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Control of *P. aeruginosa* AlgW protease cleavage of MucA by peptide signals and MucB

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Abstract

The ability of a pathogen to survive the defensive attacks 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 in the production of alginate, a surface polysaccharide that also confers resistance to many host defenses and antibiotic treatments. The alginate response is regulated by proteolytic cleavage of MucA, a transmembrane protein that inhibits the transcription factor AlgU, and by the periplasmic protein MucB. Here we show that specific peptides bind to the periplasmic AlgW protease and activate its cleavage of MucA. We 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 strategies 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 activation increases production of periplasmic chaperones and other stress-response proteins, and also leads to a mucoid phenotype because of overproduction of alginate-synthesis enzymes (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 caused by mutations in the *mucA* gene, which is immediately downstream of *algU* (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, *mucB*, encodes a protein that is exported to the periplasm, binds to the periplasmic region of MucA, and functions as a negative regulator of the alginate response (Goldberg *et al.*, 1993; Schurr *et al.*, 1996; Mathee *et al.*, 1997; Rowen and Deretic, 2000).

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). Regulation of σ^E activity occurs via the transmembrane protein

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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 subsequent 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). Hence, *E. coli* RseA and *P. aeruginosa* MucA serve comparable functions. Stress conditions in *E. coli* are sensed by DegS, which is activated by the binding of the C termini of misfolded outer membrane porins (OMPs) to its PDZ domain, and possibly by RseB (a MucB homolog), which is a protein that inhibits DegS cleavage of RseA (Walsh *et al.*, 2003; Cezairliyan and Sauer, 2007).

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). Activation can be induced by overexpression of the periplasmic protein MucE, which has no *E. coli* homolog, in a process that depends upon the C-terminal residues of MucE (Qiu *et al.*, 2007). To understand how the mucoid phenotype of *P. aeruginosa* arises, it is important to determine how proteolysis of MucA is controlled by stress signals. 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, and investigate the inhibitory effect of MucB on AlgW cleavage of MucA.

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 His₆-tagged variants of AlgW and the periplasmic domain of MucA (MucA^{peri}). AlgW is a member 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). As expected based on other characterized members of this subgroup (Li *et al.*, 2002; Walsh *et al.*, 2003; Wilken *et al.*, 2004; Zeth, 2004; Mohamedmohaideen *et al.*, 2008), AlgW ran as a trimer during gel-filtration chromatography (data not shown). MucA^{peri} had a circular-dichroism spectrum consistent with a largely unfolded random-coil structure between 4 °C and 95 °C (Figure 1B). The periplasmic domain of RseA (RseA^{peri}) has similar properties (Walsh *et al.*, 2003).

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 1C). 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 1D). 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 $159 \text{ }\mu\text{M}$, 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 $2.2 \text{ M}^{-1}\text{s}^{-1}$ (not shown). This value is roughly 2000-fold smaller than the rate constant for AlgW cleavage in the presence of MucE peptide ($4.3 \cdot 10^3 \text{ M}^{-1}\text{s}^{-1}$).

We analyzed the initial products of AlgW cleavage of MucA^{peri} by reverse-phase HPLC, N-terminal sequencing, and mass spectrometry. Two major fragments were recovered, corresponding to cleavage between Ala¹³⁶ and Gly¹³⁷ (Figures 2A and 2B). *E. coli* DegS

cleaves *E. coli* RseA at the Val¹⁴⁸-Ser¹⁴⁹ peptide bond (Walsh *et al.*, 2003). Although the residues flanking the MucA/AlgW and RseA/DegS cleavage sites are quite different, both sites are approximately 30 residues from the start of the periplasmic domain.

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 and 2D). Hence, AlgW and DegS have a common ability to recognize RseA^{peri}, but only AlgW recognizes MucA^{peri}. Thus AlgW appears to have broader substrate specificity than DegS. Indeed, we observed AlgW cleavage of MucA at sites in addition to the primary scissile peptide bond when higher enzyme-substrate ratios or longer incubation times were used (Figure 2B).

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 ($K_D = 3 \mu\text{M}$), bound moderately well to the YYF peptide ($K_D = 22 \mu\text{M}$), but did not bind detectably to 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 occurred 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 ^{Δ 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 and purified AlgW ^{Δ PDZ} and found that it cleaved MucA^{peri} very slowly compared to peptide-activated AlgW (Figure 3). Nevertheless, the activity of the truncated enzyme was peptide independent and higher than the activity of peptide-free AlgW (Figure 3C). 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 ^{Δ PDZ} does not complement an *algW*⁻ strain (Qiu *et al.*, 2007). In

DegS, arginine 256 is near the start of the PDZ domain, and mutation of this residue to alanine results in peptide-independent cleavage of RseA at a substantial level (Sohn *et al.*, 2007). However, when we mutated the homologous arginine in AlgW (R279→A), the mutant cleaved MucA extremely slowly both with peptide (Figure 3C) and in the absence of peptide. Although we cannot be certain that the Δ PDZ and R279A mutations do not affect the folding of the AlgW protease domains, our 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.

The LA loop of AlgW inhibits MucA binding

Sequence alignments show that the LA loop in the protease domain of AlgW resembles loops in homologs that have two PDZ domains 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 μ M) 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 concentrations, 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). RseA binding stabilizes active DegS relative to inactive DegS (Sohn and Sauer, 2009), and thus tighter MucA binding to AlgW $^{\Delta$ LA may result in a higher fraction of the active mutant enzyme compared to wild type.

MucB binds MucA and inhibits cleavage by AlgW

MucB is a homolog of *E. coli* RseB, which binds RseA and inhibits cleavage by DegS (Cezairliyan and Sauer, 2007). Likewise, addition of MucB slowed AlgW cleavage of MucA^{peri} (Figure 6A). To study MucA-MucB interactions, we constructed a MucA^{peri} Ser¹⁵⁴→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 $1.4 \cdot 10^{-3} \text{ s}^{-1}$ (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 mixtures of dimers and hexamers. In the case of *E. coli* RseB, only the dimer binds RseA (Cezairliyan and Sauer, 2007). We mixed fl-MucA^{peri} with MucB and analyzed the mixture by gel filtration to determine the size of the molecular complex (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 was observed 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^{ΔPDZ} and peptide-stimulated DegS have similar levels of activity (Cezairliyan and Sauer, 2007; Sohn *et al.*, 2007). In AlgW, the PDZ domain appears to play both positive and negative roles in controlling protease activity, as the AlgW^{ΔPDZ} and AlgW R279A mutants are more active than unliganded AlgW but far less active than the peptide-stimulated enzyme. However, AlgW and DegS could still operate by similar mechanisms. For DegS, both peptide binding to the PDZ domain and RseA substrate binding tip the allosteric equilibrium to favor the active conformation (Sohn *et al.*, 2007; Sohn and Sauer, 2009). A similar allosteric mechanism could also explain our AlgW results, with the proviso that the PDZ domain plays an additional role, either in helping to bind the MucA substrate or in making contacts that help stabilize the active conformation of the protease domain. Indeed, our finding of positive cooperativity both in substrate binding and in peptide stimulation of AlgW cleavage of MucA supports an allosteric model of some kind. 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 (Sohn and Sauer, 2009).

Wood *et al.* (2006) observed that the overexpression of AlgW *in vivo* is sufficient to activate alginate production under non-stress conditions. This observation may simply be a

consequence of a greater amount of AlgW cleaving MucA at a low rate in the absence of activating peptides, but it can also be explained by the cooperative nature of the enzyme's activity. High concentrations of AlgW will result in both increased binding to the basal quantities of accessible C-terminal activators and increased binding to substrate, which would contribute to some degree of activation of AlgW even under non-stress conditions.

The basic domain structures of AlgW and DegS are similar, and both proteases are trimeric and membrane bound. However, AlgW possesses some structural features, such as a long LA loop and short L2 loop, that are more akin to features found in the soluble DegQ and DegP proteases, which form hexamers and higher oligomers (Krojer *et al.*, 2002; Kim and Kim, 2005; Krojer *et al.*, 2008; Jiang *et al.*, 2008). 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. The primary AlgW cleavage of MucA occurred at an Ala-Gly peptide bond, but additional cleavages at certain Ile-Xxx, Ala-Xxx, and Ser-Xxx bonds were also observed. Interestingly, the MucA mutants from mucoid CF isolates of *P. aeruginosa* are truncations near the first site of AlgW cleavage (Martin *et al.*, 1993c). It appears that Prc, another periplasmic protease, is responsible for the activation of AlgU in these mutants (Reiling *et al.*, 2005) although Prc is not essential for the activation of AlgU in the presence of full-length MucA (Qiu *et al.*, 2007).

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. Consistent with their model, we find that deleting part of the LA loop of AlgW results in substantially higher apparent affinity for MucA and in a higher rate of MucE-independent cleavage. It is possible, therefore, that interactions of other regulatory proteins with the LA loop of AlgW also play roles in regulating its activity in the cell. 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 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 MucB/RseB sequence homology, MucB fails to bind RseA and RseB fails to bind MucA. Moreover, RseB binding to RseA and variants can block 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 prevents cleavage of MucA both at the primary cleavage site and at secondary sites, one of which is more than 50 residues away. 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 fold. *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 chromatographs 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. aeruginosa* 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 between the NdeI and XhoI sites of pET15b, adding the sequence MGSSH₆SSGLVPRGSHM 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 LEH₆ tag to the C terminus of the protein. AlgW (residues 24 to 389) lacking the membrane anchor was cloned into the NdeI and XhoI sites of pET15b, adding the sequence MGSSH₆SSGLVPRGSHM to the N terminus of the protein. To construct AlgW^{ΔLA}, the gene sequence encoding residues 77–92 (KPSHPLFDDPMFRRFF) was deleted. To construct AlgW^{ΔPDZ}, 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 a *H. influenzae* RseB variant lacking the periplasmic localization sequence (residues 2–23) was cloned between the NdeI and XhoI sites of pET21b, appending an LEH₆ 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 OD₆₀₀ 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, ³⁵S-methionine was added. ³⁵S-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}, Ser¹⁵⁴ was mutated to cysteine; the mutation did not affect purification. Purified MucA^{peri}-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 ³⁵S-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 a 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 spectrofluorimeter (excitation 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 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)** Circular dichroism spectra of MucA^{peri} (3 μ M) at different temperatures. **(C)** Cleavage of MucA^{peri} by AlgW assayed by SDS-PAGE. MucA^{peri} (20 μ M) was incubated with AlgW (0.5 μ M trimer) in the absence or presence of MucE peptide (35 μ M) for the times indicated. **(D)** Rates of cleavage of 35 S-labeled MucA^{peri} by AlgW (0.5 μ M trimer) in the presence of MucE peptide (35 μ M). The curve is fit to the Hill form of the Michaelis-Menten equation with $K_m = 159 \mu\text{M}$, $V_{\max} = 1.2 \text{ s}^{-1}$, and Hill constant = 1.3.

Figure 2.

Cleavage sites and specificities. **(A)** Reverse-phase HPLC demonstrated that AlgW initially cleaves MucA^{peri} to produce two discrete fragments. Mass spectrometry and peptide sequencing revealed that these fragments corresponded to MucA^{peri} residues 53–110 (first peak) and 2–52 (second peak). **(B)** Sequence alignment of periplasmic domains of MucA from *P. aeruginosa* and closely related species. The large arrow indicates the initial AlgW cleavage site. Small arrows indicate subsequent cleavages by AlgW. **(C)** DegS (11 μM trimer) cleavage of RseA^{peri} (20 μM) and MucA^{peri} (20 μM). Reactions were performed in the absence or presence of YYF peptide (60 μM) at room temperature for 16 hours. **(D)**

AlgW (0.5 μ M trimer) cleavage of RseA^{peri} (20 μ M) in the absence or presence of MucE peptide (35 μ M). Cleavage was less efficient than observed for MucA^{peri} (see Fig. 1C).

Figure 3. Peptide binding and activation of AlgW. **(A)** AlgW binding to fluorescently labeled peptides (50 nM) with different C termini was assayed by changes in fluorescence anisotropy. **(B)** Peptide activation of cleavage of ^{35}S -labeled MucA^{peri} (74 μM) by AlgW (0.5 μM trimer). Cleavage rates were determined by change in TCA-soluble radioactive counts over time. **(C)** Rates of cleavage of MucA^{peri} (592 μM) by AlgW, AlgW^{ΔPDZ}, and AlgW R279A (0.5 μM trimer). When present, the MucE peptide concentration was 35 μM. Numbers above the bars are substrate molecules cleaved per second per enzyme trimer.

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

Figure 5.

Role of the LA loop in proteolysis. **(A)** Cleavage of MucA^{peri} (20 μM) by AlgW^{ΔLA} (0.5 μM trimer) in the absence or presence of MucE peptide (35 μM) assayed by SDS-PAGE. Disappearance of the upper band over the time course is due to cleavage at secondary sites. **(B)** Rates of cleavage of different concentrations of ³⁵S-labeled MucA^{peri} by AlgW^{ΔLA} (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 $K_m = 6.3 \mu\text{M}$, $V_{\max} = 1.2 \text{ s}^{-1}$, and Hill constant = 1.2. **(C)** Time course of cleavage of ³⁵S-labeled MucA^{peri} (592 μM) by AlgW or AlgW^{ΔLA} (0.5 μM trimer) 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 and AlgW^{ΔLA} respectively.

Figure 6.

Interactions between MucA and MucB. (A) MucB (80 μ M) inhibited cleavage of MucA^{peri} (20 μ M) by AlgW (0.5 μ M trimer) in the presence of MucE peptide (35 μ M). (B) Binding of MucB to fl-MucA^{peri} (50 nM). The curve is a fit to the quadratic form of a hyperbolic binding equation ($K_D = 120$ nM). (C) Dissociation of fl-MucA^{peri} (50 nM) from MucB (3.5 μ M) upon addition of unlabeled MucA^{peri} (38 μ M). The curve is an exponential fit with a rate constant of 0.0014 s^{-1} . (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 fl-MucA^{peri} (0.15 nmol) or the fl-MucA^{peri}/MucB complex (0.15 nmol fl-MucA^{peri} mixed with 1.35 nmol MucB) was monitored by fluorescein absorbance.