Versatile modes of peptide recognition by the ClpX N domain mediate alternative adaptor-binding specificities in different bacterial species

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Abstract: ClpXP, an AAA+ protease, plays key roles in protein-quality control and many regulatory processes in bacteria. The N-terminal domain of the ClpX component of ClpXP is involved in recognition of many protein substrates, either directly or by binding the SspB adaptor protein, which delivers specific classes of substrates for degradation. Despite very limited sequence homology between the *E. coli* and *C. crescentus* SspB orthologs, each of these adaptors can deliver substrates to the ClpXP enzyme from the other bacterial species. We show that the ClpX N domain recognizes different sequence determinants in the ClpX-binding (XB) peptides of *C. crescentus* SspBα and *E. coli* SspB. The *C. crescentus* XB determinants span 10 residues and involve interactions with multiple side chains, whereas the *E. coli* XB determinants span half as many residues with only a few important side chain contacts. These results demonstrate that the N domain of ClpX functions as a highly versatile platform for peptide recognition, allowing the emergence during evolution of alternative adaptor-binding specificities. Our results also reveal highly conserved residues in the XB peptides of both *E. coli* SspB and *C. crescentus* SspBα that play no detectable role in ClpX-binding or substrate delivery.

Keywords: regulated proteolysis; AAA+; adaptor; SspB; ClpX N domain; ClpP

Introduction

Proteolysis of damaged or misfolded proteins by AAA+ proteases is essential for quality control and recycling of amino acids for new protein synthesis. Protein degradation also plays a regulatory role in numerous cellular processes, including responses to DNA damage and cell-cycle progression.[1] Because proteolysis occurs in crowded cellular environments with thousands of potential substrates, it is important to understand how the proper proteins are chosen for destruction. For bacterial systems, peptide signals (called degradation tags or degrons) in substrates and adaptor proteins play central roles in determining the specificity of proteolytic recognition. How adaptor proteins and degrons are recognized by AAA+ proteases is an active area of study, but only a handful of these interactions have been characterized in detail.

ClpX and ClpP assemble to form ClpXP, one of the best understood AAA+ proteases. Most biochemical studies have focused on ClpXP from *Escherichia coli*, a member of the γ-proteobacteria, but orthologs from other bacteria and mitochondria appear to have similar structures and mechanisms.[2–4] ClpP is

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a multisubunit serine peptidase, in which the proteolytic active sites reside within a barrel-shaped structure.[5] ClpX is a hexameric AAA+ enzyme (*A*TPases *a*ssociated with a variety of cellular *a*ctivities), which recognizes substrates and uses cycles of ATP-powered conformational changes to unfold the native protein and to translocate the denatured polypeptide into the proteolytic chamber of ClpP for degradation.[6–8]

ClpX typically identifies substrates by binding to degrons located near the protein termini. For example, when ribosomes stall during translation, the ssrA tag is appended onto the C-terminus of incomplete polypeptides and subsequently targets these failed translation products to ClpXP and other proteases.[6,7] The 11-residue ssrA tag can be recognized directly by ClpX but is also bound by an adaptor protein, SspB, which aids in delivery of substrates to ClpXP.[8] Indeed, adaptor proteins facilitate ClpXP degradation of numerous substrates.[9–11] Each ClpX subunit consists of an AAA+ domain and a ClpX-family-specific N-terminal domain, which binds zinc via a conserved set of cysteine residues and forms a stable dimer.[12,13] ClpX lacking the N domain ($ClpX^{\Delta N}$) can still bind ClpP and power degradation of some substrates,[12,14] establishing that the N domain is not required for the basic enzymatic functions of ClpX. However, $ClpX^{\Delta N}$ fails to recognize certain substrates and does not support degradation mediated by many adaptors.[10,12,14–18]

SspB consists of a dimeric substrate-binding domain, followed by a flexible linker and a C-terminal peptide that binds to the ClpX N domain.[8,15,19–23] By binding to ClpX and specific substrates, simultaneously, SspB increases the local concentration of substrate relative to the protease.[8,11,15,21,24] As a consequence of this tethering-mediated avidity increase, SspB enhances the rate of ClpXP degradation at sub-K_M substrate concentrations. SspB orthologs were first identified in the γ- and β-proteobacteria[8] and were later discovered in α-proteobacteria, including *Caulobacter crescentus*.[25,26] The domain organization and structure of all SspB proteins are similar, but those from α-proteobacteria comprise a distinct and more distant subfamily and are therefore called SspBα. For example, the orthologs from *E. coli* (^{Ec}SspB) and *C. crescentus* (^{Cc}SspBα) share only 16% sequence identity. Nevertheless, ^{Cc}SspBα delivers substrates efficiently to *E. coli* ClpXP (^{Ec}ClpXP).[17] The C-terminal residues of ^{Ec}SspB are known to bind the isolated N domain of ^{Ec}ClpX,[23,27] and a co-crystal structure has been solved (PDB ID: 2DS8) [Fig. 1(A)].[23] The N domain of ^{Cc}ClpXP and the five C-terminal amino acids of ^{Cc}SspBα are also required for adaptor-mediated substrate delivery,[25] suggesting a corresponding binding relationship.

Here, we probe the fine specificity of the interaction of ^{Cc}SspBα with ^{Cc}ClpX and ^{Ec}ClpX. In both cases, the 10 C-terminal residues of ^{Cc}SspBα comprise the ClpX-binding (XB) region that tethers the adaptor to the N domain. Mutational analyses show that seven side chains in ^{Cc}SspBα XB contribute to adaptor-enzyme recognition, and all SspBαs have homologous sequences that maintain the chemical character of these residues. Surprisingly, however, the corresponding XB peptide of ^{Ec}SspB is shorter, shares little meaningful homology, and displays a radically different mutational profile with just a few residues playing major roles in recognition. Again, these features appear to be shared by SspB orthologs in other γ- and β-proteobacteria. Nevertheless, we find that ^{Ec}SspB delivers substrates to ^{Cc}ClpXP for degradation. Thus, the N domains of both ^{Ec}ClpX and ^{Cc}ClpX have the ability to recognize two different XB peptides. Apparently, these domains possess distinct peptide-binding specificities and have adopted alternative but nonexclusive modes of adaptor-binding during the evolution of different bacterial lineages. We also find that some highly conserved amino acids in the XB peptides of ^{Cc}SspBa and ^{Ec}SspB play no obvious roles in substrate delivery or ClpX-binding and suggest that these amino acids may help protect the adaptors from degradation during substrate delivery.

Results

Phylogenetic comparisons suggest use of different adaptor tethering contacts

A multiple sequence alignment of the C-terminal regions of more than 100 SspBα orthologs revealed a conserved block of residues [Fig. 1(B)]. Within this region, ^{Cc}SspBα residues 153, 154, 156, 157, 159, 160, and 161 were most highly conserved. Deletion of a portion of this C-terminal region prevents substrate delivery by ^{Cc}SspBα to ClpXP.[25] Sequence conservation near the C-terminus of SspB orthologs from γ- and β-proteobacteria revealed a very different pattern of homology [Fig. 1(C)], suggesting that SspBα interacts with ClpX in a fashion distinct from their γ and β counterparts.

Previous studies showed that the ^{Ec}ClpX N domain binds to the ^{Ec}SspB XB peptide and is important for efficient substrate delivery by ^{Cc}SspBα,[17,23,27] suggesting that the ^{Cc}SspBα XB region binds directly to the N domains of ^{Ec}ClpX and ^{Cc}ClpX. To test this idea, a peptide consisting of the C-terminal decapeptide of ^{Cc}SspBα preceded by a fluorescent dye and tyrosine was synthesized for binding studies monitored by fluorescence anisotropy. As shown in Figure 1(D), this ^{Cc}SspBα peptide was bound with similar affinity ($K_D \sim 25\ \mu M$) by the purified ^{Cc}ClpX and ^{Ec}ClpX N domains. Thus, the N domains of both ClpX enzymes, which share ~60% sequence identity (Supporting Information Figure 1),

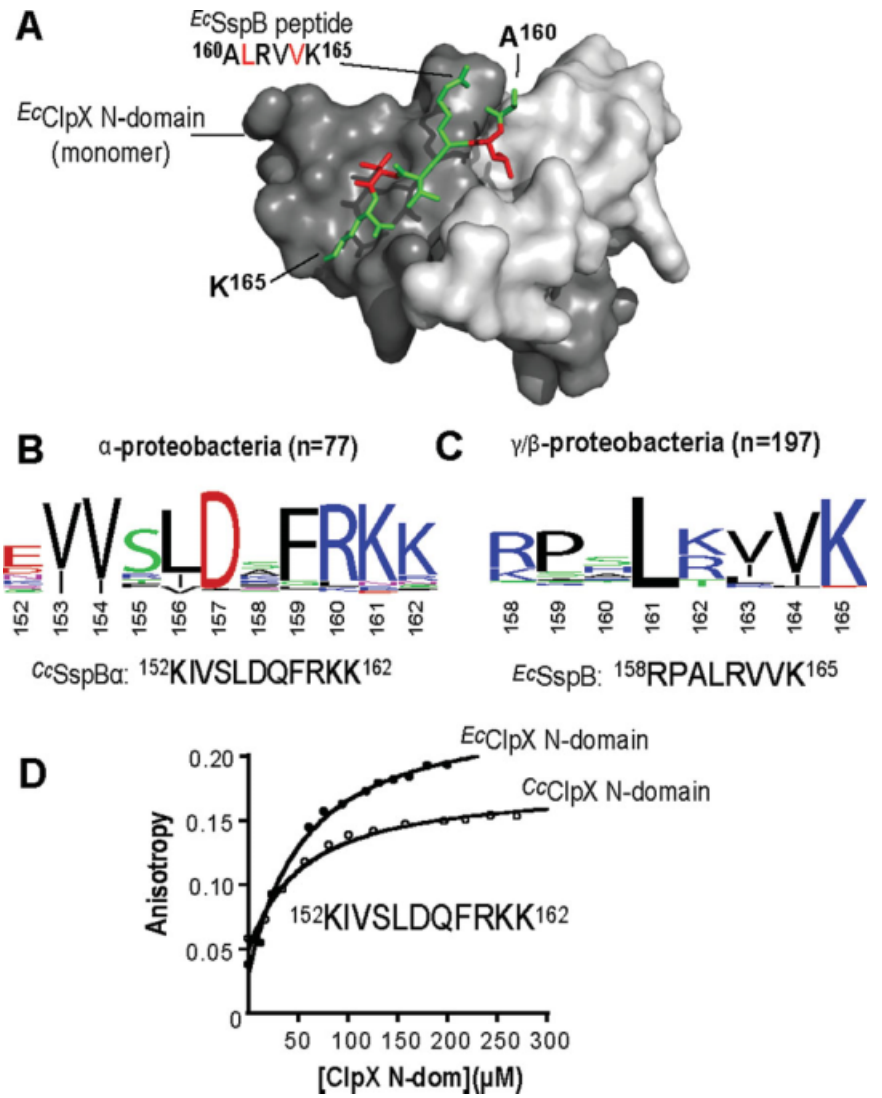


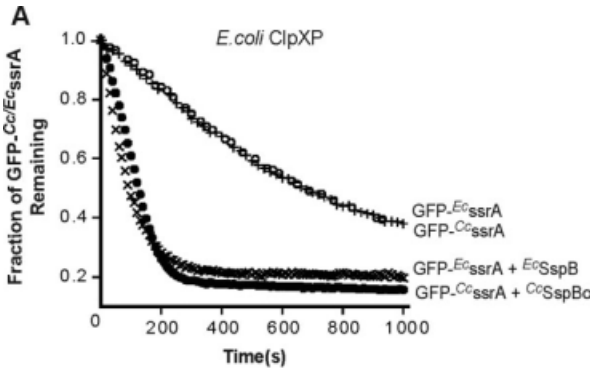
Figure 1. XB conservation and cross-species interaction of SspB and ClpX from α- and γ/β-proteobacteria. A: The structure of the *E. coli* ClpX N-domain dimer bound to the C-terminal peptide of *E. coli* SspB (PDB ID: 2DS8) shows the hydrophobic pockets of the N domain monomers occupied by the L^{161} and V^{164} residues of the peptide.[23] The N-domain monomers are shown in dark and pale gray and the peptide in green with L^{161} and V^{164} highlighted in red. B: Weblogo[28] depiction of sequence conservation within the C-terminal regions of 77 SspB proteins from α-proteobacteria. Alignments were performed using Jalview.[29] The C-terminal region of the α-proteobacteria *C. crescentus* SspB is also depicted. C: Sequence conservation in the C-terminal regions of 197 SspB proteins from γ/β-proteobacteria reveals a very different pattern than observed in panel A. The C-terminal region of the γ-proteobacteria *E. coli* SspB is also shown. D: Binding of the N domains from *C. crescentus* (K_D 25 μ*M*) or *E. coli* (K_D 25 μ*M*) ClpX to a fluorescein-labeled peptide (60 n*M*) corresponding to the XB region of ^{Cc}SspBα.

have the ability to recognize very different XB-peptide sequences.

Adaptor delivery of cognate substrates to ^{Cc}ClpXP and ^{Ec}ClpXP

For studies of adaptor stimulation of degradation, we used green fluorescent protein (GFP) bearing either a *C. crescentus* ssrA tag (AANDNFAEEFAVAA; GFP-^{Cc}ssrA), which binds well to ^{Cc}SspBα, or an *E. coli* ssrA tag (AANDENYALAA; GFP-^{Ec}ssrA), which binds well to ^{Ec}SspB.[17,21] As anticipated,[17,25] ^{Cc}SspBα stimulated degradation of GFP-^{Cc}ssrA by the ^{Cc}ClpXP protease and by the ^{Ec}ClpXP enzyme (Fig. 2). Importantly, ^{Ec}SspB also stimulated degradation of GFP-^{Ec}ssrA by both ^{Cc}ClpXP and ^{Ec}ClpXP [Fig. 2(B,C)]. Thus, despite minimal XB-sequence homology, the adaptors from *E. coli* and *C. crescentus* were both able to stimulate degradation of cognate substrates by the ClpXP enzyme from the other species.

We also constructed a chimera, consisting of the substrate-binding domain of ^{Ec}SspB followed by the ^{Cc}SspBα C-terminal linker and XB region. This chimeric adaptor enhanced ^{Ec}ClpXP degradation of GFP-^{Ec}ssrA [Fig. 3(A)], establishing that tethering interactions mediated by ^{Cc}SspBα XB can replace the interactions normally mediated by ^{Ec}SspB XB.



B

Degradation Rates of GFP-ssrA by ClpXP

Reaction Components	^{Cc}ClpXP (μM sub/min/ClpX$_6$)	^{Ec}ClpXP (μM sub/min/ClpX$_6$)
GFP-^{Cc}ssrA	0.020 ± 0.001	0.053 ± 0.002
GFP-^{Cc}ssrA + ^{Cc}SspBα	0.087 ± 0.003	0.259 ± 0.008
GFP-^{Ec}ssrA	0.013 ± 0.001	0.047 ± 0.001
GFP-^{Ec}ssrA + ^{Ec}SspB	0.071 ± 0.013	0.331 ± 0.002

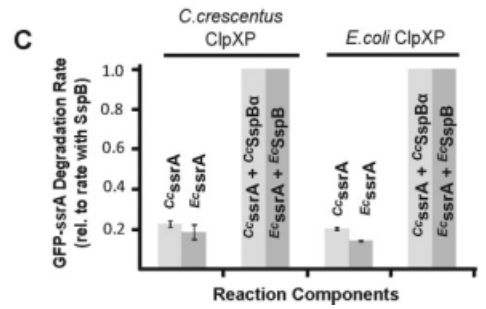


Figure 2. *C. crescentus* and *E. coli* ClpXP interact with both ^{Cc}SspBα and ^{Ec}SspB. A: ^{Cc}SspBα and ^{Ec}SspB (1.2 μ*M*) both enhanced degradation of their cognate GFP-ssrA by ^{Ec}ClpXP as monitored by decreases in fluorescence. ClpXP concentration in each case was 0.1 μ*M* and the substrate concentration was 0.1 μ*M*. This low substrate concentration (sub-K_M) was used to help ensure that degradation was adaptor-stimulated. B: Both ^{Cc}ClpX and ^{Ec}ClpXP were able to degrade GFP containing either the ^{Cc}ssrA or the ^{Ec}ssrA tags. Protein concentrations used were as in (A). The rate of substrate degradation was enhanced by the cognate adaptor SspB. Under the purification conditions used in this study, ^{Cc}ClpX was less active compared to ^{Ec}ClpX. C: Normalized degradation rates of GFP-^{Cc}ssrA (pale gray bars) or GFP-^{Ec}ssrA (dark gray bars) by ^{Cc}ClpXP and ^{Ec}ClpXP in the presence or absence of ^{Cc}SspBα or ^{Ec}SspB; in this case, the adaptor species (Cc vs. Ec) matched that of the ssrA tag sequence on the substrate. Protein concentrations were as described in (A).

To address the importance of residues near the C-terminus of ^{Cc}SspBα, we constructed truncated variants and assayed their adaptor activity. The last two lysine residues ($K^{161}K^{162}$) could be deleted without a major effect on delivery, whereas deletion of additional upstream residues eliminated activity [Fig. 3(B)]. However, the substrate-binding domain of ^{Ec}SspB followed by the C-terminal residues ^{158}QFRKK^{162} of ^{Cc}SspBα was inactive as an adaptor (data not shown), establishing that the ^{158}QFR sequence may be necessary but is not sufficient for adaptor function.

Residues involved in ^{Cc}SspBα tethering to ClpX

To determine which residues in the XB region of ^{Cc}SspBα are important for ClpX binding, we individually mutated the 10 C-terminal residues to alanine and purified these variants. In one set of assays, we tested stimulation of ^{Cc}ClpXP degradation of 0.1 μ*M* GFP-^{Cc}ssrA (Fig. 4). This substrate concentration is below K_M (~1 μ*M*) for unassisted ^{Cc}ClpXP degradation, allowing adaptor-mediated stimulation to be observed. Alanine substitutions at I^{153}, V^{154}, L^{156}, R^{160}, and K^{162} caused the largest defects in substrate delivery [Fig. 4(A)]. Milder effects were observed for substitutions at the other positions, with mutations at S^{155} and D^{157} having essentially no effect on delivery activity. The alanine mutations in ^{Cc}SspBα had generally similar effects on adaptor-mediated degradation by ^{Ec}ClpXP. However, V154A appeared to be more active with the ^{Ec}ClpXP protease than with ^{Cc}ClpXP [Fig. 4(A,B)], suggesting that this residue plays a somewhat different role in binding the two enzymes.

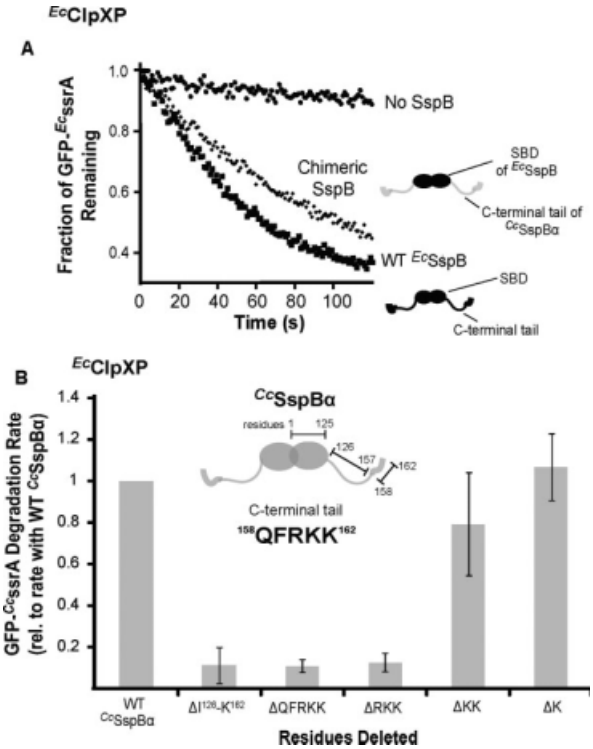


Figure 3. ^{Cc}SspBα interacts with ClpXP via its C-terminal region. A: A chimeric SspB (0.3 μ*M*), obtained by substituting the C-terminal region of ^{Ec}SspB with that of ^{Cc}SspBα, enhanced the degradation of GFP-^{Ec}ssrA (0.1 μ*M*) by ^{Ec}ClpXP (0.05 μ*M*). B: Different segments of the C-terminal region of ^{Cc}SspBα were removed and the variant adaptors tested for their ability to enhance degradation of GFP-^{Ec}ssrA (0.1 μ*M*) by ^{Ec}ClpXP (0.05 μ*M*).

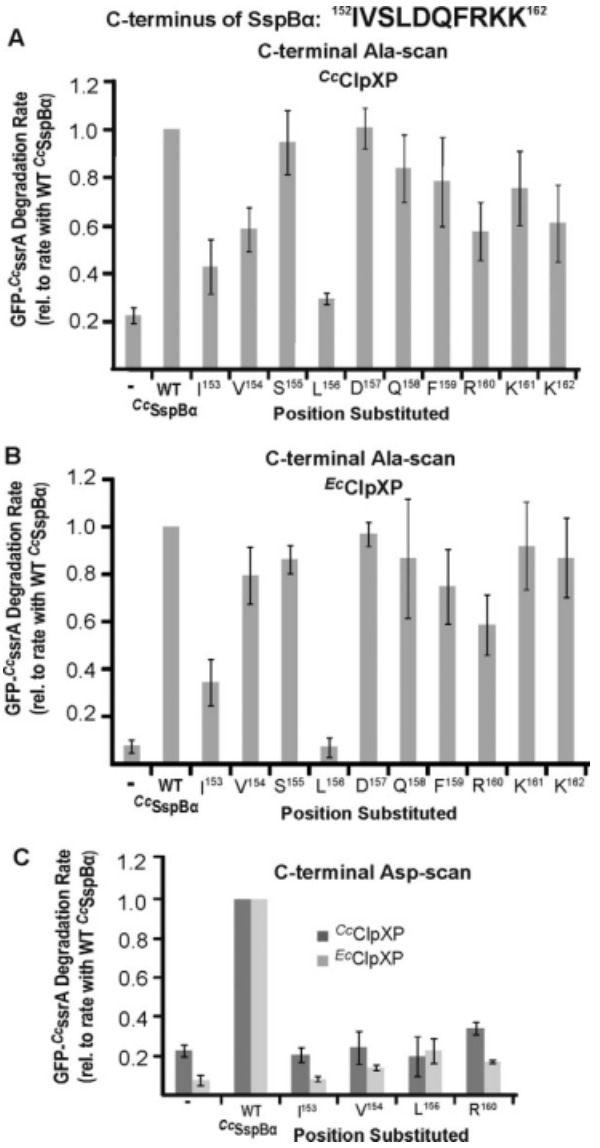


Figure 4. Substrate-delivery activity of ^{Cc}SspBα variants with substitution mutations. A: Variants of ^{Cc}SspBα with Ala substitutions in the C-terminal residues were assayed for their delivery activity by determining how well they enhanced degradation of GFP-^{Cc}ssrA (0.1 μ*M*) by ^{Cc}ClpXP (0.2 μ*M*). All rates were normalized to the degradation rate of GFP-^{Cc}ssrA in the presence of WT ^{Cc}SspBα. Wild-type ^{Cc}SspBα or variants were present at 0.3 μ*M*. B: Analysis is the same as in (A), except that the protease was ^{Ec}ClpXP (at 0.05 μ*M*). Similar results were observed, however the V154A variant appeared to be more active with ^{Ec}ClpXP than with ^{Cc}ClpXP. C: Aspartate-substitutions were made at four positions (I^{153}, V^{154}, L^{156}, and R^{160}) in ^{Cc}SspBα and the variants (0.3 μ*M*) tested for adaptor function with ^{Cc}ClpXP (0.2 μ*M*) and ^{Ec}ClpXP (0.05 μ*M*). The rates were normalized to the degradation rate of GFP-^{Cc}ssrA in the presence of WT ^{Cc}SspBα.

Most alanine substitutions did not reduce activity to the level of unassisted ClpXP degradation, suggesting that these mutations weaken but do not eliminate tethering. To test if more dramatic mutations had larger effects, we also constructed and purified variants in which I^{153}, V^{154}, L^{156}, and R^{160} were changed to aspartic acid. Except for R160D, these substitutions decreased ^{Cc}ClpXP degradation to the level of the no-SspB control when assayed with ^{Cc}ClpXP [Fig. 4(C)]. The aspartate substitutions were also more severe than the alanine substitutions in ^{Ec}ClpXP degradation assays [Fig. 4(C)].

Assays of ^{Cc}SspBα-mediated stimulation of GFP-^{Cc}ssrA degradation have limited dynamic range because this substrate is degraded reasonably well ($K_M \sim$ 1–2 μ*M*) by ClpXP alone [Fig. 5(B)]. To address this concern, we changed the C-terminal residues of this substrate from VAA to DAS (GFP-^{Cc}DAS) [Fig. 5(A)]. This substitution weakens ^{Ec}ClpX recognition of the ssrA tag and increases the adaptor-dependence of degradation.[30] When we assayed ^{Cc}SspBα stimulation of ^{Cc}ClpXP degradation of GFP-^{Cc}DAS, the I153A, V154A, L156A, R160A, and K162A mutations caused substantial reductions in the stimulated degradation rate whereas the D157A, F159A, and K161A substitutions had only modest effects [Fig. 5(C)].

Substitution of alanine for S^{155} or D^{157} in ^{Cc}SspBα did not have a large effect on substrate delivery. It seemed possible, however, that proline substitutions at these positions might interfere with binding to ClpX by disrupting conformations (for example, an α-helix or β-strand) of the entire XB peptide and thus interfering with contacts made by residues flanking these positions. However, proline substitutions at either position caused only minor reductions in the ability of these ^{Cc}SspBα variants to stimulate ^{Cc}ClpXP degradation (Fig. 6). It appears, therefore, that the XB peptide of ^{Cc}SspBα binds in a conformation compatible with the restrictions of the backbone dihedral angle that would be enforced by proline at these positions.

^{Cc}SspBα XB mutations decrease N-domain affinity

We anticipated that the alanine substitutions in the XB region of ^{Cc}SspBα would reduce affinity for the ^{Cc}ClpXP N domain. To test this ideal directly, we synthesized fluorescent XB-peptide variants and assayed binding. Alanine substitutions for I^{153}, V^{154}, L^{156}, F^{159}, R^{160}, and K^{162} decreased affinity to varying extents (Fig. 7, Table I). Substitutions at the remaining positions had very small effects. These results largely mirror the defects in substrate delivery for the corresponding substitutions in full-length ^{Cc}SspBα.

Effects of ^{Ec}SspB XB mutations on substrate delivery and N-domain affinity

As shown in Figure 1(B,C), phylogenetic comparisons reveal very different patterns of sequence conservation for the XB peptides of SspB orthologs from

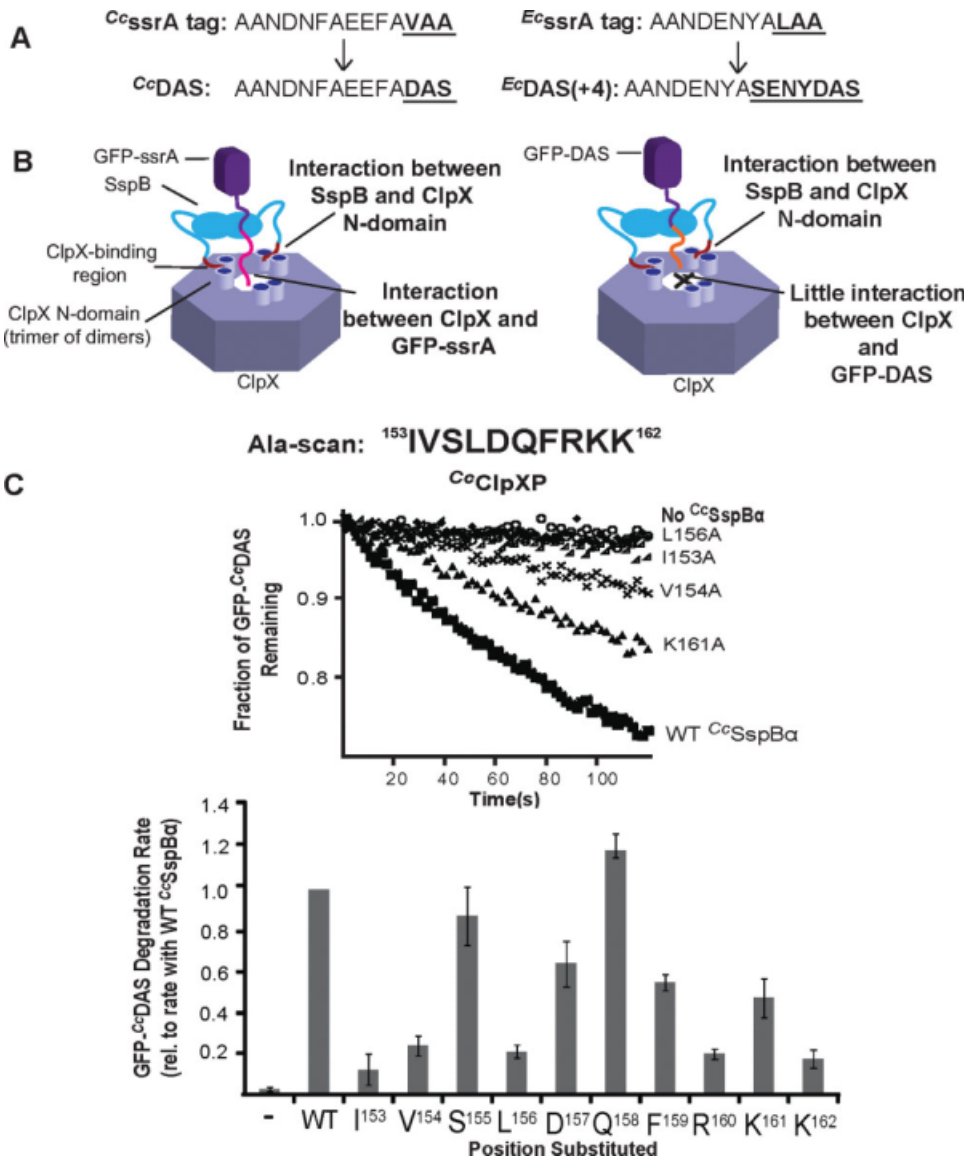


Figure 5. Degradation of GFP-^{Cc}DAS by ^{Cc}ClpXP. A: The ssrA tag in *C. crescentus* (^{Cc}ssrA) consists of 14 amino acids, the last three (VAA) resembling the terminal "LAA" residues of the 11-residue *E. coli* ssrA tag (^{Ec}ssrA). Substituting the last three residues of either tag with the sequence "DAS" made the tag a weaker degron, and thus proteolysis of tagged protein more adaptor-dependent. For ^{Ec}ssrA, the tag was also elongated (to remove an SspB-ClpX clash) to generate the DAS + 4 tag. B: Cartoon depicting ssrA-DAS recognition. The efficiency of substrate degradation depends on the protein-protein interactions occurring in the ternary complex formed between the substrate (GFP-ssrA), the adaptor (SspB), and ClpX (left panel). When the tag is modified such that there is only a weak substrate-ClpX interaction (e.g. the DAS tag), adaptor-ClpX interactions dictate the efficiency of degradation (right panel). C: The ^{Cc}SspBα C-terminal residues were individually changed to alanine and assayed for their ability to enhance degradation of GFP-^{Cc}DAS (0.1 μ*M*) by ^{Cc}ClpXP (0.2 μ*M*). ^{Cc}SspBα variants were present at 0.3 μ*M*. (Top) Degradation traces for representative ^{Cc}SspBα variants. (Bottom) Summary of the alanine-scan results of the C-terminal region of ^{Cc}SspBα. The rates were normalized to the degradation rate of GFP-^{Cc}DAS (0.1 μ*M*) in the presence of WT ^{Cc}SspBα (0.3 μ*M*).

the γ- and β-proteobacteria as opposed to those from α-proteobacteria. To probe the functional importance of residues in the ^{Ec}SspB XB peptide, we purified alanine-substituted variants and assayed their ability to deliver GFP-^{Ec}DAS to ^{Ec}ClpXP. The largest defects were observed for the L161A and V164A variants [Fig. 8(A)]. For comparison, this figure also shows the relative abilities of the same variants to deliver a normal ssrA-tagged substrate, a similar but less sensitive assay.[21]

To determine the effects of the alanine substitutions on the affinity of the ^{Ec}SspB XB peptide for the ^{Ec}ClpX N domain, we carried out peptide-binding experiments [Fig. 8(B)]. The L161A substitution made N-domain binding too weak to measure, the V164A substitution decreased binding approximately

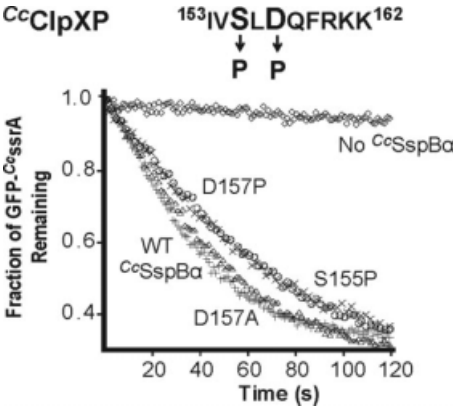


Figure 6. Secondary structure in the ^{Cc}SspBα C-terminal region is not critical. S^{155} or D^{157} (shown in bold in sequence, Top) in the C-terminal region of ^{Cc}SspBα were substituted with proline, which disrupts secondary structure, and the proteins were assayed for their ability to deliver GFP-^{Cc}ssrA (0.1 μ*M*) to ^{Cc}ClpXP (0.2 μ*M*). Neither substitution inhibited adaptor function.

seven-fold, whereas smaller effects were detected for the remaining substitutions. These results agree well with the functional studies. Importantly, they establish that the N domain of ClpX recognizes CcSspBa XB peptides in a substantially different manner than the XB peptides from c- and b-proteobacterial SspB adaptors.

Different XB sequences compete for binding to the N domain

Because ^{Cc}XB and ^{Ec}XB sequences have such distinct features, we sought to determine if they bound to distinct sites in the N domains of ^{Cc}ClpX and ^{Ec}ClpX. Therefore, degradation of GFP-^{Ec}DAS by either ^{Cc}ClpXP or ^{Ec}ClpXP was performed in the presence of ^{Ec}SspB with or without high concentrations of ^{Cc}XB peptide. As shown in Figure 9(A), this peptide inhibited degradation by both proteases, indicating that it competes with ^{Ec}SspB for interaction with these enzymes. Furthermore, GFP-^{Ec}ssrA degradation, which is less adaptor-dependent, was also inhibited by the ^{Cc}XB peptide [Fig. 9(B)]. Importantly, ^{Cc}XB only inhibited reactions in which substrate delivery was promoted by an adaptor (^{Ec}SspB). Thus, these results indicate that the *C. crescentus* and *E. coli* XB peptides bind to the same or overlapping sites on the N domain of ClpX.

Table I. *K_D Values of ^{Cc}SspBα C-Terminal Peptides Binding to C. crescentus ClpX N-Domain (Dimer)*

Peptide	Sequence	K_D (μM)
WT	YKIVSLDQFRKK	26.0 ± 4.8
I153A	YK**A**VSLDQFRKK	>400
V154A	YKI**A**SLDQFRKK	>400
S155A	YKIV**A**LDQFRKK	32.1 ± 2.3
L156A	YKIVS**A**DQFRKK	>400
D157A	YKIVSL**A**QFRKK	50.9 ± 12
Q158A	YKIVSLD**A**FRKK	23.1 ± 3.9
F159A	YKIVSLDQ**A**RKK	66.9 ± 13
R160A	YKIVSLDQF**A**KK	200 ± 82
K161A	YKIVSLDQFR**A**K	39.3 ± 5.4
K162A	YKIVSLDQFRK**A**	303 ± 100

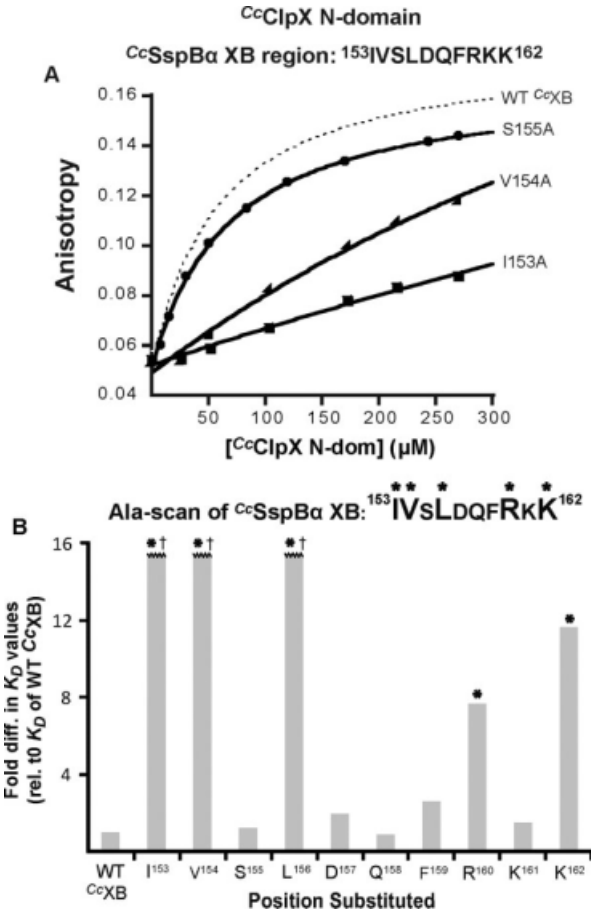


Figure 7. Binding interaction between ^{Cc}SspBα ClpX N domain and ^{Cc}SspBα. A: An alanine-scan of a peptide consisting of the C-terminal residues of ^{Cc}SspBa was done and the peptides were tested for their ability to bind the ^{Cc}ClpX N domain. Binding of the wild-type sequence is shown in dashed gray [see Fig. 1(C)]. Peptide concentration was 60 n*M*. K_D values of all the ^{Cc}SspBα C-terminal peptide variants are shown in Table I. B: Comparison of the change in K_D value caused by the Ala-substitution (relative to the value of WT ^{Cc}SspBα XB). Residues I^{153}, V^{154}, L^{156}, R^{160}, and K^{162} (marked with asterisk) were most important for binding ^{Cc}ClpX N domain. The K_D values of Ala-substitutions at I^{153}, V^{154}, and L^{156} (marked with †) were >16-fold higher than that of WT adaptor and could not be depicted within the scale of the *y*-axis.

Discussion

As previously shown for *E. coli* SspB, the C-terminal residues of *C. crescentus* SspBα mediate its binding to ClpX. Surprisingly, however, phylogenetic conservation and studies of mutant adaptors and XB peptides indicate that ClpX recognizes ^{Cc}SspBα and ^{Ec}SspB in very different ways. For instance, the XB

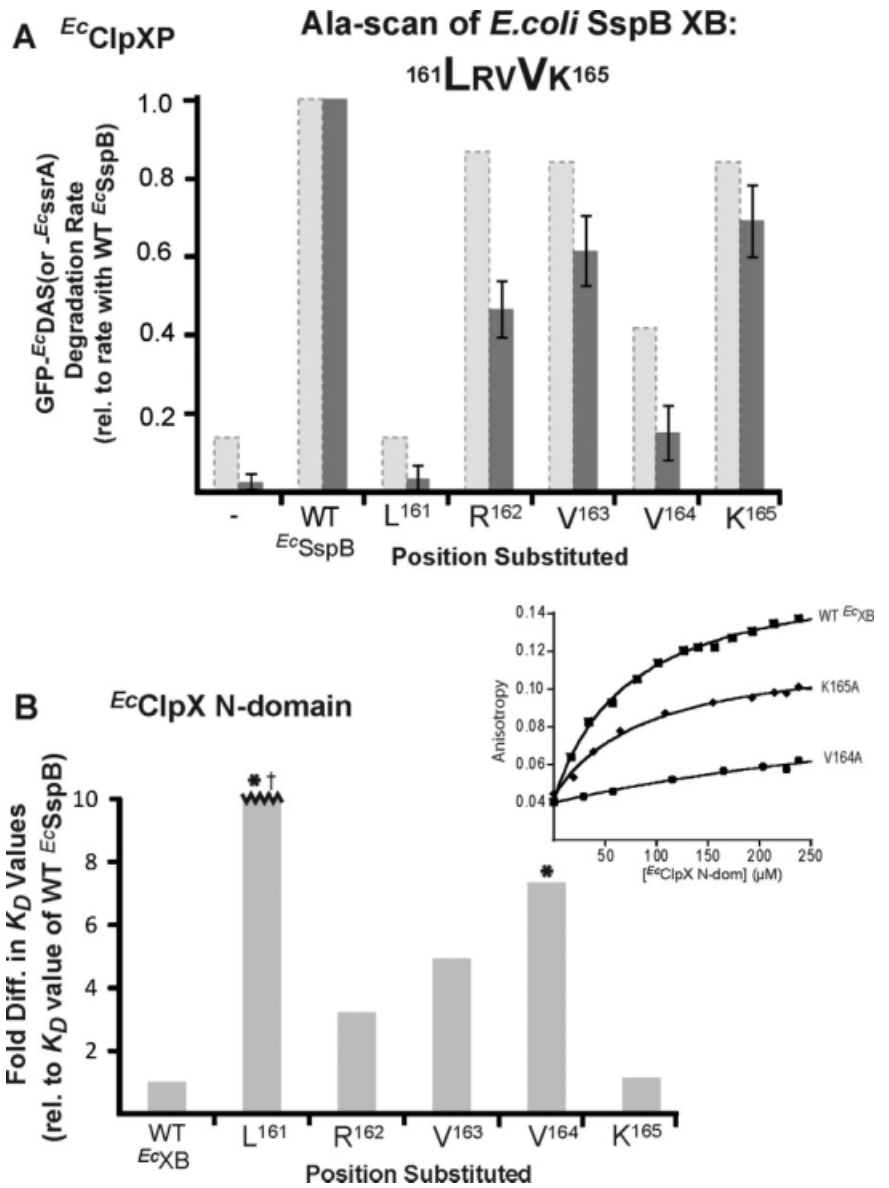


Figure 8. Functional and binding interactions between ^{Ec}ClpX N domain and ^{Ec}SspB. A: The five C-terminal residues of ^{Ec}SspB were changed individually to Ala and the variants tested for function by monitoring degradation of GFP- ^{Ec}DAS (dark gray bars). Reactions contained 0.05 μ*M* ^{Ec}ClpXP, 0.3 μ*M* ^{Ec}SspB variant and 0.1 μ*M* substrate. Rates were normalized to the rate of degradation of GFP-^{Ec}DAS in the presence of WT ^{Ec}SspB. The L161A and V164A variants were the most defective. A similar result was observed by Wah *et al*.[21] who used GFP-^{Ec}ssrA as a substrate to test the function of these Ala-variants (shown in pale gray/dashed bars). As expected, the activities of all variants were higher for degradation of WT GFP-ssrA than the DAS variant. B: Peptides (60 n*M*) corresponding to an alanine-scan of the ^{Ec}SspB C-terminal region were tested for binding to the ^{Ec}ClpX N domain. The K_D values were determined by fluorescence anisotropy (inset). Residues L^{161} and V^{164} (marked with asterisk) were the most important for the interaction. The K_D value of L161A (marked with †) was >10-fold higher than that of WT adaptor and could not be depicted within the scale of the *y*-axis.

regions of orthologs from α-proteobacteria are longer and appear to make many more side chain contacts with ClpX than the XB regions of orthologs from γ- and β-proteobacteria. Nevertheless, the N-terminal domain of ClpX from either *E. coli* or *C. crescentus* is able to bind both XB peptides. As a consequence, both ^{Cc}SspBα and ^{Ec}SspB can deliver cognate substrates to the ClpXP proteases from *E. coli* or *C. crescentus*. These results establish that the N domains of ClpX from both species have at least two distinct peptide-binding specificities. One of these modes of binding appears to be exclusively used for SspBα recognition in the α-proteobacteria, whereas the other seems to be employed for SspB tethering in all γ- and β-proteobacteria.

Mutant studies presented here and previously[21] indicate that just two residues in the XB peptide of ^{Ec}SspB, L^{161} and V^{164} (*L*R*VV*K^{165}), play major roles in ClpX recognition, with the leucine side chain being most important. Because the XB peptide of

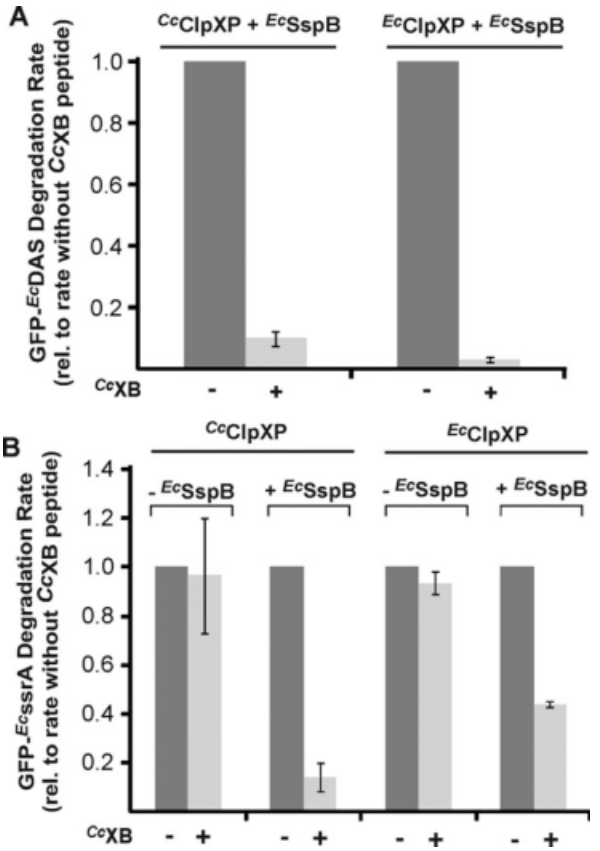


Figure 9. Competition between ^{Cc}SspB XB and ^{Ec}SspB XB motifs for binding ^{Cc}ClpX and ^{Ec}ClpX. A: GFP-^{Ec}DAS (0.1*M*) degradation reactions were set up using either ^{Ec}ClpXP (0.05 l*M*) or ^{Cc}ClpXP (0.2 l*M*) as the protease. When a high concentration of ^{Cc}XB peptide (200 μ*M*) was added, GFP-^{Ec}DAS (0.1 μ*M*) degradation was inhibited. Degradation rates were normalized to that of GFP-^{Ec}DAS in the presence of WT ^{Ec}SspB without any competitor peptide. B: ClpXP-mediated degradation of GFP- ^{Ec}ssrA (0.1 μ*M*) in the presence of ^{Ec}SspB (0.3 μ*M*) was inhibited when 200 μ*M* ^{Cc}XB (YKIVSLDQFRKK) peptide was added to the reaction. This result was observed with ClpXP from both *C. crescentus* and *E. coli*. In the absence of ^{Ec}SspB, inhibition was not observed, indicating that peptide-inhibition was due to disruption of the ^{Ec}SspB-ClpX interaction rather than a direct effect on the enzyme.

^{Cc}SspBα (IVS*L*DQ*F*RKK^{162}) contains a leucine separated by two residues from a phenylalanine, it might be argued that these peptides bind the N domain of ClpX in generally similar ways. However, several results are difficult to reconcile with this model. First, the LxxF sequence in the ^{Cc}SspBα peptide is still present in the ΔRKK chimeric variant, which fails to deliver substrates [see Fig. 3(B)], and in the ΔRKK ^{Cc}SspBα XB peptide, which fails to bind the N domain of ^{Cc}ClpX (data not shown). Second, the crystal structure of a complex of ^{Ec}ClpX N domain with ^{Ec}XB peptide shows that the residue immediately following L^{161} (*L***R***VV*K) adopts dihedral angles that would be inaccessible to proline.[23] By contrast, our results show that the ^{Cc}SspBα XB variant IVS*L***P**Q*F*RKK is active in delivering substrates to ClpXP (see Fig. 6). Third, the side chain of V^{164} in the ^{Ec}SspB XB peptide packs into a hydrophobic pocket in the ^{Ec}ClpX N domain that is too small to accommodate a phenylalanine side chain.[23] It seems most likely, therefore, that these peptides bind the N domain in fundamentally different fashions. However, competition experiments suggest that both XB peptides bind the same or overlapping sites in the ClpX N domain (see Fig. 9). Thus, the same general peptide-binding pocket may have an unusual amount of flexibility in potential modes of binding specificity.

One minor anomaly in analysis of the ^{Cc}SspBα XB region concerns differences between experiments using deletions and alanine substitutions. Specifically, we found that one (K^{162}) or two (K^{161}K^{162}) C-terminal residues could be deleted from the ^{Cc}SspBα XB peptide without causing significant defects in substrate delivery (see Fig. 3). We also synthesized ΔKK and ΔRKK XB peptides and assayed binding to the N domain of ^{Cc}ClpX. The ΔKK peptide bound the N domain with reduced but substantial affinity, whereas almost no binding was detected for the ΔRKK peptide (data not shown). By contrast, alanine substitutions at positions 161 and 162 reduced substrate-delivery activity in some assays [see Fig. 5(C)]. These results could be reconciled if contacts between these lysine side chains and ClpX stabilize the complex, whereas contacts mediated by the peptide backbone of these residues destabilize binding to a roughly comparable extent. Prior studies have also shown that deletion of the C-terminal lysine (K^{165}) of ^{Ec}SspB does not affect substrate delivery.[21] However, the co-crystal structure shows that this lysine side chain makes numerous intimate contacts with the ^{Ec}ClpX N domain.[23] Indeed, based on the structure alone, it would be reasonable to suggest that this lysine plays an important role in ClpX-binding, and yet we detected only a marginal decrease in ClpX N domain affinity when this residue was changed to alanine, indicating that the side chain contacts are not critical.

If the C-terminal lysine residues of ^{Ec}SspB and ^{Cc}SspBα are not needed for binding ClpX or for delivery of substrates for degradation, then why have these residues been conserved in adaptors and most of their orthologs? We propose that these terminal amino acids might function to help protect SspB from degradation. SspB is a dimer, and both C-terminal tails normally bind N domains in the ClpX hexamer.[27] However, ^{Ec}SspB tethered via a single XB tail also functions as an adaptor,[27,31] which would potentially allow the second XB peptide of a dimer to be engaged by the translocation pore of ClpX, leading to degradation of that subunit. However, replacing the C-terminal residue of the ssrA degron with lysine makes it an exceptionally poor

degradation tag for ClpXP.[32] Thus, having lysine at the C-terminus of the XB peptide should minimize inadvertent ClpXP-mediated degradation. Similar considerations may explain the strong phylogenetic conservation of D^{157} in SspBα orthologs [see Fig. 1(A)]. This acidic amino acid is present in more than 95% of all SspBα XB sequences, but we detected no effects of an alanine substitution either in substrate delivery or in ClpX-binding [see Figs. 4(A,B), 5(C), and 7(B); Table I]. Numerous experiments have shown that mutation of residues in degrons to aspartic acid also weakens binding to the translocation pore of ClpX.[7,33] Testing these ideas will require further analysis as the ^{Cc}SspB variants used in this study are not ideally suited for degradation experiments because they carry N-terminal affinity tags.

Why has recognition of the two peptide-binding motifs, exemplified by the XB peptides of ^{Ec}SspB and ^{Cc}SspBα, been retained by ClpX orthologs that no longer need to interact with the other class of adaptor? The obvious possibility is that these binding sites in the ClpX N domain are maintained because they are also used in recognition of other substrates. For example, an *L*RE*I*^{12} sequence in *E. coli* UmuD helps mediate ClpXP degradation[10,34] and is a good match to the β/γ- XB consensus motif. Similarly, a peptide from the λO substrate, which binds to the N domain of ClpX, contains an *L*LA*I*^{56} sequence.[16] Moreover, the C-terminal residues of the phage MuA protein (*L*D*IL*EQN*RRK*KAI^{662}) target it for ClpXP degradation in a partially N-domain-dependent manner and share homology with the ^{Cc}SspBα XB peptide.[12,18,35]

Peptide-binding domains (PDZ, WW, SH2, SH3, PTB, FHA, 14-3-3, EVH1, etc.) are used in modular fashions in an enormous number of biological processes to ensure specificity. In virtually all of these cases, each type of domain has a single binding specificity. Thus, it is somewhat unusual that the N domain of ClpX has at least two peptide-binding specificities. Similarly, the SspB adaptor has more than one binding specificity.[11,17,19,20,33,36] For example, crystal structures show that the peptide-binding groove of ^{Ec}SspB binds to a peptide sequence in the ssrA tag in one way and binds to a nonhomologous recognition sequence in the RseA protein in a completely different fashion. Nevertheless, ssrA and RseA peptides compete for SspB because the binding sites for these peptides overlap.[36] Our competition experiments suggest that the binding sites for the β/γ-XB peptides and α-XB peptides in the ClpX N domain also overlap, although structural experiments will be needed to confirm this surmise.

ClpXP has hundreds of natural substrates, which are recognized via five classes of degrons.[37] Moreover, other AAA+ proteases interact with multiple types of peptide signals to identify the correct substrates.[7,38–45] The AAA+ p97 protein also employs its N domain to interact with disparate sequences in a wide variety of adaptors.[46] The peptide-binding versatility exhibited by the ClpX N domain and SspB ensures that ClpXP can recognize many different substrates and adaptors in different ways but with high specificity. This feature allows ClpXP to carry out quality-control surveillance of a large fraction of the proteome and to participate in numerous regulatory circuits without the need for a single type of degron. Moreover, the ability of ClpXP and other AAA+ proteases to recognize multiple classes of degrons permits the recognition of several weak sequence signals to be coupled via avidity effects. These properties of the system free protein substrates to evolve sequence signals that are both compatible with function and only result in degradation under specific circumstances, such as unfolding, complex dissociation, complex assembly, chemical, or proteolytic modification.[47] Competition of different substrates and/or adaptors for distinct but overlapping binding sites provides an additional level of potential regulation of intracellular proteolysis.

Materials and Methods

Buffers

PD buffer contained 25 m*M* HEPES-KOH (pH 7.6), 5 m*M* KCl, 15 m*M* NaCl, 5 m*M* $MgCl_2$, 0.032% NP-40, and 10% glycerol. The ATP-regeneration system contained 5 m*M* ATP, 50 μg/mL creatine kinase, and 5 m*M* creatine phosphate. Buffers S1, W20, and W500 contained 50 m*M* sodium phosphate (pH 8.0), 300 m*M* NaCl, and imidazole at concentrations of 10 m*M*, 20 m*M*, or 500 m*M*, respectively. For purification of the ^{Cc}ClpX N domain, buffer S1 contained 5 m*M* imidazole and buffers S1 and W20 were supplemented with 10 m*M* β-mercaptoethanol (BME) and 10% glycerol. Buffer A contained 20 m*M* HEPES-KOH (pH 8.0), 150 m*M* KCl, 10% glycerol, and 10 m*M* BME. Buffer S contained 20 m*M* HEPES-KOH (pH 8.0) and 100 m*M* KCl.

Protein and peptide purification

^{Ec}ClpX, ^{Ec}ClpP, ^{Ec}SspB, ^{Cc}ClpX, ^{Cc}ClpP, ^{Cc}SspBα, and GFP proteins bearing the *E. coli* or *C. crescentus* ssrA tags were purified as described.[8,25,35,48,49] GFP-^{Ec}DAS(+4) protein was a gift from J.S. Butler (MIT).

The ^{Cc}SspBα variants with an N-terminal His_6 tag were cloned into a pET28b vector under T7-promotor control and transformed into *E. coli* strain BL21(DE3)/pLysS. The N-terminal His_6-tagged ^{Ec}SspB variants, cloned in pET14b vector, were expressed in BL21(DE3) strains (strains provided by laboratory of RT Sauer). Cells were grown at 37°C to $OD_{600} \approx 0.5$ in Luria-Bertani broth containing 50 μg/mL kanamycin. Protein expression was induced for 2 h by addition of 0.5 m*M* isopropyl β-D-

thiogalactoside. The culture was harvested by centrifugation, resuspended in 10 mL of buffer S1 per liter of initial cell culture, and 1 μL/mL protease inhibitor cocktail set III (Novagen, Madison, WI) was added. Cells were frozen in liquid nitrogen, stored, thawed, and lysed by incubating with lysozyme. The lysate was treated with benzonase nuclease (Novagen), cleared by centrifugation for 20 min at 30,000*g* at 4°C, and incubated with Ni-NTA agarose beads (Qiagen, Valencia, CA) equilibrated in S1 buffer for 1 h at 4°C. The beads were collected by centrifugation, resuspended, and washed sequentially with buffer S1 and buffer W20. Bound protein was eluted in five fractions using buffer W500. Fractions containing SspB variants were identified by SDS-PAGE, buffer-exchanged into buffer S using PD-10 desalting columns (GE Healthcare, Piscataway, NJ), pooled, and the concentration determined by UV absorption at 280 nm ($\varepsilon = 13{,}000\ M^{-1}\ cm^{-1}$).

GFP-^{Cc}DAS was constructed using a Gateway cloning system as previously published[50] and the protocol described earlier used to purify the protein ($\varepsilon = 55{,}000\ M^{-1}\ cm^{-1}$).

^{Cc}ClpX (residues 1–61) and ^{Ec}ClpX (residues 1–64) N domains with cleavable N-terminal His_6 tags were expressed in *E. coli* strains BLR(DE3) (provided by S. Glynn, MIT) and BL21(DE3)/pLysS, respectively, using the protocol described for expression of ^{Cc}SspBα variants. Harvested cells were resuspended in 10 mL S1 buffer plus 10 m*M* BME and 10% glycerol per liter of initial culture and lysed using a French Press (25,000 psi) at 4°C. The protocol for purification of ^{Cc}SspBα variants was then followed up to the wash step. After washing with buffer W20 plus 10 m*M* BME and 10% glycerol, the Ni-NTA beads were resuspended in wash buffer, recombinant thrombin (Novagen) was added, and the mixture was incubated overnight at 4°C to cleave the His_6 tag. The Ni-NTA resin was removed by centrifugation, and the supernatant was chromatographed on a Superdex-75 gel filtration column (GE Healthcare) equilibrated in buffer A. Fractions containing the ClpX N domain were identified by SDS-PAGE, pooled, concentrated using Amicon (MWCO 5k) (Millipore, Billerica, MA) tubes, and the protein concentration was determined by UV absorption at 280 nm.

Fluorescein-labeled peptides corresponding to the XB regions of ^{Cc}SspBα (YKIVSLDQFRKK), ^{Ec}SspB (RGGRPALRVVK), and variants containing single alanine substitutions were synthesized by using FMOC techniques on an Apex 396 solid-phase synthesizer.

Protein degradation assays

GFP substrates (100 n*M*) were incubated with ^{Ec}ClpXP (50 n*M* $^{Ec}ClpX_6$; 100 n*M* $^{Ec}ClpP_{14}$) or ^{Cc}ClpXP (200 n*M* $^{Cc}ClpX_6$; 400 n*M* $^{Cc}ClpP_{14}$) in the presence or absence of adaptor (300 n*M* monomer) at 30°C in PD buffer plus an ATP-regeneration system.[33] Degradation was monitored by decreased fluorescence (excitation 488 nm; emission 511 nm) using a Photon Technology International fluorimeter (Birmingham, NJ). The rates of reaction were determined by the slopes of linear fits to the decrease in fluorescence within the first 10–30 s of reaction. The error bars indicate the standard deviation of three or more independent measurements.

Peptide-binding assay

Fluorescein-labeled ^{Cc}SspBα XB peptides (60 n*M*) were incubated with increasing amounts of ^{Cc}ClpX N domain in buffer A at 30°C, and fluorescence anisotropy was measured using a Photon Technology International fluorimeter (excitation 490 nm; emission 515 nm). The binding of fluorescent ^{Ec}SspB XB peptides to the ^{Ec}ClpX N domain was assayed in the same way. The K_D values from individual experiments were determined by fitting binding data to the quadratic equation determined from the following equilibrium:

$$[\text{ClpX N domain}] + [\text{XB peptide}] \leftrightarrow [\text{ClpX N domain} \cdot \text{XB peptide}]$$

The binding equation used was $y = a + ((b - a)((d + x + c) - (\text{SQRT}((d + x + c)^2 - 4dx)/2d)))$, where y (y-axis) = anisotropy, x (x-axis) = [ClpX N domain], a = anisotropy of free peptide, b = anisotropy when all peptide is bound to the N domain, $c = K_D$, and d = [total peptide].

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