe the roles of structural features of AlgW, including a key regulatory loop and its PDZ domain, in regulating substrate binding and cleavage.

Introduction

The pathogenic bacterium *Pseudomonas aeruginosa* uses sophisticated schemes to survive in human hosts. One such mechanism is the production of the external polysaccharide alginate, which inhibits detection of the bacterium by the immune system and provides a barrier that allows the pathogen to survive treatment with most common antibiotics (Govan and Deretic 1996). This pathway is linked to the extracytoplasmic stress response, which serves as an additional adaptation in combating efforts by the host to destroy the bacterium (Martin et al. 1994). The AlgU transcription factor was identified as an activator of alginate production (Flynn and Ohman 1988). AlgU is similar to the *Escherichia coli* transcription factor σ^E, which regulates transcription of periplasmic stress-response genes (Erickson and Gross 1989, DeVries and Ohman 1994, Rouviere et al. 1995). AlgU activation results in the increased production of periplasmic chaperones, alginate synthesis proteins, and other stress-response proteins, and leads to a mucoid phenotype (Martin et al. 1993a).

P. aeruginosa infection is particularly problematic in cystic fibrosis (CF) patients, whose lungs provide an environment where this bacterium can thrive (Govan and Deretic 1996). *P. aeruginosa* isolated from CF patients often exhibit a mucoid phenotype. Genetic studies revealed that mutations in the *muc*A gene, which is immediately downstream of *alg*U, are responsible for the mucoid phenotype (Martin et al. 1993c). MucA is a transmembrane protein containing an N-terminal cytoplasmic domain that binds AlgU and represses its activity (Schurr et al. 1996), a segment that spans the inner membrane, and a C-terminal periplasmic region. Another gene in the algU operon, *muc*B, encodes a protein that is exported to the periplasm and binds the periplasmic region of MucA (Schurr et al. 1996, Mathee et al. 1997, Rowen and Deretic 2000).

Regulation of the activity of σ^E in *E. coli* occurs via the transmembrane protein RseA, which binds to and inhibits the activity of σ^E under non-stress conditions (De Las Penas et al. 1997, Missiakas et al. 1997). Under stress conditions RseA is initially cleaved by the membrane-bound periplasmic protease DegS and is then cleaved within the transmembrane region by the intramembrane protease RseP (YaeL), resulting in the degradation of the cytoplasmic region of RseA by additional proteases and liberation of σ^E (Ades et al. 1999, Alba et al. 2002, Kanehara et al. 2002, Chaba et al 2007). Stress conditions are sensed by DegS, which is activated by the binding of the C termini of misfolded outer membrane porins (OMPs) to its PDZ domain (Walsh et al. 2003).

P. aeruginosa contains DegS and RseP homologs, which have been implicated in the cleavage of MucA and the subsequent activation of AlgU (Wood et al. 2006, Qiu et al. 2007). Moreover, activation can be induced by overexpression of the periplasmic protein MucE (Qiu et al. 2007), which has no *E. coli* homolog, in a process that depends upon the C-terminal residues of MucE. To better understand how the mucoid pheotype of *P. aeruginosa* arises, it is critical to determine how the proteolysis of MucA is controlled by stress. Here we study the regulation of AlgW, the *P. aeruginosa* homolog of DegS, which we show cleaves the periplasmic domain of MucA when activated by C-terminal MucE peptides. We also investigate the inhibitory effect of MucB on the cleavage of MucA by AlgW.

Results

AlgW cleaves MucA

MucA functions analogously to *E. coli* RseA (Schurr et al. 1996, Xie et al. 1996, Mathee et al. 1997, Rowen and Deretic 2000), although the periplasmic regions of these proteins share only 25% sequence identity (Figure 1A). We hypothesized that AlgW, the apparent *P. aeruginosa* homolog of *E. coli* DegS, cleaves MucA. To test this model, we purified tagged variants of AlgW and the periplasmic domain of MucA ($MucA^{peri}$) and assayed cleavage *in vitro*. As monitored by SDS-PAGE, AlgW efficiently cleaved $MucA^{peri}$ in the presence of a peptide corresponding to the C terminus of the MucE protein (Figure 1B). Cleavage was inefficient without added peptide. To probe the reaction in greater detail, we radiolabeled $MucA^{peri}$ and determined steady-state rates of cleavage at different substrate concentrations in the presence of MucE peptide (Figure 1C). Fitting these data to the Hill form of the Michaelis-Menten equation gave a V_{max} of 1.2 s^{-1}, a K_M of 159 μM, and a Hill constant of 1.3. In the absence of activating peptide, AlgW cleaved MucA very slowly with a second-order rate constant of $2.2 \cdot 10^{-6}$ $M^{-1}s^{-1}$ (not shown). This value is roughly 2000-fold smaller than the rate constant for AlgW cleavage in the presence of MucE peptide ($4.3 \cdot 10^{-3}$ $M^{-1}s^{-1}$).

As assayed by circular dichroism, $MucA^{peri}$ had a spectrum consistent with a largely unfolded random-coil structure between 4°C and 95°C (Figure 2A). The periplasmic domain of RseA ($RseA^{peri}$) has similar properties (Walsh et al. 2003). We analyzed the products of AlgW cleavage of $MucA^{peri}$ by reverse-phase HPLC, peptide sequencing, and mass spectrometry. Multiple fragments were recovered, corresponding to cleavage after serines, isoleucines, and alanines (Figure 2B). Among these cleavage sites, the most conserved in close orthologs was

between A136 and G137 and between A189 and S190. The A136-G137 site is roughly 33 residues from the beginning of the periplasmic domain, approximately the same distance as the V148-S149 scissile peptide bond in *E. coli* RseA, which is the sole site of cleavage by *E. coli* DegS.

Under conditions where DegS cleaved $RseA^{peri}$ efficiently, it did not cleave $MucA^{peri}$ (Figure 2C). By contrast, AlgW cleaved $RseA^{peri}$ in a MucE-peptide dependent manner (Figure 2D), albeit more slowly than it cleaved $MucA^{peri}$. The $RseA^{peri}$ fragments produced by DegS or AlgW cleavage migrated at similar positions during SDS-PAGE (Figures 2C, 2D). Hence, AlgW and DegS have a common ability to recognize $RseA^{peri}$, but only AlgW recognizes $MucA^{peri}$. We conclude that AlgW has broader substrate specificity than DegS, consistent with our finding that AlgW cleaves $MucA^{peri}$ at multiple sites.

Peptide Binding and Activation

Binding of C-terminal residues of peptides or proteins to the PDZ domain of DegS is a key event in proteolytic activation (Walsh et al. 2003). To better understand AlgW activation, we immobilized its PDZ domain on a column, passed a randomized peptide library over this resin, and analyzed bound fractions from the PDZ and control columns by sequential Edman degradation (Songyang et al. 1997). Among specifically bound peptides, Phe, Ile, and Leu were preferred at the C terminus; Trp, Tyr, Phe, Ile, and Val were most common at the penultimate position; and Tyr, Phe, Trp, and Ile were favored at the antepenultimate position (not shown). A search of periplasmic *P. aeruginosa* proteins for C-terminal sequences matching these preferences revealed MucE ending in WVF, two porins ending in YVF (Swiss-Prot accessions

P05695 and P32977), a flagellar P-ring protein ending in IVI (Q9I4P5), and a putative inner-membrane protein ending in IFL (P25254). We synthesized fluorescently labeled peptides ending in WVF, IVI, IFL, and YYF (a very strong activator of *E. coli* DegS) and assayed binding to AlgW by fluorescence anisotropy (Figure 3A). AlgW bound well to the WVF peptide (K_D = 3 μM), bound moderately well to the YYF peptide (K_D = 22 μM), and did not bind the IVI or IFL peptides.

AlgW-dependent activation of the alginate response *in vivo* was found to be induced by overexpression of MucE but not by a variant with a C-terminal WVF→YYF substitution (Qiu et al. 2007). In our $MucA^{peri}$ cleavage assay *in vitro*, half-maximal stimulation of AlgW activity was observed using 5 μM of the WVF peptide, whereas roughly 20-fold higher concentrations of the YYF peptide were required for 50% activation (Figure 3B). Interestingly, however, maximal activation of AlgW activity was at least 2-fold higher for the YYF than the WVF peptide. Although the MucE C-terminal sequence is clearly the superior activator *in vivo* and *in vitro*, our results suggest that the C-terminal sequences of other proteins, perhaps porins, could also contribute to activation in the cell. As expected for an allosteric activation mechanism, stimulation of AlgW cleavage of $MucA^{peri}$ by the WVF and YYF peptides was positively cooperative with Hill constants of 1.8 and 1.9, respectively.

A variant of DegS lacking the PDZ domain ($DegS^{\Delta PDZ}$) is constitutively active, showing that the PDZ domain inhibits proteolytic activity in the absence of peptide binding (Walsh et al. 2003, Cezairliyan and Sauer 2007, Sohn et al. 2007). We constructed $AlgW^{\Delta PDZ}$, purified the enzyme, and found that it cleaved $MucA^{peri}$ in a peptide-independent fashion at a rate about 25-fold

greater than AlgW without WVF peptide but still almost 40-fold slower than peptide-activated AlgW (not shown). These results suggest that the PDZ domain of AlgW plays a role in repressing proteolytic activity but is also required, in some fashion, for efficient proteolysis. The latter result explains the observation that $AlgW^{\Delta PDZ}$ does not complement an *algW* strain (Qiu et al. 2007). In DegS, arginine 256 is part of a short linker between the protease domain and the PDZ domains (Wilken et al. 2004). R256 makes a salt bridge that stabilizes the inactive conformation of DegS, and the R256A mutation results in peptide-independent cleavage of RseA at a level similar to peptide-stimulated activity (Sohn et al. 2007). We mutated the homologous arginine in AlgW (R279A) and found that this mutant cleaved MucA extremely slowly both in the absence and presence of peptide (not shown). These results are consistent with a model in which the PDZ domain of AlgW plays important roles both in repressing proteolytic activity when appropriate peptide signals are absent and in stimulating cleavage when such peptides are present.

AlgW is a trimer

AlgW and DegS are members of a subgroup of the DegP/HtrA2 family of serine proteases that contain an N-terminal membrane anchor, a protease domain, and a single C-terminal PDZ domain (Waller and Sauer 1996, Ehrmann and Clausen 2004,Wood et al. 2006, Qiu et al. 2007). Two subgroup members, DegS and HtrA2, are trimeric (Li et al. 2002, Walsh et al. 2003, Wilken et al. 2004, Zeth 2004). Other family members, including DegP and DegQ, lack the membrane anchor, have a second PDZ domain, and are hexamers (Krojer et al. 2002, Kim and Kim 2005). As expected, gel-filtration chromatography of AlgW was consistent with a trimeric structure and not with a hexamer (not shown).

The LA loop of AlgW inhibits MucA binding

Sequence alignments show that the LA loop in the protease domain of AlgW resembles loops in the hexameric proteases more than in DegS (Figure 4). For example, the AlgW LA loop is longer than that of DegS, is similar in length to the LA loop of DegQ, and contains multiple phenylalanines like the LA loops of DegP and DegQ. The LA loop in the crystal structure of *Thermotoga maritima* HtrA hinders access to the active site (Kim et al. 2003, Kim et al. 2008), and we suspected that the LA loops of AlgW might influence its proteolytic activity. To test this model, we deleted 16 amino acids from the LA loop of AlgW ($AlgW^{\Delta LA}$). We observed rapid cleavage of full-length $MucA^{peri}$ as well as of intermediate cleavage products by $AlgW^{\Delta LA}$ in the presence of MucE peptide (Figure 5A). $AlgW^{\Delta LA}$ cleaved $MucA^{peri}$ with a V_{max} comparable to that of intact AlgW, but K_M was roughly 20-fold lower for the mutant (6 μM) than for the wild-type enzyme (Figure 5B). These results suggest that the LA loop of AlgW normally inhibits substrate binding, perhaps by steric occlusion. MucE peptide was still required to fully activate $AlgW^{\Delta LA}$, however at high substrate concentration the basal rate of cleavage without MucE peptide was more than 100-fold higher for $AlgW^{\Delta LA}$ than for wild-type AlgW (Figure 5C).

MucB binds MucA and inhibits cleavage by AlgW

MucB functions as a negative regulator of the alginate response (Goldberg et al. 1993, Martin et al. 1993b, Schurr et al. 1996, Mathee et al. 1997, Rowen and Deretic 2000) and is a homolog of the RseB protein of *E. coli*. RseB binds RseA and inhibits its cleavage by DegS *in vitro* (Cezairliyan and Sauer 2007). Likewise, addition of MucB halted AlgW cleavage of $MucA^{peri}$ (Figure 6A). To study MucA-MucB interactions, we constructed a $MucA^{peri}$ $Ser^{154}\rightarrow$Cys mutant

and attached a maleimide-fluorescein dye (fl-MucA^{peri}). In a fluorescence-anisotropy assay, MucB bound fl-MucA^{peri} with an equilibrium dissociation constant (K_D) of 120 nM (Figure 6B). Upon addition of unmodified MucA^{peri}, the anisotropy decreased over time, indicating that binding of MucB to fl-MucA^{peri} is specific and reversible (Figure 6C). Fitting of the kinetics gave a dissociation rate constant of $1.4 \cdot 10^{-3}$ s^{-1} (half-life $\approx$ 8 min). We did not observe binding of RseB to fl-MucA^{peri} or binding of MucB to fl-RseA^{peri} (not shown).

In gel-filtration chromatography, MucB eluted in two peaks with sizes consistent with globular monomers and dimers (Figure 6D). *E. coli* RseB and its *Haemophilus influenzae* homolog, which are essentially the same size as MucB (301-304 residues), exist as a mixture of dimers and hexamers. In the case of *E. coli* RseB, only the dimer binds RseA (Cezairliyan and Sauer 2007). To study the size of the molecular complex, we mixed fl-MucA^{peri} with MucB and analyzed the mixture by gel filtration (Figure 6E). The fl-MucA^{peri}/MucB complex eluted slightly ahead of the position of the MucB dimer, suggesting that MucA^{peri} binds the MucB dimer and causes only a slight increase in its hydrodynamic radius (as is the case with RseA^{peri} and RseB; Cezairliyan and Sauer 2007).

Discussion

Our experiments show that purified AlgW protease can be activated by peptides with MucE-like C termini to cleave the periplasmic domain of MucA. These results, homology with the *E. coli* system, and recent evidence that AlgW is an important component of the envelope-stress response in *P. aeruginosa* (Wood et al. 2006, Qiu et al. 2007) all suggest that a major biological function of AlgW is to detect C-terminal sequence signals that become accessible during stress and to initiate a proteolytic cascade that inactivates MucA thereby activating the AlgU transcription factor.

The PDZ domains of AlgW and DegS, which bind C-terminal peptide sequences, allow these proteases to sense the folding/assembly status of proteins in the periplasm. DegS preferentially binds YxF C-terminal sequences, which are found in numerous *E. coli* outer-membrane porins (Walsh et al. 2003). Because these C-terminal sequences are only accessible in denatured or unassembled porins, they serve as an indicator of protein unfolding or diminished assembly capacity. Importantly, the peptide-binding specificities of the PDZ domains of AlgW and DegS differ. We found that a peptide with the C-terminal WVF sequence of MucE, whose overexpression induces the AlgU transcriptional response in *P. aeruginosa* (Qiu et al. 2007), is an excellent activator of AlgW cleavage of MucA *in vitro*. The structure of MucE is unknown, but we assume that the accessibility of its C-terminal sequence also serves as a gauge of folding/assembly. Peptides ending with YYF, the preferred sequence for DegS, also activated AlgW but at 20-fold higher concentrations than the MucE peptide. Whether AlgW senses only levels of unfolded/unassembled MucE or responds to a broader range of activation sequences in the cell is presently unclear. Unlike *E. coli* porins, most porins in *P. aeruginosa* terminate with

LL, IW, or VW C-terminal motifs, and it is possible that these sequences may also function as AlgW activators.

In DegS, the unliganded PDZ domain acts mainly to inhibit the protease domain, as evidenced by the finding that $DegS^{\Delta PDZ}$ and peptide-stimulated DegS have similar levels of activity (Cezairliyan and Sauer 2007, Sohn et al. 2007). In AlgW, peptide binding to the PDZ domain must play both positive and negative roles in controlling protease activity, as the $AlgW^{\Delta PDZ}$ and AlgW R279A mutants are more active than unliganded AlgW but less active than the peptide-stimulated enzyme. However, AlgW and DegS could still operate by the same basic mechanism. Peptide binding to the PDZ domain of DegS appears to alter an equilibrium between active and inactive allosteric conformations (Sohn et al. 2007). The same allosteric mechanism can also explain our AlgW results, with the proviso that contacts mediated by the peptide-bound PDZ domain are now required to tip the equilibrium balance towards the active conformation in this system. Indeed, our finding of positive cooperativity in peptide stimulation of AlgW cleavage of MucA supports an allosteric model. Interestingly, the YYF peptide stimulated maximal AlgW protease activity to roughly twice the level as the WVF peptide, despite the fact that the latter peptide bound more tightly. This result can also be explained by the allosteric model. At peptide saturation, protease activity will be determined by the equilibrium ratio of the active and inactive conformations, which, in turn, will depend on the molecular details of specific peptide contacts with each conformation. Hence, peptide-binding affinity need not be correlated with the degree of activation when binding is saturated.

The basic domain structures of AlgW and DegS are similar, and both proteases are trimeric and membrane bound. However, AlgW posesses some structural features, such as a long LA loop and short L2 loop, that are more akin to features found in the soluble, hexameric DegQ and DegP proteases. DegS cleaves RseA at a single Val-Ser peptide bond, whereas DegP and DegQ have broader substrate specificity, cleaving many but not all Val-Xxx or Ile-Xxx bonds (Kolmar et al. 1996). AlgW also has broader cleavage specificity than DegS, cleaving at some Ile-Xxx, Ala-Xxx, and Ser-Xxx bonds. Kim et al. (2005) found that the LA loop in the structure of a DegP ortholog blocked substrate access to the catalytic triad and suggested that changes in the conformation of this loop may be important in controlling the protease activity of DegP (2008). Consistent with their model, we find that deleting part of the LA loop of AlgW results in substantially tighter apparent affinity for MucA. Moreover, this AlgW mutant appeared to cleave MucA at a greater number of positions (not shown), suggesting that the LA loop plays some role in substrate discrimination. AlgW and MucD are the the only DegP/HtrA2 family proteases in *P. aeruginosa*. MucD, like *E. coli* DegP, is upregulated in response to thermal stress and is required for high-temperature survival (Boucher et al. 1996), leaving *P. aeruginosa* without an obvious DegQ ortholog. Given the similarity of some aspects of their structure, it is possible that AlgW performs the roles of both DegS and DegQ in *P. aeruginosa*.

The inhibitory role of MucB in the alginate-production response of *P. aeruginosa* has been well-established, although its magnitude varies depending on growth conditions (Martin et al. 1993b, Schurr et al. 1996, Mathee et al. 1997, Rowen and Deretic 2000). We find that MucB binds tightly to MucA and inhibits its cleavage by AlgW. Although this result was anticipated given the AlgW/DegS, MucA/RseA, and MucB/RseB homologies and prior studies of the *E. coli*

system (Cezairliyan and Sauer 2007, Kim et al. 2007), there are interesting similarities and differences. For example, despite substantial sequence homology, MucB fails to bind RseA and RseB fails to bind MucA. Moreover, RseB binding to RseA and variants can blocks DegS cleavage at a site that is 11 to 35 residues from the minimal RseA•RseB interaction site. We have not mapped the MucA•MucB binding site, but MucB binding prevents cleavage of MucA at sites that are more than 50 residues apart. Free MucA and RseA have little if any native structure, but it is possible that MucB/RseB binding stabilizes more ordered conformations that shield the scissile peptide bonds from AlgW/DegS. Indeed, gel-filtration chromatography indicates a very small size difference between MucB and the MucB•MucA complex, consistent with the possibility that MucB provides a scaffold upon which the unstructured periplasmic domain of MucA might collapse. *E. coli* RseB can form a dimer or higher oligomer, with only the dimer being active (Cezairliyan and Sauer 2007, Kim et al. 2007, Wollmann and Zeth 2007). We find that the RseB ortholog from *H. influenzae* also purifies as a mixture of dimers and a higher oligomer. By contrast, *P. aeruginosa* MucB chromoatographs as a mixture of monomers and dimers, with the dimer again appearing to be the MucA-binding species. The existence of multiple oligomeric forms of RseB/MucB with different activities from several bacterial species raises the possibility that modulation of quaternary structure plays a role in controlling inhibition of RseA/MucA cleavage *in vivo*.

The studies presented here provide an important initial step in dissecting the biochemical mechanisms that regulate a critical signal transduction pathway that impacts *P. aeruinosa* pathogenesis. This work also furthers our understanding of the roles of the PDZ domain and LA

loop in HtrA-family proteases, shedding light on how these proteases are regulated and identify substrates.

Materials and Methods

Plasmids and clones

P. aeruginosa genes were PCR amplified from genomic DNA of strain PAO1. $MucA^{peri}$ (residues 106 to 194 of MucA) was cloned into the NdeI and XhoI sites of pET15b, adding the sequence MGSSHHHHHHSSGLVPRGSHM to the N terminus of the protein. The stop codon was changed from TGA to TAA to avoid read-through during overexpression in *E. coli*. DNA encoding a MucB variant lacking the periplasmic localization sequence (residues 2-21) was cloned between the NdeI and BamHI sites of pET21b, appending an LEHHHHHH tag to the C terminus of the protein. AlgW lacking the membrane anchor (residues 24 to 389) was cloned into the NdeI and XhoI sites of pET15b, adding the sequence MGSSHHHHHHSSGLVPRGSHM to the N terminus of the protein. To construct $AlgW^{\Delta LA}$, the gene sequence encoding residues 77-92 (KPSHPLFDDPMFRRFF) was deleted. To construct $AlgW^{\Delta PDZ}$, a stop codon was cloned after the sequence encoding residue R279. Genomic DNA from *H. influenzae* was a gift from Igor Levchenko (MIT). DNA encoding an *E. coli* RseB variant lacking the periplasmic localization sequence (residues 2-23) was cloned between the NdeI and XhoI sites of pET21b, appending an LEHHHHHH tag to the C terminus of the protein.

Proteins and peptides

For purification of *P. aeruginosa* MucA, MucB, AlgW, and variants, *E. coli* strain X90(DE3) containing an appropriate overproducing plasmid was grown at 37 °C in LB medium plus ampicillin (100 μg/mL) to an OD_{600} of approximately 0.6, and protein expression was induced by addition of IPTG (100 μg/mL). Cells were harvested after 2 h, resuspended in 1/50 volume of lysis buffer (50 mM sodium phosphate (pH 8), 500 mM KCl, 20 mM imidazole), and lysed by

sonication. The cell lysate was centrifuged for 30 min at 23,000 × g, and the supernatant was applied to a Ni-NTA column pre-equilibrated in lysis buffer. The column was washed with 60 volumes of lysis buffer and then with elution buffer (50 mM sodium phosphate (pH 8), 500 mM KCl, 500 mM imidazole). Fractions containing the most concentrated protein according to the Bradford-stain assay were combined, dialyzed overnight against 1000 volumes of degradation buffer (50 mM sodium phosphate (pH 7.4), 200 mM KCl, 10% glycerol), dialyzed again against fresh buffer, and stored frozen at –80 °C. To obtain radiolabeled $MucA^{peri}$, cells were grown in a defined rich medium (TekNova) lacking methionine. Upon induction with IPTG, ^{35}S-methionine was added. $^{35}S\text{-}MucA^{peri}$ protein was purified as described above except that cell lysis was accomplished by suspending the cell pellet in buffer containing 6M guanidinium hydrochloride.

E. coli $RseA^{peri}$ and DegS were purified as described (Walsh et al., 2003). Solid-phase synthesis of peptides with the N-terminal sequence DNRDGNV or fluorescein-DNRDGNV followed by the C-terminal sequence YYF (OMP peptide), WVF (MucE peptide), IVI, or IFL was performed by the MIT Biopolymers lab. Peptides were purified by reverse-phase HPLC using a Vydac C18 column. For fluorescein-labeling of $MucA^{peri}$, Ser^{154} was mutated to cysteine; the mutation did not affect purification. Purified $MucA^{peri\text{-}C154}$ was incubated with TCEP, mixed with a 10-fold molar excess of fluorescein-5-maleimide, and incubated overnight at 4 °C. Products were separated by reverse-phase HPLC. The fluorescein-modified protein ran at the same position as $MucA^{peri}$ in SDS-PAGE but fluoresced when illuminated with UV light. The labeled protein was lyophilized and resuspended in degradation buffer.

Cleavage assays

Cleavage assays were performed in degradation buffer at 25 °C. For analysis by SDS-PAGE, reactions were quenched by addition of loading buffer, boiled, electrophoresed on 12% or 15% Tris-tricine gels, and stained with Coomassie brilliant blue. To determine kinetics, assays were performed using ^{35}S-labeled substrate, quenched by dilution in 10% trichloroacetic acid, incubated for 30 min on ice, and centrifuged at 20,000 × g for 20 min at 4 °C. Acid-soluble radioactivity was measured in a scintillation counter and compared to total counts from unprecipitated control samples. For identification of cleavage sites, products were separated by reverse-phase HPLC on a Vydac C18 column and analyzed by MALDI-MS (MIT Biopolymers) and sequential Edman degradation (Tufts University Core Facility).

Circular-dichroism spectroscopy

MucA^{peri} (404 μM in degradation buffer) was diluted into water to a concentration of 3 μM and circular-dichroism spectra were taken in a 1 cm path-length cuvette with a 1 s integration time at different temperatures using an Aviv 60DS instrument.

Size-exclusion chromatography

Gel filtration was performed on a SMART system (Amersham Biosciences) at 4 °C using an Superdex 200 column. Elution of MucB was monitored by absorbance at 280 nm, whereas elution of fluorescein-labeled MucA^{peri} and fluorescein-labeled MucA^{peri}•MucB was monitored by absorbance at 490 nm. Molecular-weight standards were from BioRad (#151-1901).

Fluorescence anisotropy

Binding of fl-MucA^{peri} to MucB was monitored by changes in fluorescence anisotropy at 25 °C using a PTI QM-2000-4SE spectrofluorometer (exitation 467 nm; emission 520 nm). The fl-MucA^{peri} protein was diluted in degradation buffer to a final concentration of 50 nM in a 60 μL cuvette with a 0.3 cm path. MucB protein was serially diluted into degradation buffer, and 1 μL aliquots of increasingly concentrated MucB were titrated into the cuvette. Anisotropy was calculated based on the scattering correction of a sample containing an identical amount of MucB but no fl-MucA^{peri}. AlgW binding to synthetic peptides containing a N-terminal fluorescein was assayed analogously.

Sequence alignments

Sequence alignments were performed with CLUSTALW using BLOSUM matrices and formatted in BioEdit (Hall 1999).

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Figure legends

Figure 1 Cleavage of MucA by AlgW. (**A**) Sequence alignment of the periplasmic domains of *P. aeruginosa* MucA and *E. coli* RseA. Conserved residues are highlighted. (**B**) Cleavage of $MucA^{peri}$ by AlgW. $MucA^{peri}$ (20 μM) was incubated with $AlgW_3$ (0.5 μM) in the absence or presence of MucE peptide (35 μM). (**C**) Rates of cleavage of radiolabeled $MucA^{peri}$ by $AlgW_3$ (0.5 μM) in the presence of MucE peptide (35 μM). The curve is fit to the Hill form of the Michaelis-Menten equation with K_m = 159 μM, V_{max} = 1.2 s^{-1}, and Hill constant = 1.3. (**D**) Time course of cleavage of 592 μM radiolabeled $MucA^{peri}$ by 0.5 μM $AlgW_3$ in the absence of MucE peptide. The linear fit corresponds to 0.0013 molecules of $MucA^{peri}$ cleaved per $AlgW_3$ per second.

Figure 2 Cleavage site specificity of AlgW. (**A**) Circular dichroism spectra of $MucA^{peri}$ (3 μM) at different temperatures. (**B**) Sequence alignment of periplasmic domains of MucA from *P. aeruginosa* and closely related species. Arrows indicate observed cleavages by AlgW by mass spectrometry and peptide sequencing. (**C**) Cleavage of $RseA^{peri}$ (20 μM) and $MucA^{peri}$ (20 μM) by DegS (11 μM trimer) in the absence or presence of YYF peptide (60 μM). Reactions were performed at room temperature for 16 hours. (**D**) Cleavage of $RseA^{peri}$ (20 μM) by AlgW (0.5 μM trimer) in the absence or presence of MucE peptide (35 μM).

Figure 3 Peptide binding and activation of AlgW. (**A**) Binding of peptides with different C termini to AlgW. 50 nM fluorescein-labeled peptides with varying C terminal residues as noted were incubated with increasing amounts of AlgW and fluorescence anisotropy was observed. (**B**)

Effect of different concentrations of peptides on the rate of AlgW cleavage of $MucA^{peri}$. 74 μM radiolabeled $MucA^{peri}$ was incubated with 0.5 μM $AlgW_3$. Cleavage rates were determined by change in TCA-soluble radioactive counts over time.

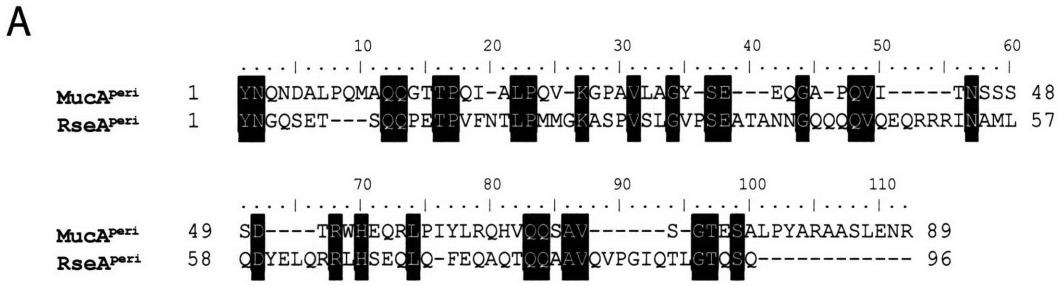
Figure 4 Sequence alignment of AlgW and homologous proteases. The LA loop and L2 loop regions are labeled.

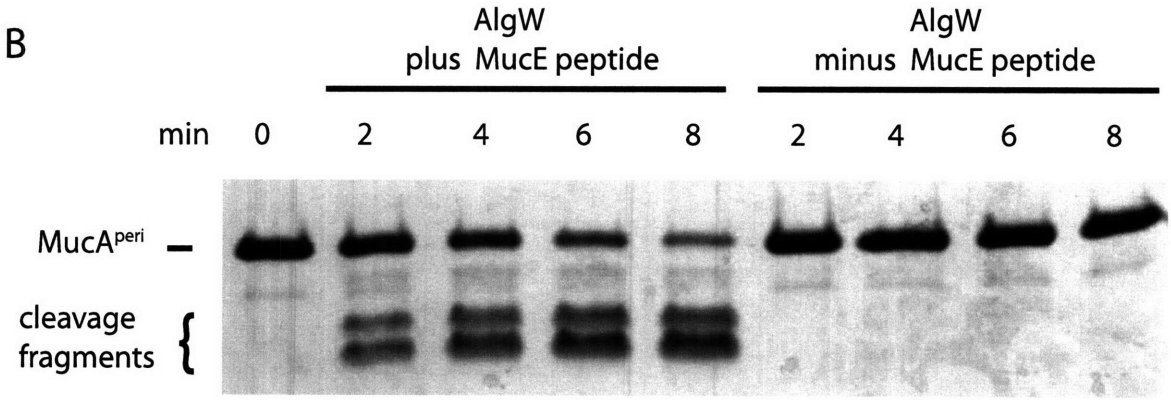
Figure 5 Role of the LA loop in proteolysis. (**A**) Cleavage of $MucA^{peri}$ by $AlgW^{\Delta LA}$. $MucA^{peri}$ (20 μM) was incubated with $AlgW^{\Delta LA}{}_3$ (0.5 μM) in the absence or presence of MucE peptide (35 μM). (**B**) Rates of cleavage of radiolabeled $MucA^{peri}$ by $AlgW^{\Delta LA}$ (0.25 μM trimer) in the presence of MucE peptide (35 μM). The curve is fit to the Hill form of the Michaelis-Menten equation with K_m = 6.3 μM, V_{max} = 1.2 s^{-1}, and Hill constant = 1.2. (**C**) Time course of cleavage of 592 μM radiolabeled $MucA^{peri}$ by 0.5 μM $AlgW_3$ or $AlgW^{\Delta LA}{}_3$ in the absence of MucE peptide. The linear fits correspond to 0.0013 and 0.16 molecules of $MucA^{peri}$ cleaved per protease trimer per second for $AlgW_3$ and $AlgW^{\Delta LA}{}_3$ respectively.

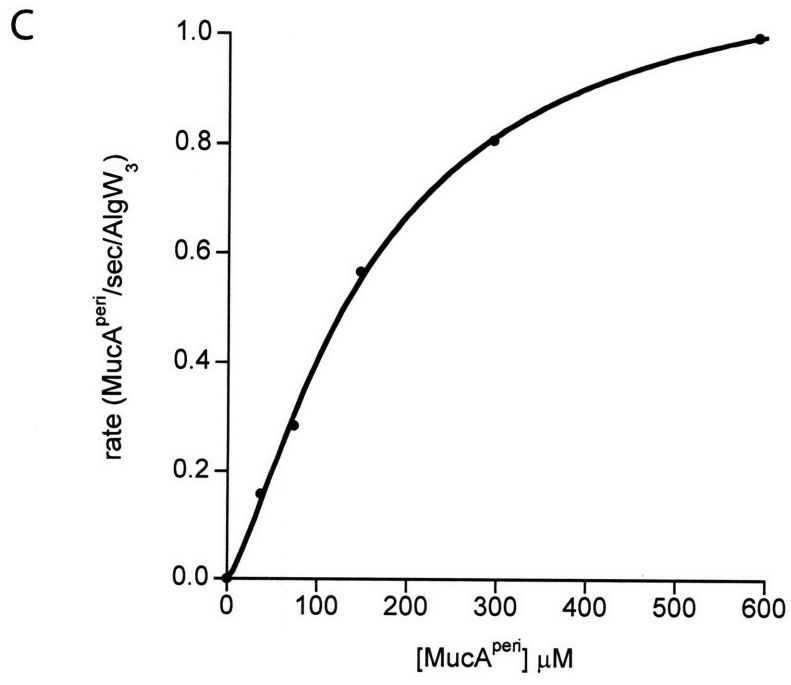
Figure 6 Properties of the interaction between MucA and MucB. (**A**) MucB (80 μM) inhibition of cleavage of $MucA^{peri}$ (20 μM) by $AlgW_3$ (0.5 μM) in the presence of MucE peptide (35 μM). (**B**) Binding of MucB to fl-$MucA^{peri}$ (50 nM). K_d = 120 nM. (**C**) Dissociation of fl-$MucA^{peri}$ (50 nM) from MucB (3.5 μM) upon addition of nonlabeled $MucA^{peri}$ (38 μM). (**D**) Gel filtration of MucB orthologs (4.3 nmol monomer). MucB = *P. aeruginosa* MucB, RseB = *E.coli* RseB, HiB = *H. influenzae* RseB. (**E**) Gel filtration of the fl-$MucA^{peri}$ (0.15 nmol) and the fl-$MucA^{peri}$/MucB

complex (0.15 nmol fl-MucA^{peri} mixed with 9-fold molar excess of MucB. The line corresponds to a linear fit of the logarithm of the molecular masses of globular protein standards.

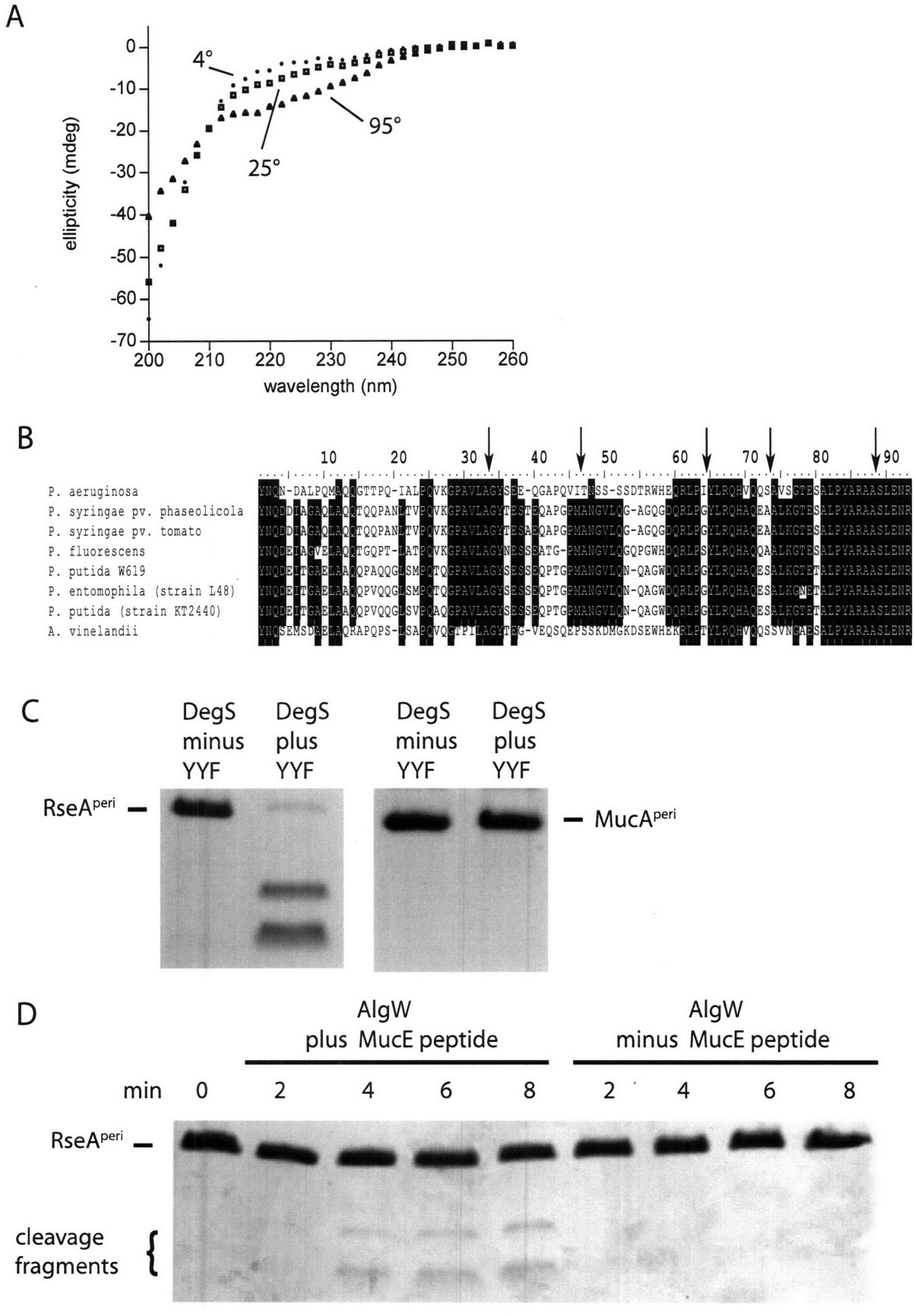
Cleavage of MucA by AlgW



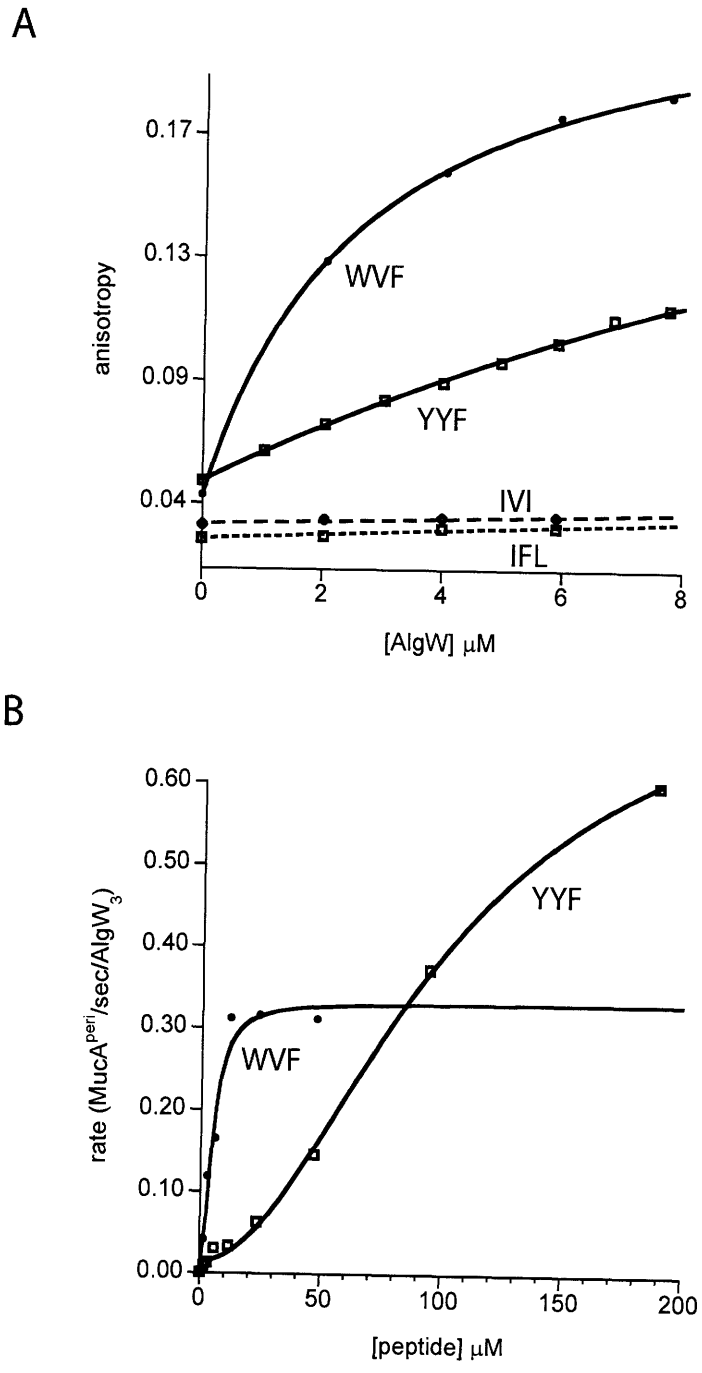




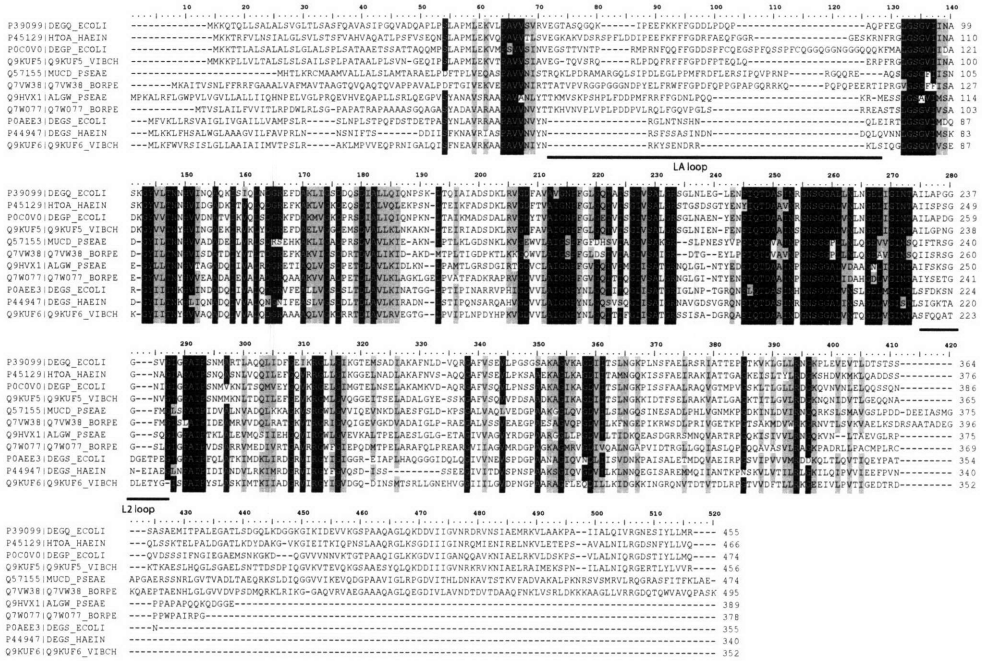
Cleavage site specificity of AlgW



Peptide binding and activation of AlgW



Sequence alignment of AlgW and homologous proteases



Role of the LA loop in proteolysis

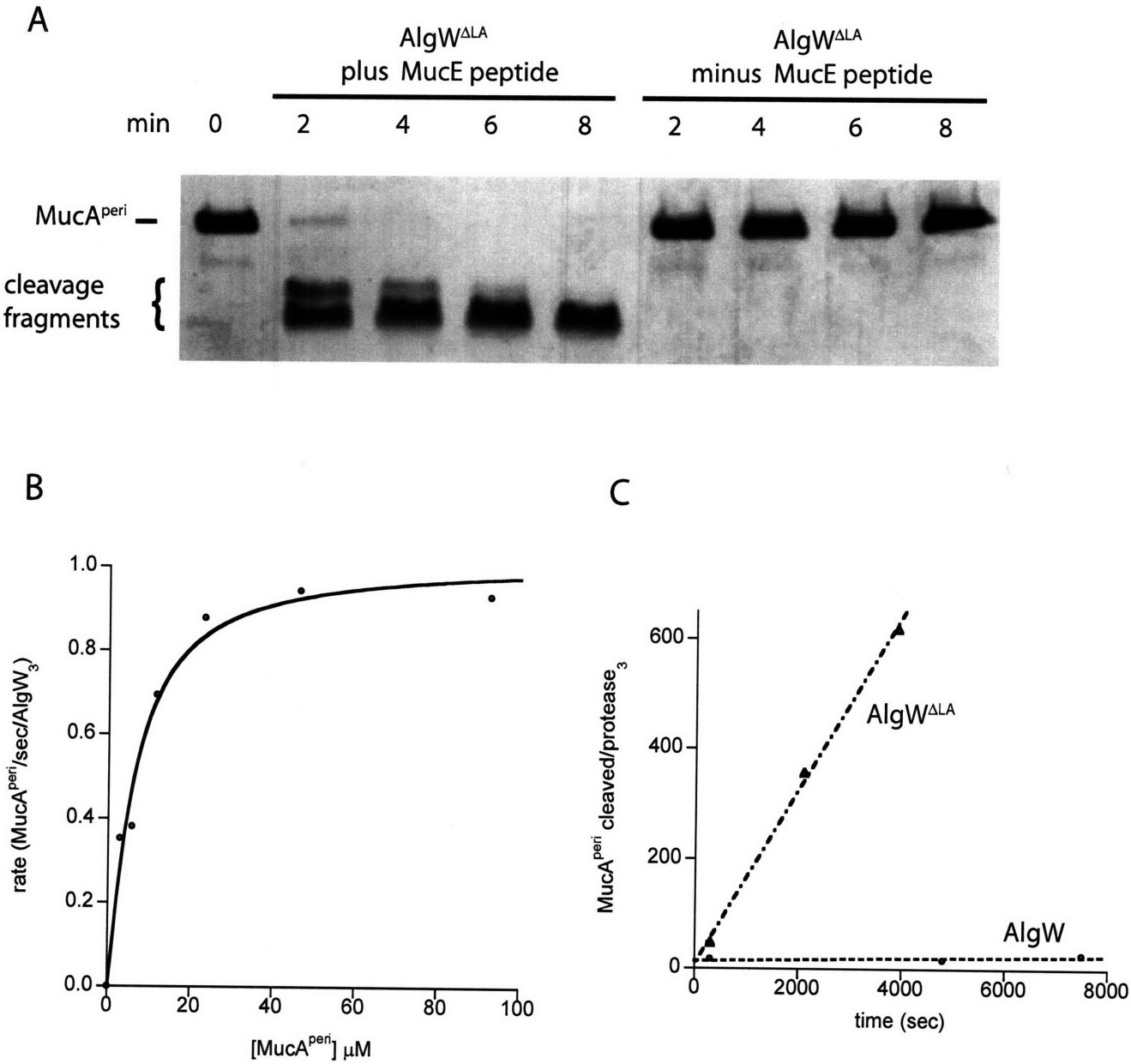
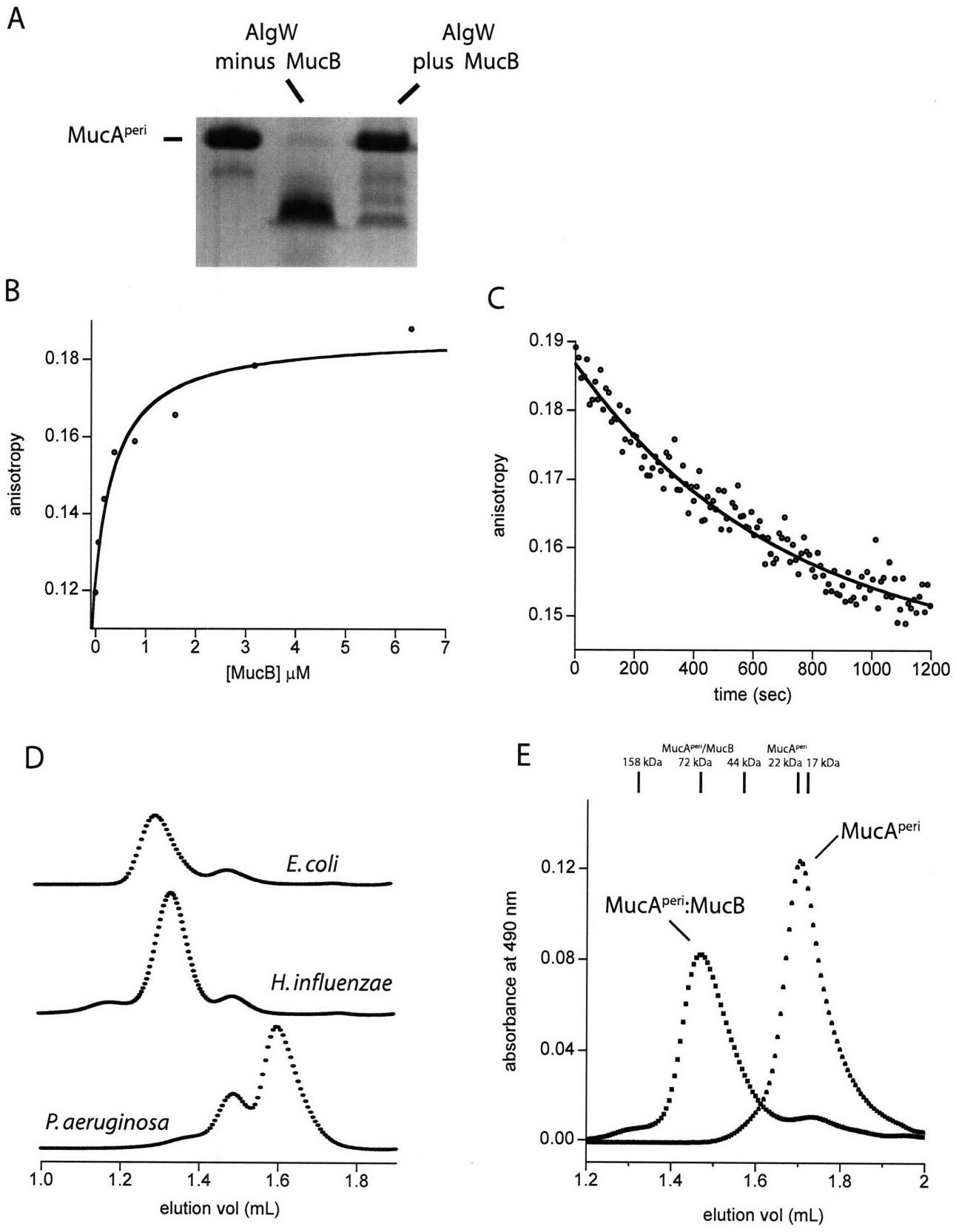


Figure 6

Properties of the interaction between MucA and MucB



CHAPTER FOUR

Prospectus

A model for the periplasmic stress response

The aim of the stress-sensing pathway is to activate transcription of stress-response genes. A number of protein molecules play a role in this complex transmembrane signal transduction pathway in *E. coli* and *P. aeruginosa*. Porins have been shown to be important for activation of DegS (Walsh et al. 2003), and related C-terminal sequences have been shown by me and others to be important for activation of AlgW (Qiu et al. 2007). In addition to protease activation, the response appears to be controlled by RseA/MucA binding to RseB/MucB. The observation in *E. coli* that RseB inhibits RseA cleavage by DegS and in *P. aeruginosa* that MucB inhibits MucA cleavage by AlgW suggests that there are multiple regulatory mechanisms for the initiation of the periplasmic stress responses. Tables 1 and 2 summarize the phenotypic effects of deletion or mutation of proteins in these pathways in *E. coli* and *P. aeruginosa*.

A model to account for all current observations in *E. coli* requires multiple regulatory mechanisms for cleavage of RseA. Cleavage by DegS requires binding of porin C termini to the PDZ domain in order to switch it from the inactive to the active proteolytic conformation (Walsh et al. 2003). In order for RseA to be a good substrate for DegS, RseB must not be bound. This presumably occurs by way of an unknown signal that is associated with periplasmic stress, or with the overexpression of outer membrane porins. When RseB is bound to RseA, RseB inhibits the cleavage of RseA by DegS. It is not clear how this occurs. One possibility is that RseB binding to the C-terminal region of the periplasmic domain of RseA allows for contacts to be made closer to the site at which DegS cleaves, preventing access of RseA to the catalytic site of DegS (Chapter 2, Figure 6). Another possibility is that DegS requires, to some degree, the C-

terminal region of RseA for substrate recognition and that RseB binding obscures that region (Figure 1). The latter model is supported by evidence that truncated forms of RseA lacking the primary RseB-binding motif are cleaved more slowly (Chapter 2, Figure 5A), and also evidence from Kanehara et al. (2003) suggesting that constructs lacking residues 180-190 of RseA ('YELQRRLHSEQ', which is part of the RseB-binding determinant, and for which the second 'R' residue has been shown to be critical for RseA-binding (Kim et al. 2007)) are cleaved more slowly than full-length.

RseB also inhibits cleavage of RseA by YaeL (Grigorova et al. 2004). The first PDZ domain of YaeL has been shown to inhibit its proteolytic activity. Removal of this PDZ domain results in constitutive cleavage of RseA in both the absence and presence of RseB, and independent of prior cleavage of RseA by DegS. In addition, the glutamine-rich regions of the periplasmic domain of RseA have been implicated in inhibition of its processing by YaeL (Kanehara et al. 2003). Deletion of these regions, or replacement of them by other sequences, result in constitutive cleavage of RseA by YaeL. It should be noted that there are two glutamine-rich regions in RseA, and that the primary RseB-binding determinant that I have discovered lies between them. These observations are consistent with a model wherein the binding of RseB to RseA occludes access to the protease by way of clashes with the PDZ domain of YaeL (Figure 2). These clashes might prevent the protease from getting near enough to the transmembrane region of RseA to cleave it. Removal of the occluding object on either molecule would allow for cleavage to occur, even in the absence of cleavage by DegS. It is also possible that there are specific interactions between RseB and the PDZ domain of YaeL that are responsible for inhibition of RseA cleavage.

How is RseB-based activation regulated? It is possible that a factor binds to RseB and competes with the RseA binding site, thereby titrating RseB off RseA under stress conditions. It is also possible that another molecule binds RseB allosterically under stress conditions and releases RseA, perhaps by inducing it to convert into another oligomeric form that is incapable of binding RseA. In addition, it is possible that a molecule present under non-stress conditions stabilizes the RseA/B complex, and when this molecule is titrated away under stress conditions, RseB is released and RseA can be cleaved by DegS before RseB can rebind. In this respect, the soluble-protein assays I performed have a limitation. For example, it is possible that much of the affinity of DegS for RseA is dependent on interaction between their transmembrane segments. If this is the case, it would be possible to cleave RseA after release of RseB. In the solution assay, however, RseB might rebind before DegS has an opportunity to recognize RseA under these conditions. Another possibility is that RseA cleavage can be initiated in the presence of RseB by proteases other than DegS. Indeed it has been shown that YaeL can perform this function if RseB is absent, or if the first PDZ domain of YaeL is mutated or deleted. I have found that, when overexpressed, the periplasmic protease DegQ can also serve this function. Other proteases may also be involved in regulation of RseA.

The model for *P. aeruginosa* is quite similar to that of *E. coli*. The identity of the AlgW-activating signal is less clear, because although a few porins in *P. aeruginosa* possess a potential AlgW-activating motif at their C termini, many do not. Furthermore, the MucE protein, which has the potential to be a strong activator, does not possess homology to proteins in the porin class. In addition, a third protease in *P. aeruginosa* has been implicated in the processing of

truncated MucA proteins found in *P. aeruginosa* isolates from the lungs of cystic fibrosis patients. It was observed that when mucoid strains carrying *mucA* mutations are plated, a fraction of them revert to a non-mucoid phenotype. A search for multicopy suppressors of reversion in nonmucoid MucA-truncated strains resulted in the discovery of mutations in the *P. aeruginosa* gene encoding the soluble periplasmic protease Prc (Tsp) (Reiling et al. 2005). Deletion of *prc* in MucA-truncated strains was shown to eliminate the mucoid phenotype. It was shown that Prc is required for activation of AlgU in a variety of mucoid MucA-truncated strains. However, Prc did not appear to act on full-length MucA, even in the absence of MucB.

More recent results that implicated AlgW in the cleavage of MucA also investigated the importance of Prc. Qiu et al. (2007) found that Prc was not involved in mucoidy associated with the purported AlgW-dependent cleavage of MucA. Wood et al. (2006) did notice a slight effect of Prc on the induction of alginate synthesis. These results indicate that Prc is important in the processing of MucA mutants that are truncated in the periplasmic domain. This is interesting because one of the mutants, *mucA22* (also described as *mucAΔG440*), the most commonly found in isolates from CF lungs, is only slightly larger than the putative cleavage product resulting from cleavage at the conserved A/G bond in MucA. This suggests that *P. aeruginosa* MucP (the YaeL ortholog) can make subtle distinctions between fragments in this size range. In contrast, a variety of similarly short mutants of RseA were shown to be processed effectively by YaeL in *E. coli* (Kanehara et al. 2003).

Rationale for control by multiple inputs

The inhibition of DegS proteolytic activity by its PDZ domain and the inhibition of DegS cleavage of RseA by RseB suggest that the two function as a kind of dual switch, i.e. that two signals (porins through DegS and an unknown signal through RseB) must both exist in order to cleave RseA and activate the stress response. Why have two signals? Regardless of what the inputs are into the signal transduction pathway, there are some advantages to having control at different levels. Having multiple control points can serve to reduce noise in the system, for example, if for some reason one molecule that is a stress signal is abundant but another is not, so the pathway can remain off instead of being activated under conditions that do not warrant it. In addition, the presence of multiple control points can allow for better control of the magnitude of the response. For example, if a low level of stress is sensed, corresponding, for example, to the presence of excess of one type of molecule, then the stress response can be activated moderately. If an additional type of molecule is present, it may indicate that the cell is under more severe stress, which would allow for a stronger stress response.

It is also possible that RseB binding to RseA and inhibition of proteolysis serves to prevent cleavage of RseA when DegS is activated for other reasons. Although no other substrates for DegS have been discovered, it is possible that others exist. In such a case it might be desirable to be able to cleave one substrate but not another. In the absence of RseB, RseA would be cleaved whenever DegS is active. However, RseB provides a second switch by which RseA cleavage can be controlled in the presence of active DegS.

Comparison with other pathways regulated by intramembrane proteolysis

Gamma-secretase is a eukaryotic intramembrane protease that has been shown to cleave a variety of molecules including amyloid precursor protein and Notch (De Strooper et al. 1998, De Strooper et al. 1999). Unlike YaeL, gamma-secretase is a multiprotein complex, with the presenilin molecule performing the proteolytic activity. The means by which gamma-secretase recognizes its substrate has been studied in detail. Nicastrin, a subunit of gamma-secretase, recognizes free N termini of substrates close to the membrane, usually resulting from cleavage of the substrate by another protease (Shah et al. 2005). The presence of an alpha amino group is important for recognition by Nicastrin, but the identity of the residues themselves is not important. In the case of YaeL, cleavage of RseA by DegS reveals a free C terminus close to the membrane. It has not been demonstrated how YaeL recognizes its substrate, but it has been shown that YaeL can cleave a variety of molecules with different transmembrane and extramembrane segments (Akiyama et al. 2004).

Detection of stress in the eukaryotic endoplasmic reticulum also relies on a regulated intramembrane proteolysis system. ATF6 is a membrane-bound transcription factor that resides in the ER, where binding of the protein BiP to the lumenal domain of ATF6 keeps it from being translocated to the Golgi. In the presence of ER stress, unfolded proteins in the ER bind to BiP, which releases BiP from ATF6, unmasking signals for Golgi translocation (Shen et al. 2002). ATF6 is translocated to the Golgi, where it is cleaved by S1P and S2P in sequence, which frees the transcription factor domain that then enters the nucleus (Haze et al. 1999, Ye et al. 2000). The dependence on S1P cleavage of ATF6 to allow S2P cleavage has been studied and it has

been determined that bulky domains near the C terminus hinder the action of S2P, i.e. that a specific inhibitory sequence is not required to inhibit cleavage by S2P (Shen and Prywes 2004). I believe that the dependence of RseA cleavage on DegS prior to cleavage by YaeL may incorporate elements of both of these pathways. BiP and RseB may be analogous in function, although I have demonstrated that RseB will not bind to just any unfolded protein.

Future directions

Multicopy suppressors of *degS*⁻ lethal phenotype

Based on the observation that *degS* and *yaeL* are both essential genes because they provide the cell with essential σ^E activity, and that a *degS*⁻ *rseB*⁻ strain is viable because YaeL can cleave full-length RseA in the absence of of RseB, I sought to identify multicopy suppressors of the *degS*⁻ lethal phenotype. The thought was that if some protein were serving to titrate RseB off RseA, its presence in excess would free RseB from RseA, allowing RseA to be cleaved by YaeL, subsequently leading to the activation of σ^E and allowing the cells to remain viable. Although the screen was never carried out to completion, the DegQ protease was found to be such a suppressor. The PDZ domains of DegQ are essential for rescue. Multicopy *degQ* was unable to rescue lethality of a YaeL-depleted strain, suggesting that DegQ acts through RseA. I observed cleavage of RseA by DegQ in vitro. It should also be noted that overproduction of a catalytically inactive mutant of DegQ (active site serine to alanine mutation) suppressed the lethal phenotype, although this was in a background that had wild-type *degQ* on the chromosome. It is likely, therefore, that DegQ is able, to some degree, to cleave RseA in the presence of RseB.

Interestingly, multicopy *degP* was unable to suppress the *degS*⁻ lethal phenotype, even though DegP is similar in domain structure to DegQ. Both DegQ and DegP have similar cleavage site preferences for unfolded substrates *in vitro* (Kolmar et al. 1996), however, they appear to behave differently in the cell.

It will be important to characterize more fully the ability of DegQ to cleave RseA in the presence of RseB. It is possible that it can do so more effectively in the presence of a trans-activator that is present during some stress conditions. It will also be desirable to perform the screen to identify other multicopy suppressors of the *degS*⁻ phenotype. If RseB is pulled off of RseA in the presence of a non-protein molecule, it may be difficult to identify the factor by this method. Lipids and LPS, for example, are synthesized through multi-step pathways. In such a case, it is possible that a short fragment of genomic DNA present in multicopy will not code for sufficiently many enzymes to effect a change in the level of such molecules.

Identification of additional RseB-binding molecules

The identification of additional RseB-binding proteins by co-immunoprecipitation, mass spectrometry, and protein sequencing would be a reasonable approach to identify potential regulators of RseB activity. Testing such proteins for the ability to inhibit formation of the RseA/RseB complex or to inhibit inhibition of DegS proteolysis of RseA by RseB might reveal additional levels of regulation. Based on the structural homology of the N-terminal domain of RseB to lipid-binding domains, it is possible that RseB activity is regulated by a non-protein molecule. If tightly bound to RseB, such a molecule might also be found by

immunoprecipitation. It could be separated from the protein by chromatography in the presence of a chemical denaturant and identified by a combination of NMR spectroscopy, IR spectroscopy, and mass spectrometry. Chemical crosslinking is another biochemical means of enriching for RseB-interacting molecules. This would require the use of a properly reactive probe and a reactive substrate, which would require the testing of multiple crosslinking agents. Finally, one could test different purified and concentrated cellular components for the ability to inhibit the RseA/RseB interaction or RseB inhibition of RseA proteolysis by DegS. The use of both genetic and biochemical techniques should shed light on additional mechanisms of regulation of the periplasmic stress response.

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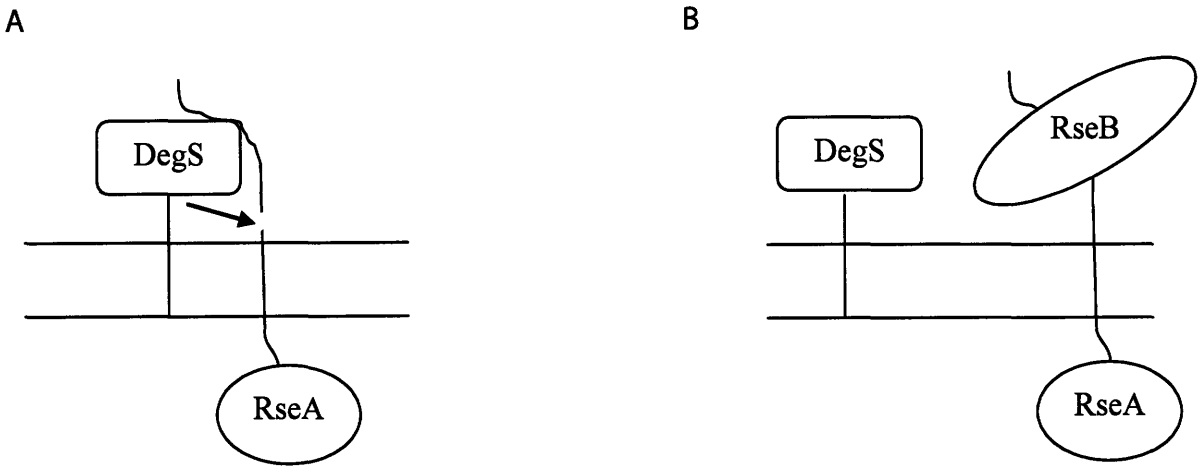
1 Phenotypes of mutations in genes involved in the σ^E pathway in *E. coli*

mutation	phenotype	references
null	lethal	De Las Penas et al. 1997
overexpression	suppresses lethal phenotype of *yaeL* null	Kanehara et al. 2002
null	increased σ^E activity; suppresses lethal phenotype of *yaeL* null	De Las Penas et al. 1997, Missiakas et al. 1997, Kanehara et al. 2002
truncations	increased σ^E activity; enhanced cleavage by YaeL in absence of DegS	Kanehara et al. 2003
overexpression	decreased σ^E activity; lethal	Missiakas et al. 1997
null	increased σ^E activity (20%-90% increase)	De Las Penas et al. 1997, Missiakas et al. 1997, Grigorova et al. 2004
overexpression	decreased σ^E activity (30%-40% decrease)	Missiakas et al. 1997
null	lethal; reduced σ^E activity	Ades et al. 1999, Alba et al. 2001
ΔPDZ	viable; reduced σ^E activity	Walsh et al. 2003
null	lethal; reduced σ^E activity	Kanehara et al. 2001, Kanehara et al. 2002
ΔPDZ	viable; increased σ^E activity	Kanehara et al. 2003, Bohn et al. 2004
null	no effect on σ^E activity; lethal at high temperatures	Raina et al. 1995
overexpression	no effect on σ^E activity or viability	Mecsas et al. 1993
null	enhancement of σ^E activity	Ades et al. 1999
overexpression	complementation of *degS* null (low levels); lethal (high levels)	Cezairliyan and Sauer, unpublished
null	enhancement of σ^E activity	Ades et al. 1999

Phenotypes of mutations in genes involved in the AlgU pathway in *P. aeruginosa*

mutation	phenotype	references
null	nonmucoid	Martin et al. 1993a
null	mucoid; constitutive AlgU activity	Martin et al. 1993b
truncations	mucoid; constitutive AlgU activity	Martin et al. 1993b, Rowen and Deretic 2000
overexpression	nonmucoid; decreased AlgU activity	Rowen and Deretic 2000
null	mucoid on some media (PIA), non-mucoid on others (LB); enhanced AlgU activity	Martin et al. 1993b, Rowen and Deretic 2000
null	nonmucoid; reduced AlgU activity	Wood et al. 2006, Qiu et al. 2007
ΔPDZ	nonmucoid; reduced AlgU activity	Qiu et al. 2007
overexpression	mucoid; inhibits mucoidy of strains with truncated MucA	Wood et al. 2006; Boucher et al. 1996
null	nonmucoid; reduced AlgU activity	Qiu et al. 2007
null	mucoid; lethal at high temperatures; sensitive to oxidative stress; reduced pathogenicity	Boucher et al. 1996, Yorgey et al. 2001, Wood and Ohman 2006, Qiu et al. 2007
null	slight increase in AlgU activity?	Wood et al. 2006

Figure 1

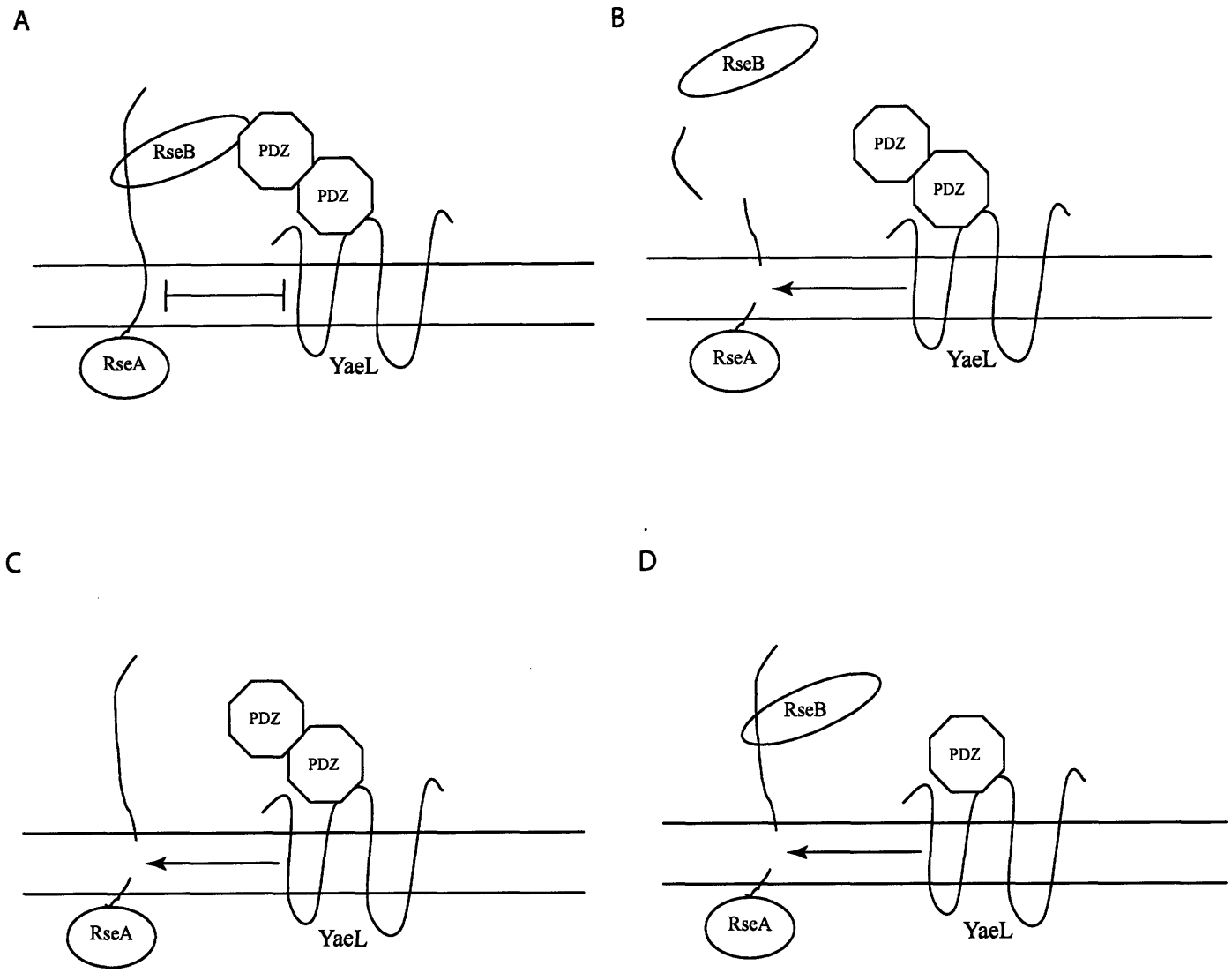


Model of RseB-controlled inhibition of RseA proteolysis by DegS based exclusively on binding to a region distal to the cleavage site

A) DegS cleavage of RseA depends on recognition of a region apart from the cleavage site

B) inhibition of DegS cleavage of RseA by RseB binding to the recognition site

Figure 2



Model of RseB-controlled inhibition of RseA proteolysis by YaeL

A) YaeL proteolysis of RseA is inhibited by its PDZ domain and RseB

B) DegS-cleavage of RseA allows YaeL to access and cleave RseA

C) The absence of RseB allows YaeL to access and cleave RseA

D) RseB does not inhibit RseA cleavage by YaeL missing one of its PDZ domains