can be differentially regulated in vivo. The rapid expression of A20 is essential for limiting inflammatory responses and the damage those responses cause in multiple tissues.

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A Specificity-Enhancing Factor for the ClpXP Degradation Machine

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Events that stall bacterial protein synthesis activate the ssrA-tagging machinery, resulting in resumption of translation and addition of an 11-residue peptide to the carboxyl terminus of the nascent chain. This ssrA-encoded peptide tag marks the incomplete protein for degradation by the energy-dependent ClpXP protease. Here, a ribosome-associated protein, SspB, was found to bind specifically to ssrA-tagged proteins and to enhance recognition of these proteins by ClpXP. Cells with an *sspB* mutation are defective in degrading ssrA-tagged proteins, demonstrating that SspB is a specificity-enhancing factor for ClpXP that controls substrate choice.

Members of the Clp/Hsp100 adenosine triphosphatase (ATPase) family are hexameric, ring-shaped proteins that catalyze the unfolding of specific target proteins (*1*–*8*). Clp/Hsp100-catalyzed unfolding reactions have been implicated in a variety of intracellular processes, including reactivating heat-damaged proteins during stress, modulating the transformation of prionlike factors, and disassembling or degrading protein complexes involved in transposition, DNA replication, and virulence (*9*, *10*). Many family members also participate directly in protein degradation by unfolding proteins and transporting the unfolded chain to an associated peptidase complex. For example, the ClpX unfoldase associates with the ClpP serine peptidase to form the multiring ClpXP protease (*6*, *11*).

Clp/Hsp100 ATPases appear to recognize their substrates by binding to short, unstructured peptide sequences displayed on otherwise native proteins (*12*–*15*). The best characterized recognition peptide is the ssrA tag, AANDENYALAA, which targets proteins to the ClpX and ClpA ATPases (*7*, *8*, *12*). Despite recent progress in identifying substrates for the Clp/Hsp100 proteins and the peptide signals important for their recognition, no simple sequence code has emerged that marks proteins as a specific substrate for a particular unfolding ATPase. Furthermore, although both ClpXP and ClpAP efficiently degrade ssrA-tagged proteins in vitro, ClpXP is largely responsible for degradation of these proteins in the cell (*12*). These observations suggested that additional cellular factors might serve to modulate substrate recognition in vivo.

Initial evidence for a ClpX-stimulatory factor was observed during purification of *Escherichia coli* ClpX, and a high-salt wash of partially purified ribosomes was found to be especially rich in this activity (*16*). Under conditions where purified ClpXP partially degraded green fluorescent protein carrying an ssrA tag (GFP-ssrA) (*17*), this activity stimulated degradation 10 times or more (Fig. 1A). We purified the stimulatory factor (*18*) until a single major protein of ~20 kD was visible by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). NH_2-terminal sequencing identified this protein as SspB (stringent starvation protein B), a molecule of unknown function that is part of an operon induced by starvation (*19*). To demonstrate that SspB was indeed the stimulatory factor, we overproduced the protein (*20*) and purified it to apparent homogeneity (Fig. 1B).

Rates of GFP-ssrA degradation by ClpXP were determined in the presence or absence of SspB (Fig. 1C). SspB reduced the Michaelis constant (K_m) for this substrate by a factor of 4 to 5, indicating that it enhances productive interactions between ClpXP and ssrA-tagged proteins. SspB also stimulated V_{max} by about 25%. Moreover, SspB stimulated degradation over many enzyme turnovers, did not stimulate degradation of other ClpXP substrates (MuA and λ O), and did not stimulate ClpAP, which also recognizes and degrades GFP-ssrA (*7*). Thus, SspB enhances substrate recognition of ssrA-tagged substrates by the ClpX ATPase in a highly specific manner.

SspB bound specifically to ssrA-tagged proteins (*21*) (Fig. 2). SspB and GFP-ssrA coeluted from a Superose 12 column (Fig. 2A), whereas SspB and untagged GFP eluted as distinct peaks (Fig. 2B). Likewise, the ssrA-tagged NH_2-terminal domain of λ repressor (λ-cI-N-ssrA) was bound by SspB (Fig. 2C). Mutagenesis of the ssrA tag revealed that residues critical for SspB binding were distinct from those recognized by ClpX. Mutations in the YALAA portion of the tag did not prevent SspB binding (Fig. 2, D to F) but severely reduced degradation by ClpXP (*8*, *12*). By contrast, the $Asn^3 \rightarrow Ala$ (N3A) mutation in the AANDEN segment of the tag obliterated binding of SspB (Fig. 3A) and eliminated SspB stimulation of ClpXP degradation without affecting unstimulated degradation (Fig. 3B). Thus, Asn^3 in the ssrA tag is a cardinal determinant of SspB recognition, and binding of SspB to the peptide tag is critical for

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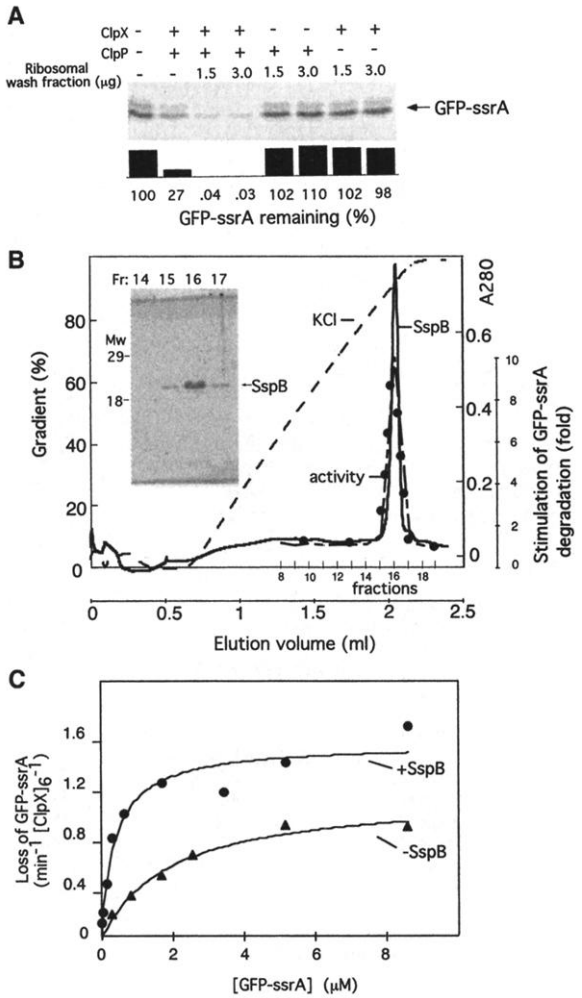

Fig. 1. SspB stimulates ClpXP degradation of GFP-ssrA. (**A**) A ribosome-associated factor stimulates proteolysis of 0.96 μM GFP-ssrA by 0.1 μM $ClpX_6$ and 0.1 μM $ClpP_{14}$. (**B**) MonoQ chromatography of SspB. (Inset) SDS-PAGE of fractions 14 to 17. (**C**) Effect of SspB on GFP-ssrA degradation. Rates were determined as described (*9*) in the absence (K_m = 1.8 ± 0.34 μM, V_{max} = 0.96 ± 0.06 min^{-1}) or presence of 0.24 μM SspB (K_m = 0.40 ± 0.10 μM, V_{max} = 1.44 ± 0.07 min^{-1}). Reactions contained 5 mM ATP, 0.3 μM $ClpX_6$, and 0.8 μM $ClpP_{14}$.

stimulation of degradation. We conclude that SspB recognizes determinants in the AANDEN portion of the ssrA tag, binding adjacent to the region recognized by ClpX.

SspB stimulated degradation of ssrA-tagged proteins in vivo. Pulse-chase experiments were used to determine the rate of degradation of an ssrA-tagged substrate in isogenic *sspB*$^+$ and *sspB*$^-$ strains. These cells carried the wild-type *clpX* allele but were *clpA*− to allow degradation by ClpXP to be specifically measured (*22*). Synthesis of a tagging substrate (λ-cI-N) was induced from a gene with a strong transcriptional terminator before a stop codon; translation of this mRNA results in efficient addition of the ssrA tag to generate λ-cI-N-ssrA (*23*, *24*). In *sspB*$^+$ cells, λ-cI-N-ssrA was degraded with a half-life of ~0.5 min (Fig. 4, A and C), whereas in *sspB*-defective cells its half-life was about 5 min. Over the short time-course of these experiments, no appreciable degradation was observed in the absence of ClpX in either *sspB*$^+$ or *sspB*$^-$ cells (Fig. 4B) (*25*).

Our results demonstrate that SspB binds to ssrA-tagged proteins and increases the efficiency with which they are recognized and degraded by ClpXP in vitro and in vivo. Moreover, the ssrA tag contains distinct sequence determinants important for recognition by SspB and by ClpX. The existence of factors, like SspB, helps explain how individual members of the Clp/Hsp100 family can efficiently recognize substrates with substantially different pep-

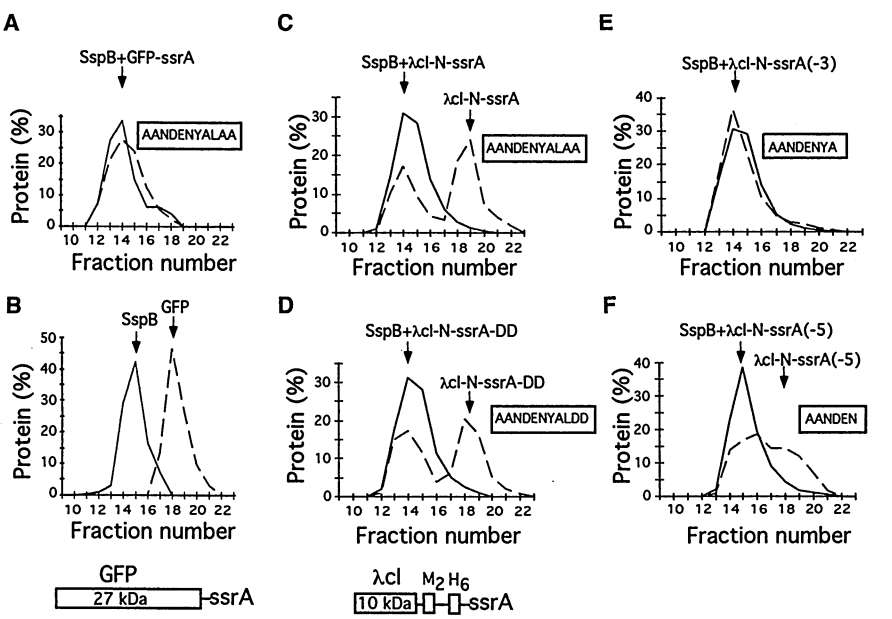

Fig. 2. Gel filtration of SspB·ssrA-tagged protein mixtures. (**A** to **F**) Elution profiles of SspB (solid line) and tagged or untagged GFP or λ-cI-N substrates (dashed line). Control experiments suggest that the unbound λ-cI-N material in (C) and (D) may have lost the ssrA tag by proteolysis during purification. Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; L, Leu; N, Asn; and Y, Tyr.

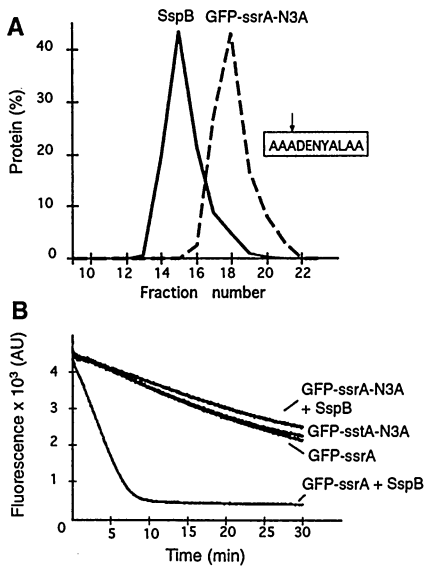

Fig. 3. Asn^3 of the ssrA tag is critical for SspB binding and function. (**A**) SspB and GFP-ssrA-N3A chromatograph independently in gel filtration. (**B**) SspB does not stimulate degradation of GFP-ssrA-N3A by ClpXP. Reactions contained 5 mM ATP, 0.48 μM GFP-ssrA or GFP-ssrA-N3A, 0.02 μM $ClpX_6$, 0.02 μM $ClpP_{14}$, and 0.48 μM SspB where indicated.

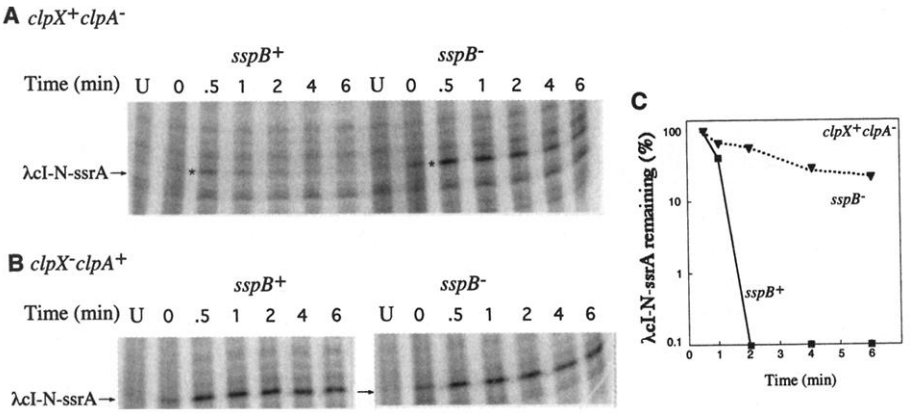

Fig. 4. SspB stimulates intracellular degradation of ssrA-tagged proteins. **(A)** Pulse-chase assays of degradation of λcI-N-ssrA in *sspB*$^+$ and *sspB*$^-$ strains in cells lacking *clpA*. λ-cI-N-ssrA is indicated by the arrow and asterisks. **(B)** Experiment identical to that in (A), except that the strain was *clpA*$^+$ and *clpX*$^-$. **(C)** Quantification of a pulse-chase experiment similar to that in (A).

tide-targeting sequences. The observation that SspB stimulates degradation by ClpXP but not ClpAP also reconciles the facts that ClpXP mediates most intracellular degradation of ssrA-tagged proteins, but both enzymes degrade these substrates with similar efficiencies in vitro (*12*). Overall, SspB functions as a specificity-enhancing factor in two ways: by preferentially stimulating degradation of ssrA-tagged proteins, and directing this specific class of substrates to ClpXP but not to other protease complexes.

SspB is encoded in an operon whose synthesis is stimulated by carbon, amino acid, and phosphate starvation (*19*), suggesting a special role during nutrient stress. Starvation is likely to increase ribosome stalling and thereby increase ssrA tagging (*26*). If starved cells contain higher levels of ribosome-bound SspB, these molecules could be poised to capture tagged proteins as they exit the ribosome, and this complex could then be targeted to ClpXP for degradation. Cells could therefore specifically stimulate degradation of the incomplete protein products of stalled translation, releasing amino acids for productive protein synthesis. Thus, induction of SspB would allow the turnover of individual ClpXP substrates to be differentially regulated. In contrast, inducing synthesis of ClpXP itself during starvation would lead to a global increase in degradation of all substrates, including some whose functions might be important under nutrient stress (*27*). Thus, recognition-enhancing specificity factors, like SspB, both help to explain how diverse substrates can be efficiently recognized by individual Clp/Hsp100 proteins and provide a way to regulate unfolding and degradation of specific protein targets in response to changing cellular environments.

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16. Ribosomes were purified from *E. coli* C600, and associated proteins were extracted with buffer containing 1.5 M NH_4Cl [M. J. Miller, A. Niveleau, A. J. Wahba, *J. Biol. Chem.* **249**, 3803 (1974)].
17. Degradation was performed at 30°C for 20 min in 50 mM Hepes-KOH (pH 7.6), 80 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 0.02% NP-40, and 5 mM ATP. Reactions were stopped with SDS-loading buffer. After electrophoresis on a 12.5% SDS gel, the amount of GFP-ssrA was quantified by fluorimaging of the unstained gel.
18. ClpXP-stimulatory activity was precipitated from the ribosomal wash fraction by ammonium sulfate (35%), collected by centrifugation, resuspended in buffer A [50 mM tris-HCl (pH 7.5), 150 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 10% glycerol], and dialyzed against buffer A. The activity flowed through heparin-Sepharose and blue Sepharose columns and bound to Q-Sepharose (all equilibrated in buffer A). The activity eluted at ~0.41 M KCl, and active fractions were dialyzed against buffer B [50 mM MES-KOH (pH 6.0), 150 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 10% glycerol], loaded onto Q-Sepharose (in buffer B), and eluted with a KCl gradient. Peak fractions (eluting at ~0.28 M KCl) were then applied to Superose 6 column in buffer C [50 mM MES-KOH (pH 5.6), 100 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 10% glycerol]. Active fractions were loaded onto MonoQ in buffer C and eluted with a KCl gradient. After this step, active fractions contained a ~20-kD protein that was identified as SspB by Edman degradation.
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20. The *sspB* gene from *E. coli* DNA was amplified by polymerase chain reaction, cloned into pET3a to generate pET3a-SspB, and transformed into HMS 174. These cells were lysed by lysozyme, and then soluble SspB was precipitated by addition of ammonium sulfate (40%), collected by centrifugation, dissolved, and desalted into buffer D [50 mM tris-HCl (pH 6.8), 100 mM KCl, 1 mM DTT, 10 mM $MgCl_2$, 5% glycerol], and loaded onto a Q-Sepharose column in buffer D. After elution with a 0.1 to 0.45 M KCl gradient, fractions containing SspB were loaded onto a Sephacryl S-100 HR column in buffer D. Finally, SspB was loaded onto a MonoQ column equilibrated with buffer E [50 mM MES-KOH (pH 6.0), 100 mM KCl, 1 mM DTT, 10 mM $MgCl_2$, 5% glycerol] and eluted with a 0.1 to 0.3 M KCl gradient. After this step, SspB was >95% pure as determined by SDS-PAGE stained with Sypro Orange.
21. Complexes of SspB with ssrA-tagged proteins were formed in 40 μl of 50 mM tris-HCl (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 5% glycerol, incubated for 10 min at room temperature, and chromatographed on a Superose 12 column in the same buffer. Fractions were analyzed by SDS-PAGE and fluorimaging of Sypro Orange–stained gels.
22. An *sspB* mutant (IL500) was constructed by transforming strain DY330 [D. Yu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5978 (2000)] with a linear DNA fragment with the *cat* gene cloned in the Bss HII site of *sspB* and selecting for drug resistance. Western blotting revealed no detectable SspB in IL500. This *sspB:cat* allele was P1 transduced into strains SG22099 (*clpA*$^-$) or SG22101 (*clpX*$^-$), to generate strains IL502 and IL503. SG22099, SG22101, IL502, and IL503 cells were transformed with pPW500 (2), which encodes λ-cI-N-trpAt protein. For pulse-chase experiments, an aliquot of cells (optical density at 600 nm ~ 0.4) was induced with 0.6 mM isopropyl-β-D-thiogalactopyranoside for 2 min and pulse-labeled with 125 μCi of ^{35}S-labeling mixture for 10 s. Excess unlabeled methionine was added at time zero, and 0.5-ml samples were withdrawn at appropriate times. ^{35}S-Labeled proteins were analyzed by 10 to 15% gradient SDS-PAGE and phosphorimaging. For Fig. 4C, a Ni–nitrilotriacetic acid pulldown assay of the His_6-tagged λ-cI-N-ssrA substrate [1 hour on ice in 25 mM tris-HCl (pH 7.5), 300 mM NaCl, 0.5% NP-40, and bovine serum albumin (5 mg/ml)] was performed before electrophoresis.
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24. The similar levels of tagged λ-cI-N-ssrA at the two 0.5-min time points in Fig. 4B indicates that the efficiency of ssrA tagging is not substantially altered by the absence of SspB.
25. Although other experiments reveal detectable degradation of ssrA-tagged proteins by ClpAP in vivo, its contribution is modest. Comparison of the degradation rates in *clpX*$^+$ *clpA*$^-$ *sspB*$^-$ and *clpA*$^+$ *clpX*$^-$ *sspB*$^+$ cells (Fig. 4) indicates that the contributions of ClpXP and ClpAP are much more similar in the absence of SspB than in its presence.
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27. SspB orthologs are present in the beta and gamma proteobacteria but appear to be absent in many other bacteria that contain ClpXP and SsrA. Perhaps other proteins replace SspB in these latter species, or SspB might play a largely regulatory role that has evolved to enhance ssrA-mediated degradation.

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