The Role of ClpXP-mediated Proteolysis in Resculpting the Proteome after DNA Damage

by

Saskia B. Neher

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Signature of Author: _____
Department of Biology
(13 May 2005)

Certified by: _____
Tania A. Baker
Professor of Biology
Thesis Supervisor

Accepted by: _____
Stephen P. Bell
Professor of Biology
Chair, Biology Graduate Committee

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ABSTRACT

When faced with environmental assaults, *E. coli* can take extreme measures to survive. For example, starving bacteria consume their own proteins, and bacteria with severe DNA damage introduce mutations into their genomes. These survival tactics require restructuring of the bacterial proteomic landscape. To reshape the proteome, bacteria alter both protein synthesis and protein degradation. For important regulatory proteins and proteins potentially harmful to the cell under non-stress conditions, these changes must be environmentally responsive and specific.

This thesis explores the role of the ClpXP protease in the response to DNA damage. First, we determine how DNA damage affects substrate selection by ClpXP. These experiments combine quantitative proteomics and use of an inactive variant of ClpP to "trap" cellular ClpXP substrates and compare their relative levels with and without DNA damage. Analysis of trapped substrates reveals that cellular stress can result in dramatic changes in protease substrate selection. Next, we explore a specific mechanism that allows coupling of an environmental signal to a change in proteolysis. When the cell senses DNA damage, it triggers autocleavage of the LexA repressor. Autocleavage creates new signals for ClpXP recognition, ensuring the timely degradation of the LexA cleavage products. The mechanism of LexA recognition became a model for cleavage-dependent recognition of other substrates. Finally, we determined the mechanism of ClpX recognition of a known, damage-inducible substrate, UmuD'. We find that UmuD directs UmuD' degradation in an SspB-like manner. These experiments show how, with the right sequence motif, an interacting partner can become a ClpXP delivery factor.

This thesis work contributes to the idea that the bacterial cell has an imperative to degrade certain stress response proteins. Substrate priorities may change throughout the stress response and cellular proteases have devised a variety of strategies to ensure selection of the right substrate at the right time with respect to cellular conditions. This allows the cell to put its best proteome forward as it meets repeated cycles of environmental stress.

Thesis supervisor: Tania A. Baker
Title: Professor of Biology

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1:
INTRODUCTION

INTRODUCTION OVERVIEW

Escherichia coli is remarkably adaptable bacterium that can survive in a variety of harsh environmental conditions. This adaptability is in part due to a number of specific stress responses evolved to optimize survival in suboptimal circumstances. The *E. coli* reaction to DNA damage, the "SOS" response, is a paradigm for environmental stress responses. In the SOS response, recognition of damage to the genome is coupled to upregulation of a specific set of genes with roles in repairing, and if necessary tolerating, DNA damage.

Because environmental stresses perturb the cellular system, they present an excellent opportunity to explore cellular regulation at multiple levels. Changes in cellular mRNA levels following DNA damage are extensively characterized. However, it is protein availability that ultimately affects cellular processes, so it is important to look beyond the transcriptome. How does the cell fine-tune the proteome? Protein levels depend on the balance between protein synthesis and degradation. Unlike eukaryotes where post-translational addition of ubiquitin converts a protein to a protease substrate, in *E. coli* protease substrates are generally recognized directly by built-in sequence motifs. This makes coordinating degradation of specific substrate proteins with cellular or environmental cues a challenge.

The work described in this thesis explores the role of proteolysis in the *E. coli* response to DNA damage. This introduction first considers the challenges that damage to the genome creates for the cell, and reviews the DNA damage response. Additionally, it covers the energy dependent proteases in *E. coli* with a special emphasis on ClpXP. Finally, some specific examples of regulatory mechanisms for the degradation of stress-responsive proteins are given. These examples reveal the importance of proteolytic control over protein levels during environmental stress responses. Furthermore, they highlight the ingenious methods that *E. coli* utilizes to ensure degradation of the right protein at the right time.

THE DNA DAMAGE RESPONSE

DNA Damage and Repair Overview

Maintaining the structural and sequence integrity of its single chromosome is essential for *E. coli's* survival. If not repaired, damage to DNA can result in replication arrest and cell death. All biological macromolecules are chemically labile, and cellular DNA damage is accelerated by challenges from a variety of exogenous and endogenous sources. For example, cells growing in normal physiological conditions suffer from hydrolytic base loss, base damage by hydroxyl radicals and damage by other endogenous genotoxic agents (Lindahl, 1993). One of the best-studied exogenous sources of damage to DNA is UV irradiation. Cells exposed to UV irradiation form a number of specific photoproducts. Pyrimidine dimers constitute the major photoproduct upon exposure to UV light (Ravanat et al., 2001). Less common photoproducts include adenine-containing dimers, deaminated cytosines and oxidated guanines (Ravanat et al., 2001). Furthermore, a large number of environmental toxins can result in a range of DNA lesions, from double stranded breaks to base modification by bulky adducts (reviewed in Freidberg et al., 1995).

To respond to these threats to the genome, organisms encode a number of proteins that function to repair and tolerate DNA damage. Several repair processes, and in some cases specific repair proteins, are conserved throughout the bacterial, archaeal, and eukaryotic superkingdoms. For example, recombinational mechanisms for double-stranded break repair are remarkably similar in these three superkingdoms (Cromie et al., 2001). A central protein in this process, RecA (Rad51), has homologs from bacteria to man (Bianco et al., 1998). The process of translesion synthesis, which allows strand extension across template lesions, is present in prokaryotes and eukaryotes. Translesion synthesis utilizes the Y-family of specialized, structurally similar polymerases (Yang, 2003). Although many repair and tolerance processes are similar in different organisms, bacteria have a specific regulatory circuit to identify and counteract DNA damage called the SOS response.

The SOS Response

The SOS response links a signal of DNA damage (exposed single stranded DNA) to a multipronged attack aimed to repair damage and ensure cell survival. The SOS model is clearest when laid out in full detail (Figure 1.1). Therefore, a brief overview of the current model is presented prior to a review of the development of this model (reviewed in Freidberg et al., 1995). In an undamaged cell, LexA, a dimeric transcription factor, represses a set of about 30 genes (the SOS regulon) with roles in repairing and tolerating damage (Table 1.1). LexA has two domains, a DNA binding and a dimerization domain. Following DNA damage, exposed single stranded DNA activates RecA. Activated RecA stimulates LexA autocleavage activity, resulting in separation of the dimerization and DNA binding domains. This relieves repression and results in upregulation of genes in the LexA regulon. As damage is repaired, less RecA is in the active, ss-DNA bound form. LexA controls its own expression, so newly synthesized LexA can restore repression of the SOS regulon.

Development of this model was complicated by several factors. First, given the number and diversity of genes under *lexA* and *recA* control, mutations in either gene cause pleotrophic phenotypes. Furthermore, several different classes of mutations for both genes exist. For example, *lexA* mutations can be uninducible or constitutively active. Mutants in *recA* can be completely defective for LexA activation, partially defective, constitutively inducible or conditionally inducible. Additionally, *recA* has roles in both activating LexA and in homologous recombination. Finally, both *lexA* and *recA* are under *lexA* control.

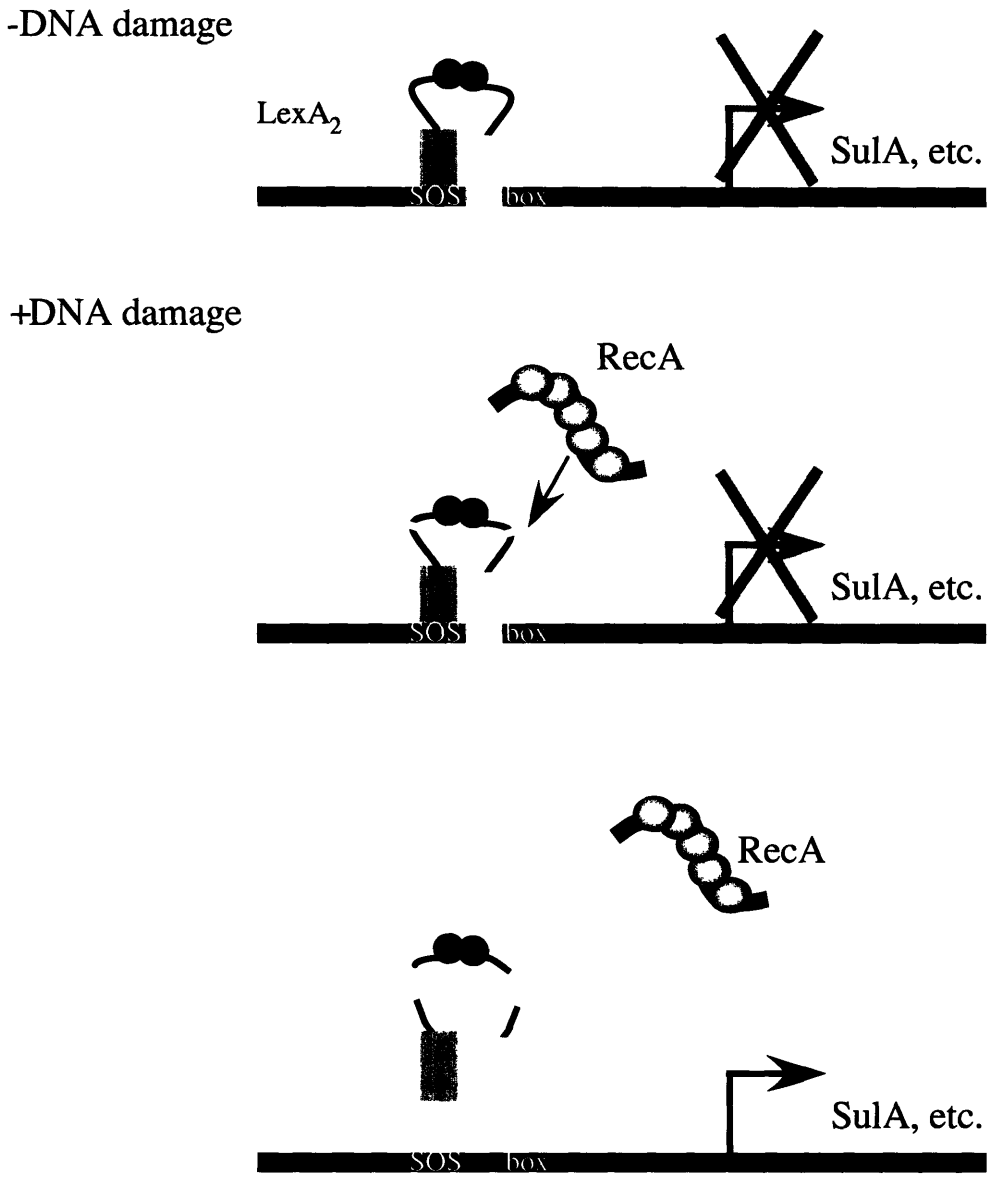


Figure 1.1: The SOS response

LexA is a dimeric transcription factor that normally represses expression of the SOS regulon. Following DNA damage, LexA autocleavage activity separates its DNA binding and dimerization domains to relieve transcriptional repression.

Table 1.1: Known Members of the SOS Regulon

Gene	Function	Reference
fsK	cell division	(Dorazi and Dewar, 2000)
dinB	Y family DNA polymerase	(Goodman and Tippin, 2000)
dinD	unknown	(Kenyon and Walker, 1981)
dinG	helicase	(Voloshin et al., 2003)
dinI	modulates RecA function	(Lusetti et al., 2004)
dinQ	unknown	(Fernandez De Henestrosa et al., 2000)
dinS	possible transposase	(Fernandez De Henestrosa et al., 2000)
hokE	toxin	(Fernandez De Henestrosa et al., 2000)
lexA	transcriptional repressor	(Brent and Ptashne, 1980)
molR	molybdate regulator	(Lee et al., 1990)
recA	SOS regulation/recombination	(Gudas and Mount, 1977)
recN	double strand break repair	(Kosa et al., 2004)
ruvAB	Holliday junction branch migration	(Benson et al., 1988)
sbmC	DNA gyrase inhibitor	(Nakanishi et al., 2002)
ssb	ss-DNA binding protein	(Brandsma et al., 1983)
sulA	cell division inhibitor	(Lee et al., 1990)
umuC	SOS mutagenesis	(Bagg et al., 1981)
umuD	SOS mutagenesis	(Bagg et al., 1981)
uvrA	nucleotide excision repair	(Kenyon and Walker, 1981)
uvrB	nucleotide excision repair	(van den Berg et al., 1981)
uvrD	nucleotide excision repair	(Finch and Emmerson, 1983)
ybfE	unknown	(Fernandez De Henestrosa et al., 2000)
ydjM	unknown	(Fernandez De Henestrosa et al., 2000)
ydjQ	UvrC like	(Fernandez De Henestrosa et al., 2000)
yebG	unknown, chaperone like	(Lomba et al., 1997)
yigN	unknown	(Van Dyk et al., 2001)
yjiW	possible restriction/alleviation	(Fernandez De Henestrosa et al., 2000)
ysdAB	unknown	(Fernandez De Henestrosa et al., 2000)

Historical perspective

The "SOS" hypothesis postulated that *E. coli* has a set of diverse but coordinately regulated functions that are induced in response to treatments that damage DNA (Radman, 1974; Radman, 1975). This hypothesis brought together a number of seemingly unrelated UV-associated phenomena (reviewed in Witkin, 1987). First, UV exposure of host *E. coli* cells enhanced the survival of UV irradiated phage (Weigle reactivation), and was necessary for phage mutagenesis (Weigle mutagenesis) (Weigle, 1953). This phage mutagenesis was proposed to be related to the mutagenic repair seen in bacteria exposed to DNA damaging agents (Kondo, 1973; Radman, 1974; Radman, 1975). Additionally, treatments that damaged DNA resulted in prophage induction in lysogenic bacteria (reviewed in Witkin, 1976). Furthermore, a number of parallels between prophage induction and the filamentous growth observed in UV-induced bacteria were previously noted (Witkin, 1967). One common factor linking these functions was the involvement of *lexA* and *recA* (Radman, 1975).

Identification of a particular temperature sensitive mutant, *tif-1*, further linked these phenomena (Kirby et al., 1967). At the nonpermissive temperature, these mutants showed prophage induction, filamentous growth, Weigle reactivation and Weigle mutagenesis without exposure to DNA damaging agents (Castellazzi et al., 1972a; Castellazzi et al., 1972b). Additionally, mutagenesis of bacteria grown at the nonpermissive temperature was higher in a *tif-1* strain than in a wild-type strain (Witkin, 1974). Furthermore, following UV exposure, mutation frequency in the thermally induced *tif-1* strains had a linear relationship with UV dose while mutation frequency in a wild-type strain showed a quadratic relationship with UV dose (Witkin, 1974). These observations suggested the need for two independent events—one to induce the signal and one to damage the DNA.

The *tif-1* mutation mapped near *recA*, and was ultimately determined to be an allele of *recA* by two-dimensional gel electrophoresis (Castellazzi et al., 1972b; Emmerson and West, 1977; Gudas and Mount, 1977). Using a newly isolated constitutively active allele of *lexA*, these experiments indicated that *recA* was under *lexA* control (Gudas and Mount, 1977; Mount, 1977). From these and other experiments a model for SOS regulation emerged: *lexA* is a repressor of *recA, lexA* and other DNA

repair genes (Brent and Ptashne, 1980; Gudas and Pardee, 1975). Important clues came from the study of the repressor of bacteriophage λ. These studies showed that the cause of RecA-dependent induction of λ phage is inactivation of λ repressor by RecA-mediated proteolytic cleavage (Roberts and Roberts, 1975; Roberts et al., 1978; Roberts et al., 1977). These studies were extended to LexA, and ssDNA was shown to activate RecA to induce LexA autocleavage (Little et al., 1980; Phizicky and Roberts, 1981).

Members of the LexA regulon were identified by a variety of methods. Initially, derivatives of phage Mu containing promoterless *lac* genes were used to identify damage inducible (*din*) genes (Kenyon and Walker, 1980). This study yielded a handful of operon fusions that showed increased β-galactosidase activity following treatment with a DNA damaging agent. Additional members of the LexA regulon were identified by computational methods that search for consensus LexA binding sites (Fernandez De Henestrosa et al., 2000; Lewis et al., 1994). More recently, microarray studies have been used to understand *lexA*-depedent changes in transcription following DNA damage (Courcelle et al., 2001).

The most comprehensive descriptions of the *E. coli* response to DNA damage are at the transcriptional level. Three studies use DNA microarrays to describe changes in transcript level following treatment with DNA damaging agents (Courcelle et al., 2001; Khil and Camerini-Otero, 2002; Quillardet et al., 2003). Results from these studies do not coincide perfectly. For example, some studies report changes in a very large (1000+) number of the genes studied whereas others report more conservative (~300) changes (Khil and Camerini-Otero, 2002; Quillardet et al., 2003). Furthermore only 5 of approximately 30 known damage-inducible genes were identified in all three studies. However, when taken individually, some experiments very accurately show damage-inducible changes in known members of the SOS regulon (Courcelle et al., 2001). Most discrepancies are likely due to the different strains, damaging treatments, array conditions and statistical methods used in the three studies. Despite inconsistencies, these studies provide a framework for exploring large-scale damage-induced transcriptional changes.

Further characterization of the response to DNA damage comes from a limited number of proteomic studies using two-dimensional gel electrophoresis. These studies were mainly used to confirm the identity of the protein responsible for a particular mutant

phenotype (Gudas and Mount, 1977; McEntee, 1977). However, one proteomic study aimed at larger scale identification of damage-induced proteins (Finch et al., 1985). The difficulty of identifying proteins in specific gels spots impeded this effort, but a few known damage inducible proteins were assigned to gel spots whose intensity changed as a result of DNA damage (Finch et al., 1985). In general, characterization of the DNA damage response at the proteomic level lags behind the relatively thorough characterization at the transcriptional level. Because transcript levels are not always a good indication of protein abundance (Griffin et al., 2002), further characterization of the DNA damage response using proteomic techniques is an attractive opportunity.

One notable result from these transcriptional and proteomic studies is that, rather than being an all-or-nothing switch, the response functions as a continuum. For example, transcriptional profiles taken at various times after DNA damage reveal that some genes are upregulated immediately while others are upregulated later in the response (Courcelle et al., 2001). Furthermore, at the same dose of a DNA damaging agent, members of the SOS regulon are induced to different extents (reviewed in Freidberg et al., 1995). These differences are generally attributed to relative promoter strength and position and strength, number and location of LexA binding sites (Schnarr et al., 1991). They allow the cell to respond incrementally dependent on the severity of DNA damage.

The tight, environmentally responsive control that is maintained over transcription is likely to be extended to protein levels. Studies of individual proteins provide anecdotal evidence that this is the case. Furthermore, loss of specific proteases results in increased sensitivity to DNA damage. A major goal of this thesis is to advance our understanding of how proteolysis contributes to this stress response.

PROTEOLYSIS IN *E. COLI*

Protease Overview

Living cells and their components are in a constant state of turnover. In the cell, proteolysis serves many purposes. Protein degradation eliminates damaged, incomplete and misfolded proteins. Degradation of unnecessary proteins provides needed amino acids during nutritional stress. Furthermore, protein degradation can regulate cellular processes by terminating the activity of key substrates. Proteolysis of individual substrates must be rapid, complete and specific. Examination of the proteases in *E. coli* reveals how their structure and mode of action meet these mandates.

E. coli has five energy-dependent proteases: Lon, FtsH, HslUV, ClpXP and ClpAP (reviewed in Gottesman, 1996). Each protease has distinct substrate preferences, but certain substrates are degraded by more than one protease. Non-substrate proteins are protected from degradation through the use of common architectural features that sequester the protease active sites. Each protease has at least one ATPase that unfolds substrates and allows entry to an otherwise inaccessible peptidase chamber where degradation occurs. Lon and FtsH combine the protein unfoldase and peptidase activities in a single polypeptide chain while ClpAP, ClpXP and HslUV divide the activities between two subunits. An in-depth description of the focus of this thesis, ClpX, is followed by a brief description of *E. coli's* other ATP-dependent proteases.

The ClpXP protease

Studies using electron microscopy and crystallography reveal the basic organization of the ClpXP complex (Figure 1.2). The ATPase subunit, ClpX, is a ring shaped hexamer (Grimaud et al., 1998). ClpP is composed of two 7-membered rings that stack together to make a barrel-shaped chamber (Flanagan et al., 1995; Kessel et al., 1995; Wang et al., 1997). Its 14 proteolytic active sites face the inside of this chamber (Wang et al., 1997). The ClpX hexamer stacks atop the ClpP barrel, and the two subunits collaborate to destroy substrate proteins. ClpX recognizes, unfolds and translocates substrate proteins into ClpP for degradation and release of resultling peptides (Kim et al., 2000).

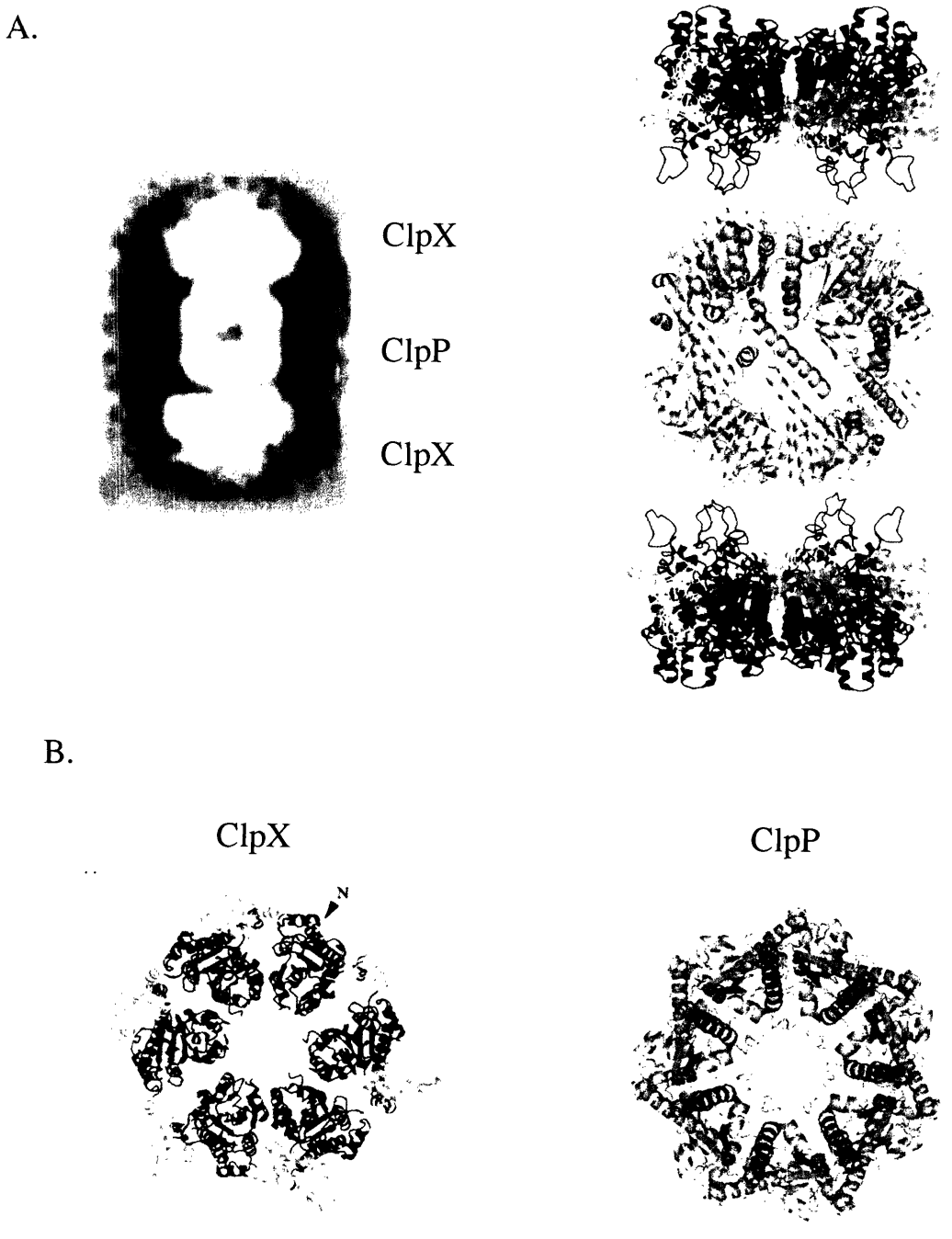


Figure 1.2: Structure of ClpXP

A. Averaged electron microscopy image of ClpXP, and crystal structures giving side views of the ClpP and modeled ClpX multimers (Grimaud et al., 1998; Kim and Kim, 2003; Wang et al., 1997).

B. Top view of ClpX and ClpP (Kim and Kim, 2003; Wang et al., 1997).

Proteolysis is irreversible and energetically expensive (Kenniston et al., 2003b). Therefore, the crucial step in protein degradation is substrate selection. In the ClpXP protease, ClpX recognizes substrate proteins by short peptide motifs located at their N or C-termini. Early indications of the importance of C-terminal residues for ClpX recognition came from deletion mutants of the phage Mu transposase, MuA (Levchenko et al., 1995). Variants lacking the last 4 or 8 amino acids were poor substrates for ClpXP degradation. The identity of C-terminal residues also proved important in destabilizing variants of the Mu repressor (Laachouch et al., 1996). Furthermore, the discovery of the ssrA tagging system showed that destabilizing C-terminal residues can be added cotranslationally to nascent polypeptides stalled on the ribosome (Keiler et al., 1996; Tu et al., 1995). The extreme C-terminal residues of the 11 amino acid ssrA tag (AANEDENYALAA) are especially important for targeting tagged proteins to ClpXP or ClpAP for destruction (Flynn et al., 2001; Gottesman et al., 1998). ClpX can also recognize sequence motifs located at the N-terminus. For example, deletion of the first 18 amino acids of λO protein dramatically slows its degradation by ClpXP (Gonciarz-Swiatek et al., 1999).

Although this handful of known proteins provided clues about substrate selection, identification of a large set of ClpXP substrates through use of a ClpP "trap" allowed consensus N- and C-terminal recognition motifs to emerge (Flynn et al., 2003). Cellular substrates are stably trapped, not degraded, when an active site mutant of ClpP ($ClpP^{trap}$) is expressed in the cell (Figure1.3). The $ClpP^{trap}$ and enclosed substrates are purified using a tandem affinity tag and the substrates are identified by mass spectrometry. Analysis of the sequence from over 50 trapped substrates revealed three classes of N-terminal recognition motifs and 2 classes of C-terminal recognition motifs (Figure 1.4) (Flynn et al., 2003).

In addition to intrinsic recognition of particular substrate sequences, use of adaptor/delivery proteins can dramatically enhance the affinity of ClpX for a substrate. When acting as delivery factors, these proteins enhance the degradation of specific substrate proteins but are not themselves degraded. Regulation of the presence or absence of delivery proteins could allow conditional substrate degradation. ClpX has three known delivery proteins: SspB, RssB, and UmuD (Gonzalez et al., 2000;

Levchenko et al., 2000; Zhou et al., 2001, Neher et al., 2003b) The most thoroughly characterized of these proteins, SspB, mediates the formation of a delivery complex comprised of a SspB dimer, two molecules of ssrA-tagged substrate, and one ClpX hexamer (Levchenko et al., 2000; Wah et al., 2002b). SspB uses its substrate binding domain to interact with a specific sequence in the ssrA tag (Flynn et al., 2001; Wah et al., 2002b). It then uses a peptide motif (the XB motif) in its flexible C-terminal region to interact with the ClpX N-terminal domain and bring these substrates into proximity with ClpX (Bolon et al., 2004; Dougan et al., 2003a; Wah et al., 2003b; Wojtyra et al., 2003a). These multiple contacts between substrate, delivery protein and unfoldase work together to enhance the degradation of ssrA-tagged substrates.

Once a substrate is selected, it must be unfolded before it can pass though the narrow pore of ClpP for degradation (Wang et al., 1997). ClpX uses the energy of ATP hydrolysis to denature even very stable substrates and translocate them into ClpP (Kenniston et al., 2003b; Kim et al., 2000; Singh et al., 2000). Substrate unfolding begins at the recognition tag, so the structure immediately adjacent to the tag affects degradation (Lee et al., 2001). Studies on a series of variants of titin revealed that destabilization of areas near the degradation tag reduced total ATP consumption and accelerated degradation (Kenniston et al., 2003b). These studies also revealed that the rate of ATP consumption was constant during denaturation regardless of substrate stability, suggesting that ClpX repeatedly attempts substrate denaturation until it succeeds.

The unfolded proteins translocated into ClpP are substrates for degradation. In the absence of an ATPase, ClpP can only act on small peptides (Woo et al., 1989). ClpP is a serine protease that uses a conserved serine, histidine, aspartic acid catalytic triad to cleave protein substrates (Maurizi et al., 1990a; Wang et al., 1997). ClpP seems to have a preference for cleaving after nonpolar and hydrophobic residues and produces small (>20 amino acid) peptides (E. Oakes, unpublished, Thompson and Maurizi, 1994).

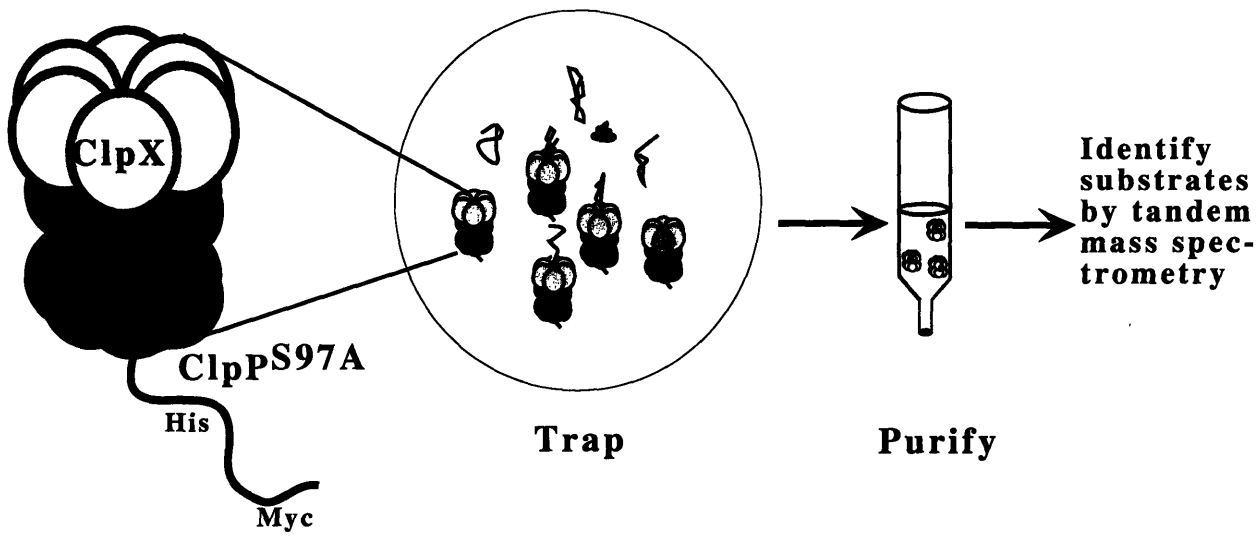


Figure 1.3: Use of ClpP^{trap} for *in vivo* substrate capture

When an inactive, epitope tagged variant of ClpP is expressed in the cell, substrate proteins are not degraded but are stably trapped. The trap and associated substrates can be purified and identified by mass spectrometry (Flynn et al., 2003).

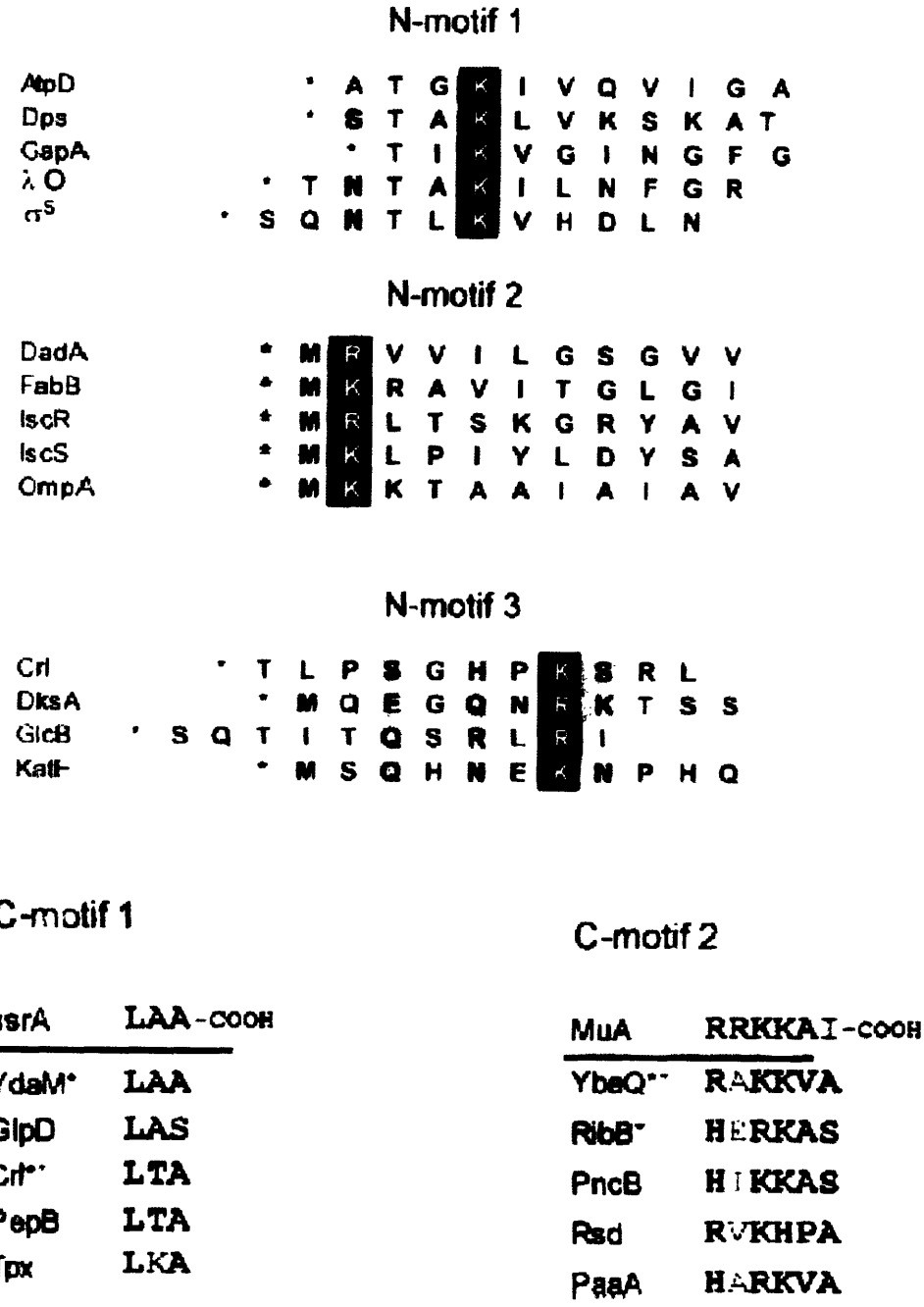


Figure 1.4: N- and C-terminal ClpX substrate recognition signals

Three classes of N-terminal signals and two classes of C-terminal signals target substrate proteins for ClpXP degradation (Flynn et al., 2003a).

Other E. coli proteases

ClpAP and ClpXP use separate ATPases but share a common peptidase. Initially, ClpAP was identified on the basis of its ability to degraded casein in an ATP dependent manner in *lon*$^-$ cells (Katayama et al., 1988; Katayama-Fujimura et al., 1987). ClpA forms 6-membered ring complexes structurally analogous to ClpX (Grimaud et al., 1998). However, these ATPases are dissimilar in sequences, as ClpA has two ATPase motifs, whereas ClpX has only one (Gottesman et al., 1990). *In vitro*, ClpAP degrades ssrA-tagged substrates, but in the cell a modulator protein, ClpS, may prevent ClpA from degrading ssrA-tagged substrates (Dougan et al., 2002; Gottesman et al., 1998). Furthermore, *in vitro* ClpAP degrades denatured proteins in the absence of a recognition tag, and ClpS enhances ClpAP-mediated disaggregation and degradation of denatured substrates (Dougan et al., 2002; Hoskins et al., 2000).

Because the Lon protease degrades substrates involved in diverse cellular processes *lon*$^-$ mutants are extremely pleiotrophic. Therefore Lon was identified separately on the basis of several different phenotypes (reviewed in Gottesman, 1996). As a protease, Lon was initially identified by its ability to suppress degradation of β-galactosidase nonsense fragments (Bukhari and Zipser, 1973). In the cell, Lon degrades specific substrates as well as abnormal and unfolded proteins (reviewed in Gottesman, 1996). A very physiologically important Lon substrate, SulA, is discussed in the next section.

FtsH is a membrane-anchored protease comprised of an N-terminal transmembrane domain and a large cytosolic domain containing AAA-ATPase and protease motifs (Krzywda et al., 2002; Niwa et al., 2002). The N-terminal membrane-associated portion is important for oligomerization and activity (Akiyama and Ito, 2000). FtsH degrades membrane as well as cytoplasmic proteins (Akiyama et al., 1996; Herman et al., 1995; Kihara et al., 1995). Degradation by FtsH is important in the heat shock response and in controlling the lysis vs. lysogeny decision of phage lambda (Herman et al., 1993; Herman et al., 1995).

Initially identified as a heat shock inducible operon, HslUV is the most recently described ATPase/protease pair in *E. coli* (Chuang et al., 1993). HslV, the protease component, is a bacterial homologue of the eukaryotic 20S proteosome (Missiakas et al.,

1996). The ATPase component, HslU, is 50% identical to ClpX (Missiakas et al., 1996). To date, the most physiologically relevant activity of HslUV is degradation of several proteins also known to be substrates for the Lon protease (Wu et al., 1999). Interestingly, HslUV transcription is upregulated following DNA damage (Courcelle et al., 2001).

Bulk Proteolysis

Estimates of the rate of bulk protein turnover in *E. coli* using a variety of methods suggest that the majority of proteins are relatively stable. Studies measuring the release of label from intact cells report that only 2-3% of cellular protein is degraded per hour (Nath and Koch, 1971; Pine, 1970). A proteomic study following a selection of proteins by 2-D electrophoresis after pulse-chase labeling classify 25% as "unstable" where unstable proteins have half-lives of 2-23 hours (Mosteller et al., 1980). A similar study described 3 of 250 proteins as unstable (Larrabee et al., 1980). In these studies, even proteins classified as "unstable" will last for several cell generations. It is useful to contrast this general stability with the extreme instability observed for some of the stress-response proteins discussed later in this thesis.

What sort of a proteolytic load do these 5 proteases share? Using data from the literature, one can make a "back of the envelope" estimate of the responsibilities of the cellular proteases. If 2-3% of the 3 million molecules in each cell are degraded per hour, 1000-1200 protein molecules must be degraded per minute (Nath and Koch, 1971; Pine, 1970; Sauer et al., 2004). Some estimates suggest that 70-80% of the proteolysis in the cell is accomplished by Lon and ClpXP, so 700-960 molecules must be degraded between the two proteases per minute (Maurizi, 1992). Naturally, the accuracy of this estimate depends on the correctness of the parameters used to make it. It is noteworthy that the measurements of bulk protein degradation were made before the mechanisms and proteases in *E. coli* were well understood. More informed choice of timepoints, strains and conditions might yield a more rapid bulk proteolysis rate.

LESSONS FROM PROTEOLYSIS DURING STRESS RESPONSES

Overview

The major goals of this thesis are to understand both how protein turnover is dependent on DNA damage and the consequences of protein turnover during DNA damage. The most relevant models for this work are detailed mechanisms describing the degradation of other *E. coli* proteins during stress responses. The following section outlines mechanisms of degradation for proteins involved in various environmental stress responses. Although these substrates are degraded by different proteases, they have something in common: they are important players in their respective stress response. Some of these proteins are major regulators, while others are toxic if they persist after the stress is resolved. These examples provide a major theme: stress-responsive proteins with important regulatory roles are most likely to have interesting mechanisms to allow control of degradation.

σ^{32} : Extreme instability allows rapid change; Interacting partners affect stability

E. coli utilizes one standard sigma factor, σ^{70}, as well as 6 alternate sigma factors that target RNA polymerase to transcribe genes required for adaptation to environmental changes. The heat shock sigma factor, σ^{32}, upregulates the transcription of a number of chaperones, proteases and regulatory factors following heat shock. Levels of σ^{32} increase transiently following a temperature upshift as a result of both increased protein synthesis and stability (Morita et al., 1999; Straus et al., 1987). Synthesis increases dramatically during the first 3-4 minutes of heat shock, but then drops off. This increase in synthesis coincides with an increase in protein stability—the normal 1 minute half life is increased at least 8 fold in this initial phase of the heat shock response (Straus et al., 1987). The extremely short half-life of σ^{32} permits the observed rapid changes in levels (Alberts et al., 2002).

FtsH is the main protease responsible for σ^{32} degradation, as σ^{32} half life increases up to 12 fold in cells lacking FtsH (Herman et al., 1995). What controls differential σ^{32} degradation under heat shock conditions? The DnaK chaperone network (DnaK, DnaJ and GrpE) is involved in regulating σ^{32} activity and stability (Straus et al., 1990). GroEL/S is also involved in regulating σ^{32} activity and stability (Guisbert et al.,

2004). In fact, a decrease in the level of GroEl or DnaK results in increased σ^{32} stability, but it is not know precisely how these chaperones promote σ^{32} degradation by FtsH (Guisbert et al., 2004; Tomoyasu et al., 1998). Additionally, depletion of the available GroEL/S or DnaK in the cell by induction of a chaperone substrate increases σ^{32} levels and activity (Guisbert et al., 2004; Tomoyasu et al., 1998). A working model for σ^{32} control during the heat shock response is that during heat shock, unfolded proteins compete with σ^{32} for the chaperone network. As cells adapt, available chaperones increase and can downregulate the availability and activity of σ^{32}.

SulA: Protein degradation rate dictates cell survival

Following UV irradiation, cells with mutations in the *lon* gene exhibit a greatly increased sensitivity and a characteristic long cell shape resulting from a defect in septation (Howard-Flanders et al., 1964). A variety of post-irradiation treatments that modulated the severity of this defect were proposed to change the relative rates of new protein synthesis and DNA repair (Witkin, 1967). Screens for mutations suppressing the UV sensitivity of *lon*$^-$ strains (or radiation sensitive *E. coli* B strains) identified a suppressor that mapped to the SulA gene (Gayda et al., 1976; Johnson and Greenberg, 1975; Witkin, 1947). Subsequently, SulA was proposed to inhibit cell division, and shown to be induced by DNA damage (George et al., 1975; Huisman and D'Ari, 1981).

In vivo degradation assays following the stability of SulA in wild-type and *lon*$^-$ cells confirmed this model. In wild-type cells, SulA has a half-life of less than 2 minutes (Mizusawa and Gottesman, 1983). This half-life is increased to 19 minutes in *lon*$^-$ cells (Mizusawa and Gottesman, 1983). SulA is also a substrate for HslUV, as SulA half-life increases to over 90 minutes in cells lacking either protease subunit (Wu et al., 1999). It is noteworthy that although HslUV contributes to SulA degradation, the rapid degradation effected by Lon is necessary for normal resistance to UV irradiation. This demonstrates that the rate of turnover for some stress-responsive proteins is important for survival.

σ^S: Use of an adaptor allows conditional proteolysis

The stationary phase sigma factor, σ^S, regulates a network of genes that enhance cell survival under starvation conditions. Multiple levels of regulation govern the activity of σ^S. These include control of transcription, translation, protein stability and activity (reviewed in Ishihama, 2000). During exponential phase, σ^S is very unstable with a half-life of 1.4 minutes (Lange and Hengge-Aronis, 1994). Changes in σ^S stability occur at the onset of starvation as σ^S half life increases to about 10 minutes during stationary phase (Lange and Hengge-Aronis, 1994). ClpXP is the protease responsible for σ^S degradation, and the response regulator RssB governs its growth-phase responsive turnover (Muffler et al., 1996; Pratt and Silhavy, 1996; Schweder et al., 1996).

In vitro experiments reveal that RssB can interact with both ClpX and σ^S in order to deliver σ^S for ClpXP degradation (Zhou et al., 2001). RssB stimulates σ^S degradation more than 10 fold and this stimulation is not seen with unrelated ClpXP substrates (Zhou et al., 2001). *In vitro*, amino acid D58 of RssB is phosphorylated by acetyl phosphate, and this phosphorylation stimulates σ^S degradation by 10 fold (Bouche et al., 1998; Zhou et al., 2001). One model for growth-phase dependent σ^S degradation is that during exponential growth, phosphorylated RssB enhances σ^S degradation but during starvation, dephosphorlyated RssB allows σ^S accumulation. However, genetic screens have not identified a specific kinase for RssB (Cunning and Elliott, 1999; Ruiz and Silhavy, 2003). Furthermore, a nonphosphorylatable D58A mutation in RssB does not eliminate growth-phase dependent changes in stability, although it does slow degradation during exponential phase (Peterson et al., 2004). Therefore, RssB contributes to growth-phase regulated σ^S degradation, but a precise mechanism remains to be determined.

RseA and LexA: A proteolytic cascade couples environmental signal to changes in substrate stability

Degradation of two proteins, RseA and LexA, is dependent upon prior processing events that create protease recognition signals. The mechanism of LexA degradation is covered in detail in chapter 3, so this section focuses on RseA. *E. coli* has a distinct pathway to recognize and respond to extracytoplasmic stress. Extracytoplasmic stress

resulting from accumulation of unfolded outer membrane proteins in the periplasm is communicated across the inner membrane to the cytoplasm and causes the activation of σ^E (reviewed in Raivio and Silhavy, 2001). Activation of σ^E results in upregulation of periplasmic proteases and protein folding factors (Dartigalongue et al., 2001).

In unstressed cells, an anti-sigma factor, RseA, controls σ^E activity (De Las Penas et al., 1997; Missiakas et al., 1997). RseA has an N-terminal cytoplasmic domain that binds σ^E, a domain that spans the inner membrane, and a C-terminal periplasmic sensor domain (De Las Penas et al., 1997; Missiakas et al., 1997). Following induction of extracytoplasmic stress, an inner membrane protease, DegS, cleaves RseA within its periplasmic domain (Ades et al., 1999; Alba et al., 2002). RseA cleavage by DegS activates cleavage by another protease, YaeL (Alba et al., 2002; Kanehara et al., 2002; Walsh et al., 2003). YaeL cleaves RseA on the cytoplasmic side of the membrane and releases RseA-bound σ^E into the cytoplasm (Figure 1.5).

The crystal structure of σ^E bound by the cytoplasmic domain of RseA reveals that, until RseA is removed, RNA polymerase is sterically blocked from interacting with σ^E (Campbell et al., 2003). Trapping experiments reveal that ClpXP and SspB work together to recognize and degrade the N-terminal RseA fragment (Flynn et al., 2004). Cleavage of RseA by YaeL occurs between amino acids 108 and 109, creating a ClpX-recognition signal (VAA) at the new C-terminus (Flynn et al., 2004). Full induction of the σ^E regulon requires both ClpX and SspB (Flynn et al., 2004). The response to extracytoplasmic stress, therefore, requires the action of a proteolytic cascade. Three proteases act sequentially, each creating a signal for the next, to transmit an environmental cue into a stress response.

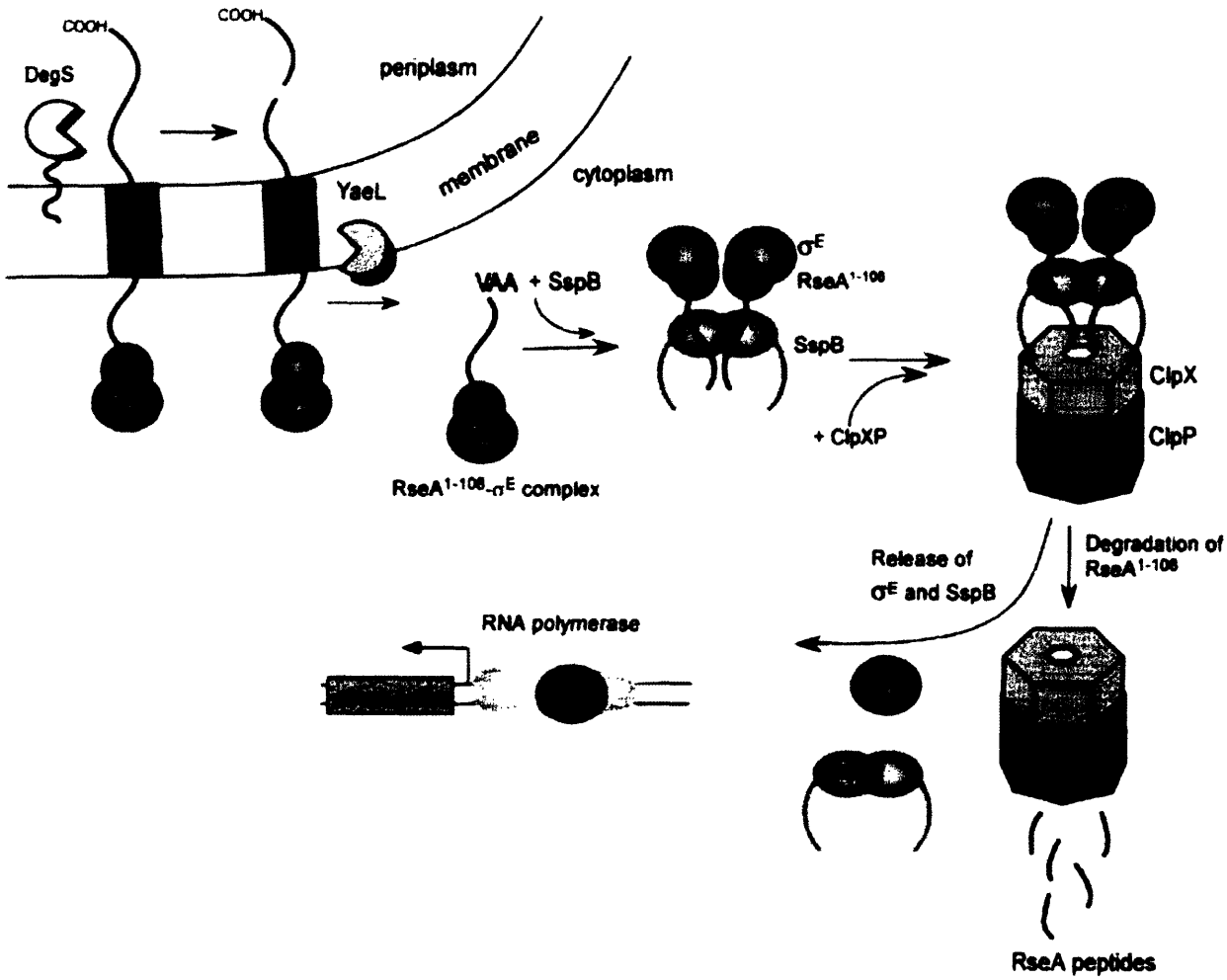


Figure 1.5: A proteolytic cascade results in release of σ^E

This schematic shows the proteolytic cascade resulting in release of RseA from the membrane and activation of the σ^E regulon (Flynn et al., 2004).

Summary of lessons from stress response proteins:

Following an environmental stress, proteolysis is vital for optimizing cell survival. The previous examples are a sampling of the important roles protein degradation can play: it can allow full induction of a response, prevent toxic effects from over abundant protein or regulate the duration of a response. These examples show how *E. coli* relies on a variety of clever strategies to ensure that the right protein is degraded at the right time. Some very toxic and transiently expressed proteins, such as SulA, are naturally unstable. The stability of others, such as σ^{32}, depends on the availability of interacting partners. Furthermore, the use of delivery proteins that are conditionally expressed or active can allow temporal control of substrate degradation. Finally, proteolytic cascades linked to an environmental signal, as in RseA, can create or expose signals for protease recognition. In summary, these examples show that challenges to the cellular system can provide rich opportunities for identifying new regulatory strategies for proteolysis.

THESIS OVERVIEW:

This thesis presents an in-depth look at the role of ClpXP in one of the first characterized bacterial stress responses, the SOS response. In chapter 2, quantitative proteomics are used to identify new ClpXP substrates and illustrate how an environmental stress alters protease substrate selection. Next, analysis of LexA degradation provides a model for linking an environmental stress to a change in protein degradation. Finally, elucidation of the mechanism of UmuD-mediated UmuD' degradation by ClpXP shows how an interacting partner can become an accessory factor to allow conditional degradation.

CHAPTER 2:

REDEPLOYMENT OF THE *E. COLI* CLPXP PROTEASE FOLLOWING DNA DAMAGE

This is a draft of a manuscript to be submitted. Dr. Judit Villet in Professor Steve Gygi's lab at Harvard Medical School performed and analyzed the quantitative mass spectrometry, and collected data for and prepared Figure 1B.

Abstract:

In harsh environmental conditions bacteria exercise precise control over levels of key proteins to enhance survival. Protein levels depend on the balance between synthesis and degradation. Our previous studies revealed that a disproportionate number of ClpXP substrates are stress response proteins. To investigate this observation, we analyzed the role of ClpXP in one of the most thoroughly characterized environmental stress responses, the SOS response. We used *in vivo* substrate trapping coupled with quantitative mass spectrometry to compare levels of proteins captured with and without DNA damage. Our results show that capture of about half of the identified proteins changed more that three-fold in response to stress. Substrates with enhanced capture include members of the SOS-regulon. ClpXP recognition of non-SOS regulated proteins was also affected by DNA damage, including the known ClpXP substrates Dps and σ^s. This dramatic redeployment of ClpXP suggests an active role for proteolytic control of protein levels during environmental stress.

Introduction

Following a switch to adverse environmental conditions, cells must be able to rapidly adjust their proteome to optimize survival. Efforts to characterize these adjustments have generally focused on the changes in transcript levels. However, controlled degradation of specific proteins is also an important regulatory strategy. In some cases, activation of transcriptional response to stress depends on proteolysis. For example, induction of the extracytoplasmic stress response relies on release of σ^E from its regulator, RseA (Campbell et al., 2003; Missiakas et al., 1997). This release requires two membrane bound proteases, DegS and RseP (YaeL), as well as the cytoplasmic ClpXP protease (Alba et al., 2002; Alba et al., 2001; Flynn et al., 2004). In other cases, proteolysis aids in ensuring appropriate levels of stress-induced proteins. DNA damage triggers the upregulation of the inhibitor of cell division, SulA. SulA is rapidly turned over by Lon protease, and in the absence of Lon the accumulation of SulA results in lethal inhibition of septation in UV-exposed cells (Mizusawa and Gottesman, 1983). Although these examples illustrate the importance of proteolysis in the cellular response to stress, the global impact of proteolysis on a stress response is unknown.

Rapid protein turnover might be especially important for the DNA damage response because many damage-regulated genes encode proteins potentially detrimental to cells during normal growth. The LexA repressor controls induction of the damage-response proteins. LexA binds to specific promoter sequences to inhibit expression of more than 30 members of the SOS regulon (Fernandez De Henestrosa et al., 2000). Upon DNA damage, exposed single stranded DNA activates RecA, which in turn triggers autocleavage of LexA. This cleavage results in relief of LexA repression. Genome-wide transcriptional profiling following DNA damage reveals changes in transcript level for a number of genes in addition to those directly under LexA control (Courcelle et al., 2001; Khil and Camerini-Otero, 2002; Quillardet et al., 2003). However, there is little information about how these changes in gene expression correlate to changes in protein levels. For a few well-studied proteins, proteolysis has also been shown to play a critical role. For example, the LexA autocleavage fragments are degraded by both ClpXP and Lon proteases (Little, 1983a; Neher et al., 2003a). Additionally, components of the

translession polymerase, DNA pol V, are substrates for the Lon and ClpXP proteases (Frank et al., 1996; Gonzalez et al., 1998b).

Protein degradation by the ClpXP protease contributes to fitness, as, at high doses of UV irradiation, loss of ClpX decreases cell survival (Neher et al., 2003b). ClpX is an ATPase that recognizes substrate proteins, generally by short sequence motifs located near the N- or C-terminus of the protein. ClpX actively unfolds these substrate proteins and translocates them into an associated serine peptidase, ClpP (Kim et al., 2000). ClpP is comprised of two 7-membered rings that stack to make a cylindrically shaped chamber (Flanagan et al., 1995; Wang et al., 1997). Its serine active sites face the inside of this cylinder, and when these active sites are mutated, ClpP can function as a substrate trap ($ClpP^{trap}$) inside the cell (Flynn et al., 2003). Using the $ClpP^{trap}$, we previously identified over 50 *in vivo* ClpXP substrates during normal cell growth (Flynn et al., 2003).

An emerging biological conclusion from this work on substrate identification is that many stress-response proteins appear to be substrates. These substrates include σS, Dps, Fnr, RseA, and LexA. Therefore, we were interested in the extent to which ClpXP is involved in a specific response, and how substrate choice changes with changes in environmental conditions. Stable isotope labeling with amino acids in culture (SILAC) allows comparison of relative levels of proteins from two samples by mass spectrometry (Ong et al., 2002). Use of SILAC in combination with the $ClpP^{trap}$ allowed us to compare ClpXP substrates captured during normal growth with those captured following DNA damage. These experiments reveal dramatic changes in substrate capture following environmental stress. Degradation experiments reveal that many of the identified proteins are unstable *in vivo*. Thus, we conclude that a common feature of many proteins whose gene expression is regulated by stress is that they are intrinsic protease substrates, allowing their levels to rapidly respond to environmental change.

Results

Quantitative proteomics reveals a large shift in ClpXP substrate selection in response to DNA damage.

To measure the changes in ClpXP substrate selection following DNA damage, we adapted a method for quantitative mass spectrometry (SILAC) to use with our *in vivo* trapping system. Briefly, half of a population of cells expressing the $ClpP^{trap}$ grew in media containing light leucine with the DNA damaging agent nalidixic acid (NA), and half grew without NA treatment in media containing heavy leucine. We subsequently mixed equal amounts of the two populations, isolated the $ClpP^{trap}$ and associated substrates and determined relative protein ratios by mass spectrometry (Figure 2.1A). *E. coli* are not auxotrophic for leucine. Therefore, to ensure that incorporation of heavy leucine was complete, we inactivated *leuB*, an essential enzyme in the leucine biosynthesis pathway. Cells lacking *leuB* grew on minimal media only when supplemented with leucine. Examination of peptides from a sample grown in heavy leucine revealed only the presence of the heavy isotope with the expected +6 mass units for each leucine present in the sequence (Figure 2.1B). Thus, ratio of heavy peptide, to the identical peptides containing light leucine in the experimental sample is an accurate means of determining the abundance of a specific protein in the $ClpP^{trap}$ in the two growth conditions.

To ensure capture of only ClpXP substrates, trapping was carried out in a *clpA*$^-$, *smpB*$^-$ strain. ClpA is an alternate ATPase that can work with ClpP. Loss of *smpB* eliminates inactivates SsrA tagging (Karzai et al., 1999). SsrA tags are added cotranslationally to poplypeptides stalled on the ribosome, and target these incomplete proteins for ClpXP degradation (Gottesman et al., 1998; Keiler et al., 1996). Additionally, to ensure that co-purification with $ClpP^{trap}$ was dependent upon interaction with ClpX, as expected for ClpXP substrates, we repeated the trapping experiment under both sets of environmental conditions in cells lacking both *clpX* and *clpA*. In the absence of the ClpX ATPase, 20 proteins reproducibly copurified with ClpP (Table S1). Some of these proteins also interact/co-purify with proteolytically competent ClpP using a different purification scheme (Butland et al., 2005).

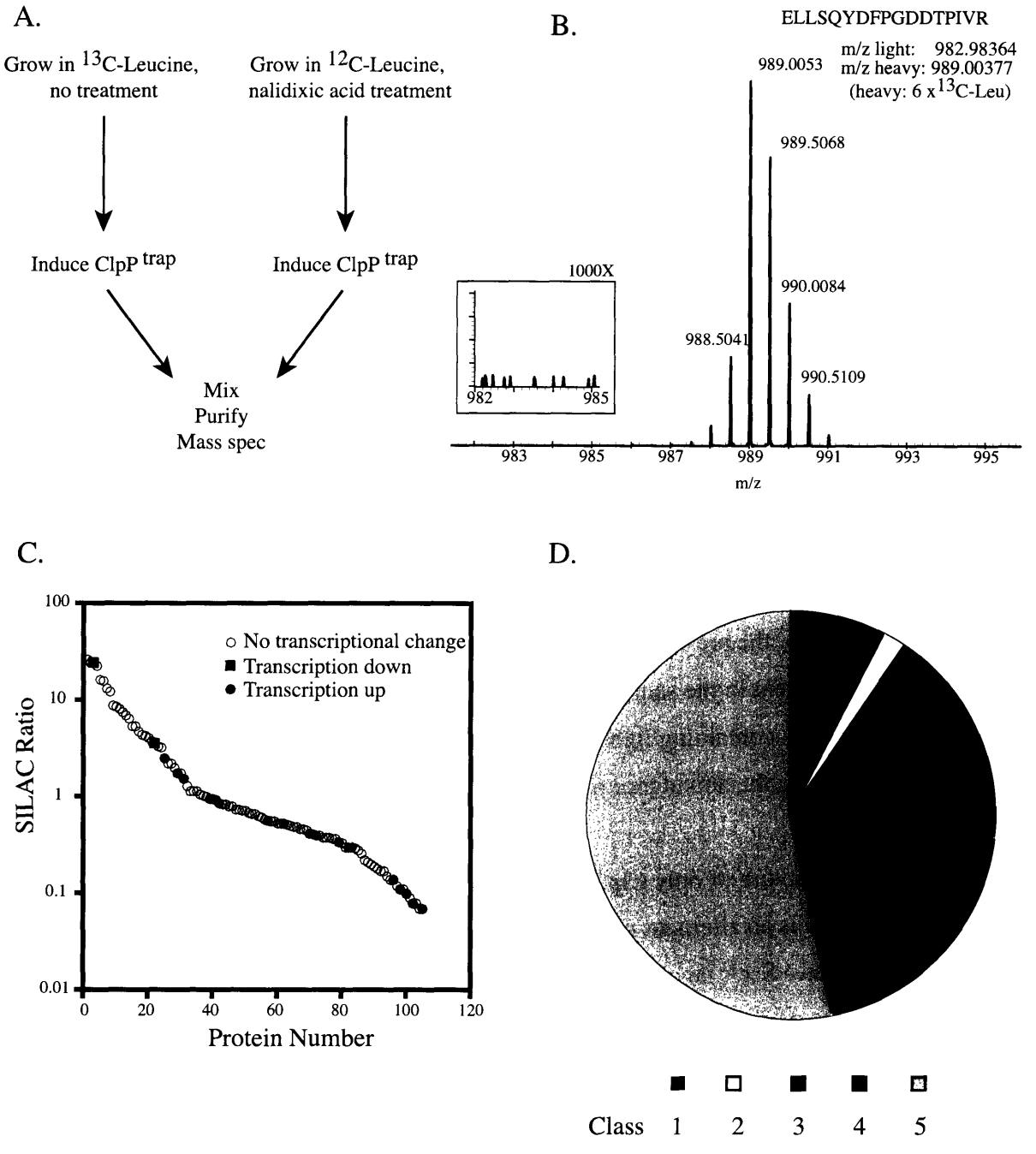


Figure 2.1: SILAC allows comparison of substrate trapping with and without DNA damage. A: An overview of the experimental design. **B.** Analysis of peptides from a sample grown in heavy media reveals no incorporation of light leucine. **C.** The distribution of ratios of heavy/light (untreated/treated) proteins captured correlates well with transcriptional changes. **D.** Trapped substrates were assigned to one of 5 classes.

We identified at least two peptides from over 100 proteins in the $ClpP^{trap}$ (Table 2.1). The SILAC ratio changed by more than three-fold for about half of these proteins. About 15% of these substrates overlap with substrates previously trapped in the absence of DNA damage (Flynn et al., 2003a). The distribution of SILAC ratios (Figure 2.1C) for the identified substrates reveals that ClpXP recognizes numerous proteins more frequently after damage (low SILAC ratio). This analysis also demonstrates that some proteins are significantly underrepresented in $ClpXP^{trap}$ in response to DNA damage.

To determine the effect of DNA-damaged induced changes in transcription on the substrate profile, we used available microarray data to determine for each identified protein how transcription changes following DNA damage. Transcriptional changes were considered significant if mRNA levels changed more than 1.5-fold as published in either of two sources (Courcelle et al., 2001; Quillardet et al., 2003). In general, changes in transcription correlated well with changes in trapping (Figure 2.1C). However, transcriptional changes could not account for all of the observed changes in trapping. To understand these changes in substrate choice, we divided the trapped proteins into 5 classes and studied representative proteins from several of these classes. The fraction of total trapped protein represented by each of these classes is shown in figure 2.1D.

Proteins were assigned to classes based on how their trapping changed and how these changes correlated to transcriptional changes reported by microarray analysis (Courcelle et al., 2001; Quillardet et al., 2003). For these classifications, we used a cut-off of three-fold or more in the ratio of proteins trapped with and without DNA damage. Proteins in class I were overrepresented by three fold or more in the trap following DNA damage and also showed induced transcription. Similarly, proteins in class II were underrepresented by three fold or more in the trap following DNA damage and had decreased transcript levels. Members of classes III and IV had no reported transcriptional change but were significantly under- and overrepresented, respectively, following DNA damage. Finally, proteins in class V increased or decreased less than three fold.

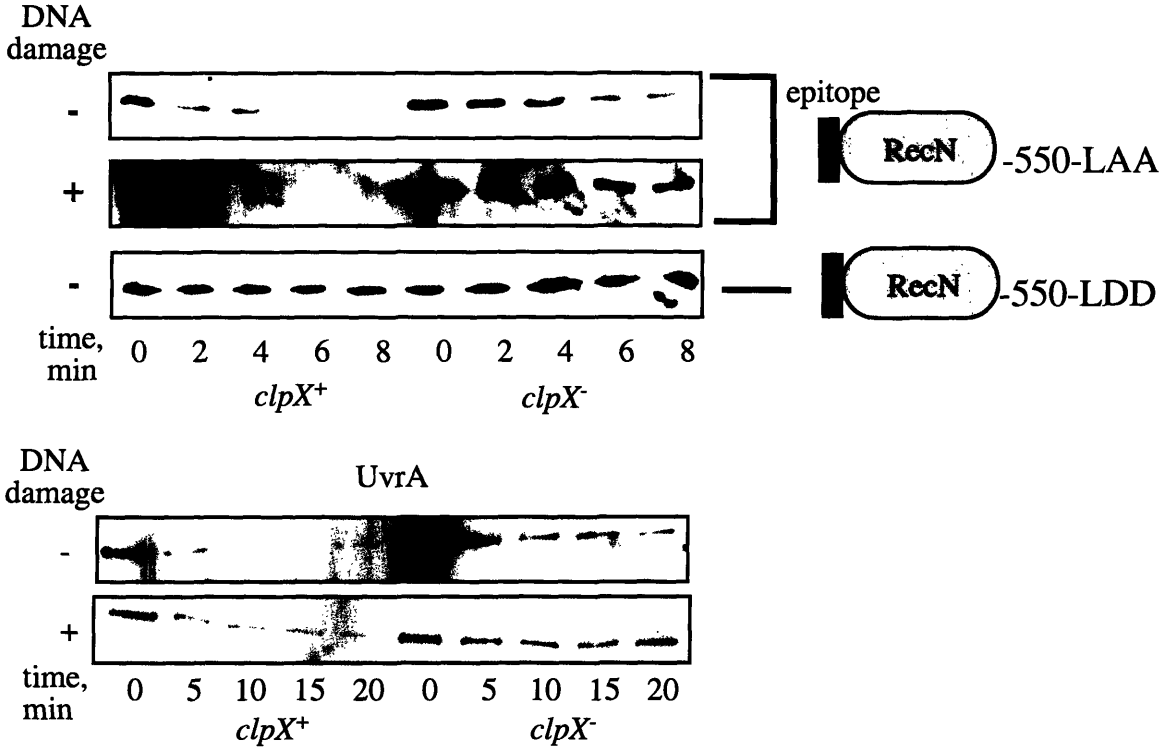
Class 1 substrates: increased expression leads to increased recognition.

Representative proteins from the Class I include two members of the LexA regulon, RecN and UvrA. RecN is involved in double strand break repair, and UvrA functions in nucleotide excision repair (Kidane et al., 2004; Kosa et al., 2004; Sancar, 1996). A simple model for their increased trapping in the light (NA-treated) culture is that they are more highly expressed. To test this idea, epitope tagged versions of the genes were expressed using an exogenous promoter and the stability of these proteins was determined in the presence and absence of NA treatment. Both proteins showed ClpXP-dependent degradation *in vivo,* and DNA damage had little or no effect on degradation (Figure 2.2A). These experiments used N-terminally epitope tagged versions of RecN and UvrA (see experimental procedures). A N-terminally affixed epitope tag did not targeted a control protein (Arc-st11) for ClpXP degradation (data not shown). The residual degradation observed in the *clpX*$^{-}$ strain is not uncommon in bacteria, where a single substrate is often the target for multiple proteases.

The extreme C-terminal residues of RecN, LAA, are identical to the last three residues of the SsrA tag (LAA) that are used for ClpX recognition (Flynn et al., 2001; Gottesman et al., 1998). To determine if these residues direct RecN degradation we mutated both alanines to aspartic acids. This version of RecN was greatly stabilized relative to wild-type RecN (Figure 2.2A). Therefore, the RecN's C-terminal alanines are important determinants for ClpX recognition. Given that ClpXP degrades both UvrA and RecN in the absence of DNA damage, it is most likely that their increased presence in the trap following DNA damage is due to increased synthesis. Other Class I members are listed in Figure 2.2B.

To identify new substrates following DNA damage we initially used conventional proteomics to identify trapped substrates. Because these trapping experiments did not utilize SILAC, they can't quantitatively describe changes in substrate capture. However, these experiments identified several potential new ClpXP substrates that are induced at the level of transcription after DNA damage (Figure 2.2B). These proteins were not identified in trapping experiments without a DNA damaging treatment (Flynn et al., 2003) and therefore are conceptually most similar to class I substrates. Some of these substrates probably weren't identified by SILAC because they are very small proteins

A.



B.

Class 1 substrates from SILAC

Gene	Function
asnA	Asparagine synthase A
deaD	RNA helicase
rapA	RNA polymerase recycling
recN	DS break repair
rplO	Ribosome subunit
uvrA	Nucleotide excision repair
yfgB	Unknown

Identified by conventional Mass Spec

Gene	Function
dinI	Modulates RecA activity
dinD	Unknown
sulA	Inhibits cell division
yebC	Unknown
yebG	Unknown

C.

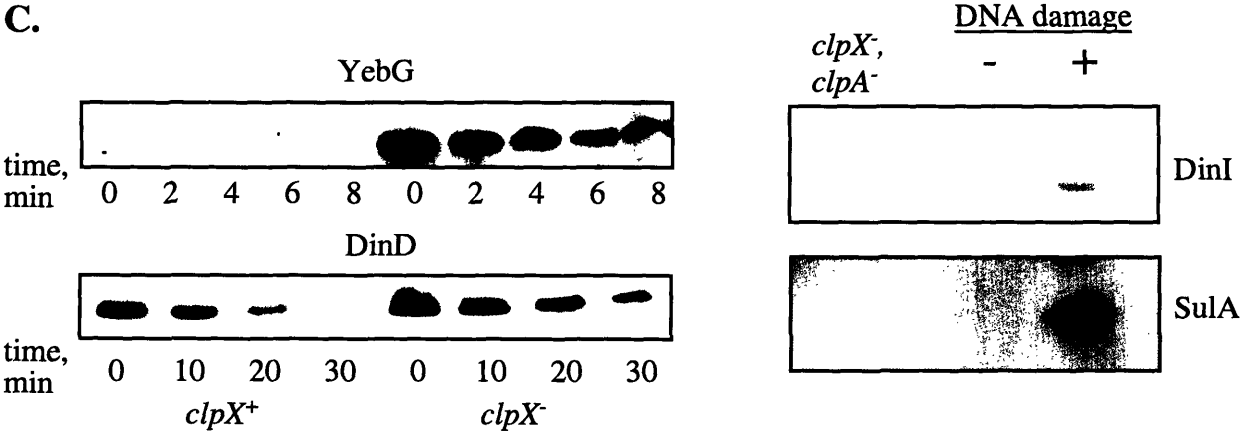


Figure 2.2: Characteristics of Class I substrates

A: The ClpX-dependence of *in vivo* RecN degradation was determined using a N-terminally epitope tagged version of the protein, as shown at right. Induction of DNA damage by addition of nalidixic acid did not affect the rate of degradation. The C-terminal alanines are important, as a variant with alanines mutated to aspartic acids is stabilized. UvrA is also a stabilized by loss of *clpX*.

B. Class I substrates identified by SILAC are listed. Additionally, Class I-like substrates identified by conventional mass spec are listed.

C. Degradation of the ClassI-like substates YebG and DinD is slowed by loss of *clpX*. Additionally, SulA and DinI are captured in the $ClpP^{trap}$ in greater amounts in cells treated with nalidixic acid.

(<100 amino acids) with few leucine-containing tryptic peptides. Indeed, western blots of trapped proteins show that DinI and SulA are overrepresented in a damage-induced trap relative to a wild type trap (Figure 2.2C). When N-terminally epitope tagged and expressed from an exogenous promoter, YebG and DinD show robust ClpX-dependent *in vivo* degradation (Figure 2.2C). Interestingly, the C-terminal residues of YebG (HAA) were again similar to the ClpX-recognition signal from the ssrA tag. YebG levels are difficult to detect in wild-type cells because degradation of YebG is so rapid. Thus, a number of DNA-damaged induced proteins are targets for ClpXP degradation. This analysis suggests that one hallmark of stress response proteins may be mechanisms to ensure their rapid degradation such that they are only present as long as the signal responsible for inducing their expression remains.

Class II substrates: decreased expression leads to decreased trapping.

Following DNA damage, some genes show reduced transcript levels, although this is a much smaller class of genes than those that are induced (Courcelle et al., 2001; Quillardet et al., 2003). Therefore it follows that there are fewer Class II than Class I substrates among the trapped proteins. This class, composed of proteins with both a decrease in transcript level and a decrease in trapping following DNA damage, contains only two members, MinD and GlgA. One of these, MinD, is involved in cell division site selection (reviewed in Lutkenhaus, 2002). Quantification of MinD protein level with and without a DNA damaging treatment revealed that, like transcript levels, protein levels decrease when DNA damage occurs (Figure 2.3A). *In vivo* degradation experiments show that MinD is degraded in a ClpX-dependent manner (Figure 2.3B). However, DNA damage did not alter the rate of degradation (Figure 2.3B). Therefore, decreased MinD trapping after DNA damage is likely a reflection of the decreased cellular levels.

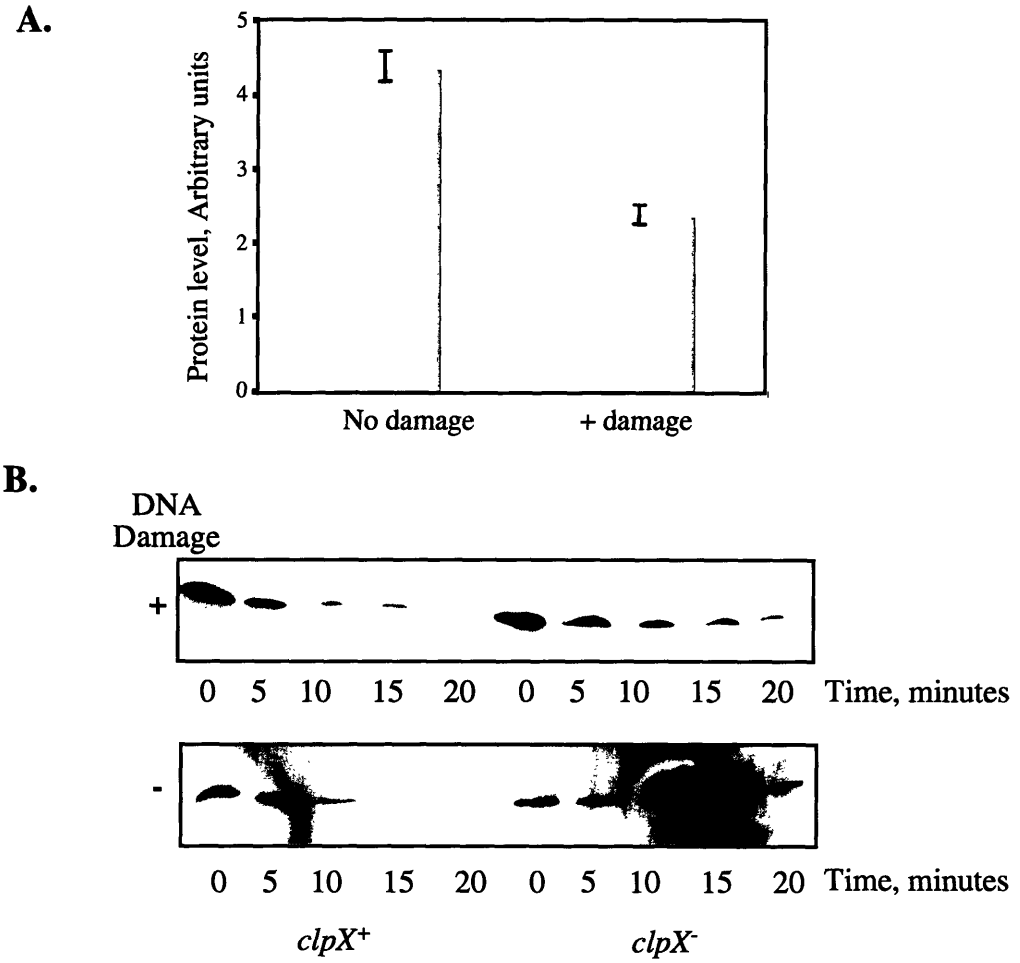


Figure 2.3: MinD degradation

A. Cellular MinD levels, as assayed by Western Blot, are decreased following DNA damage.

B. MinD is stabilized *in vivo* in *clpX*$^-$ cells relative to wild-type cells. The degradation rate is not affected by DNA damage.

Class III and IV substrates: differential trapping without changes in gene expression.

Many of the ClpXP captured proteins that were differentially trapped in the presence and absence of DNA damage are not regulated at the transcriptional level during the damage response. These proteins included substrates that were preferentially trapped in undamaged cells (Class III) as well as proteins preferentially trapped after damage (Class IV). Several possible mechanisms could underlie the changes in ClpXP-recognition of individual proteins in these classes. First, constant transcript levels may not reflect constant protein levels; alterations in translation or susceptibility to other proteases may change the cellular levels of the Class III and IV substrates after DNA damage. Furthermore, changes in interacting partners could either protect potential substrates from degradation or target specific proteins to ClpXP. Finally, a large number of potential ClpXP substrates compete for a small pool of the protease (Ortega et al., 2004) (Farrell et al., submitted). A rapid influx of new substrates following environmental stress could compete with the substrates recognized under non-stressed conditions for ClpXP recognition.

Two well-characterized ClpXP substrates, Dps and σ^S, are clearly under-represented (~4.5-fold and ~7-fold respectively) in ClpXP^{trap} after DNA damage (Figure 2.4) and are therefore members of Class III. Dps is the major DNA-binding protein in stationary phase cells and is though to protect DNA from oxidative damage (Ali Azam et al., 1999; Martinez and Kolter, 1997). σ^s is the stationary phase sigma factor, and a key transcriptional regulator of many stress-response genes (Hengge-Aronis, 1999). Cellular levels of both σ^s and Dps are regulated in part by regulated proteolysis by ClpXP (Schweder et al., 1996; Stephani et al., 2003). For example, both proteins are rapidly degraded during exponential growth and then stabilized as cells enter stationary phase (Almiron et al., 1992; Lange and Hengge-Aronis, 1994). Regulated degradation of σ^s (but not Dps) requires the adaptor protein RssB (Muffler et al., 1996; Zhou et al., 2001).

To test whether changes in Dps and σ^s protein levels after DNA damage were responsible for their under-representation in ClpXP^{trap}, we determined the intracellular protein levels by Western blotting. Levels were measured in cells lacking ClpP, as the trapping strain is *clpP*$^-$. The amount of Dps and σ^s did not change after treatment with a DNA-damaging agent, nalidixic acid (Figure 2.4). Therefore, the observed change in

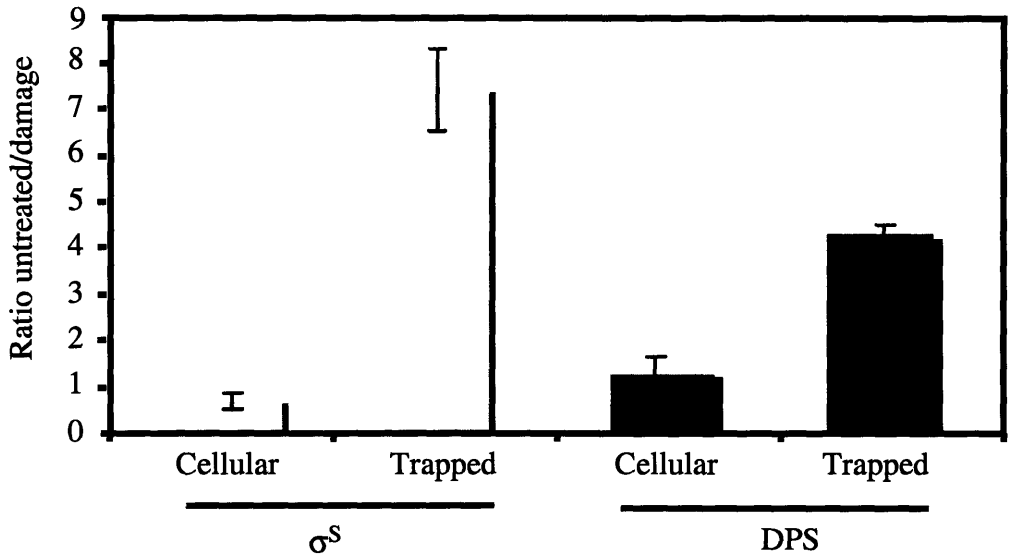


Figure 2.4: Comparison of the effects of DNA damage on cellular and trapped DPS and σ^S levels. Although DNA damage does not greatly affect cellular DPS and σ^S levels, both proteins are more present in the trap in the absence of a DNA damaging treatment.

trapping was not due to a change in protein levels, but rather reflects a redirection of ClpXP away from these two substrates (see Discussion).

In contrast to the behavior of Dps and σ^S, other ClpXP substrates were over-represented in $ClpXP^{trap}$ in the damaged culture (Class IV). This class included three ribosomal proteins: L10 (rplJ), S7 (rpsG) and L15 (rplO). We noticed that two of these proteins, L10 and S7, function as translational repressors for their respective ribosomal protein operons (Keener and Nomura, 1996). L10 is also rapidly degraded *in vivo* when overproduced in the absence of its binding partners (L7/L12) (Petersen, 1990). Furthermore, L10 has a C-terminal AA sequence, and is rapidly degraded by ClpXP *in vitro* (J. Flynn personal communication). Translational repression by free ribosomal subunits occurs to allow cells to synthesize ribosomal components in balance as well as to down-regulate the synthesis of these components when growth rate slows. Therefore, we considered the possibility that L10 and S7 (and perhaps other proteins) were over-represented in the $ClpXP^{trap}$ in the DNA-damaged culture as an indirect result of the expected decreased growth rate of cells after DNA damage. In the original experiment, trapping in both cultures was done for 3 hours, and as a result of the different growth rates, the untreated cells were harvested at a higher OD_{600} (~1.7 compared to ~1.0) and thus were likely entering stationary phase at the time of harvest.

To test whether growth-phase regulation was responsible for the differential trapping of some substrates, the SILAC experiment was repeated. Parallel cultures were again grown with either heavy or light leucine, and the light leucine culture was treated with NA. However, in this experiment, both cultures were harvested after trapping for different amounts of time but at the identical OD_{600} of 1.0. As expected for growth-phase regulators, L10 and S7 were trapped much more similarly in these two cultures, having SILAC ratios near one (Figure 2.5A). Thus, we suggest that these proteins are efficiently recognized and degraded by ClpXP when cells are in log phase, but that upon entry into stationary phase, ClpXP degradation is repressed to allow the proteins to accumulate and assume the function as repressors of translation. Five proteins of the approximately 20 class IV substrates showed significant growth-phase specific change in SILAC ratio: L10, S7, L15 and two RNA helicases, Rho and DeaD (Figure 2.5A). The majority of proteins in this class, such as CarA, did not change greatly (Figure 2.5A).

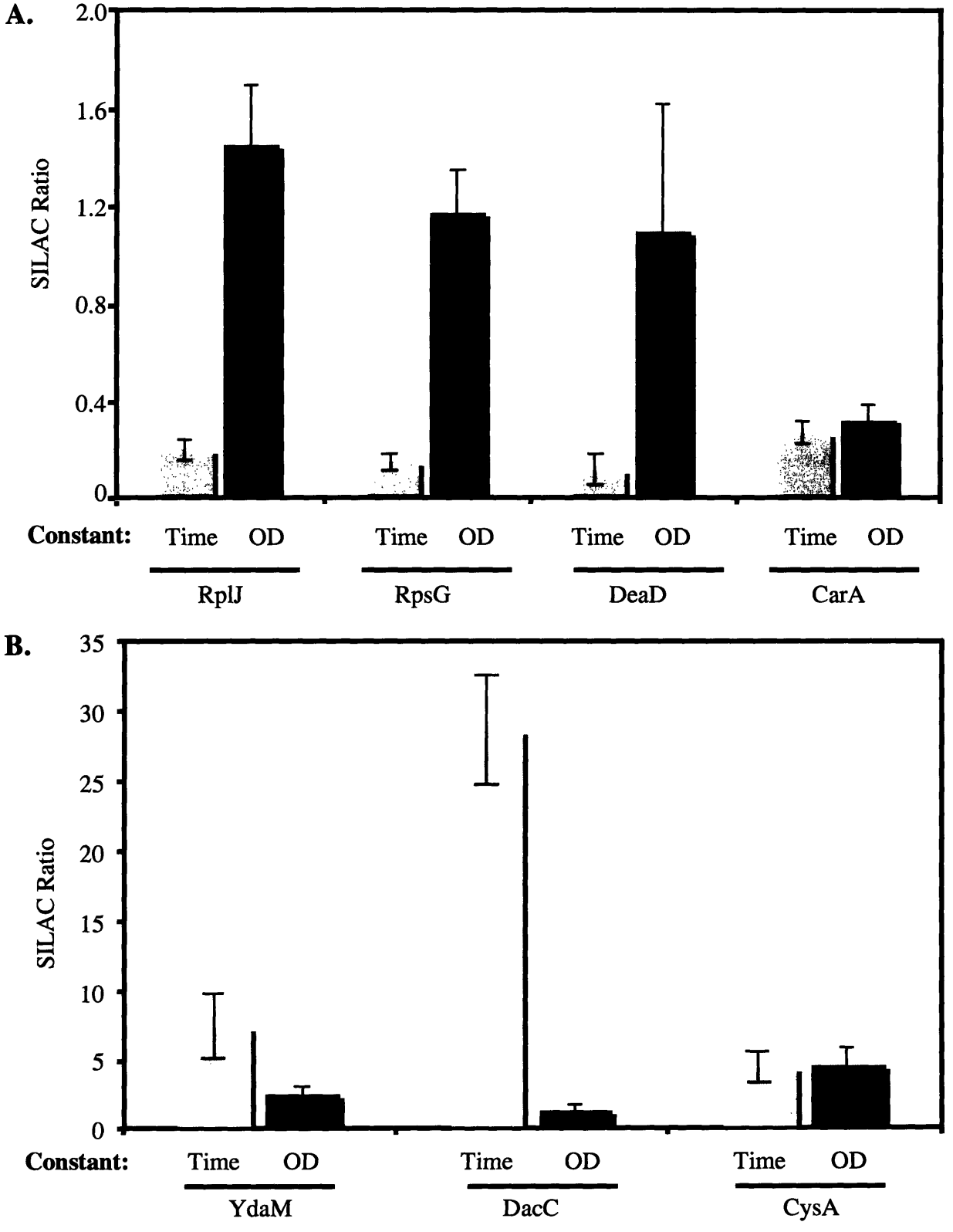

A.
2.0
1.6
1.2
0.8
0.4
0
SILAC Ratio
Constant:
Time
OD
RplJ
RpsG
DeaD
CarA
B.
35
30
25
20
15
10
5
0
SILAC Ratio
Constant:
Time
OD
YdaM
DacC
CysA

Figure 2.5: Effects of growth phase on trapping

A. The decreased trapping observed for 5 substrates from class IV was not seen when the treated and untreated samples were harvest at an equal OD. However, trapping for many substrates, such as CarA, was unaffected by OD.

B. Increased trapping in the undamaged sample for two substrates from Class III was not observed when the samples were harvested at equal ODs.

Interesting, two of the approximately 20 Class III substrates also showed significant changes in SILAC ratios when the cultures where harvested at the same OD (Figure 2.5B). These proteins included an enzyme involved in peptidoglycan synthesis, dacC , [penicillin-binding protein 6], as well as a protein of unknown function (ydaM). The implication is that these proteins may be stable in log phase cultures, but become ClpXP substrates as cell growth slows in stationary phase. In contrast, other class III substrates such as CysA did not show significant growth dependent changes (Figure 5B). Furthermore, the SILAC ratios of the Class I substrates were not dramatically different in the two experiments.

Non-degradable RecN affects cell survival

Among members of the SOS regulon, RecN is distinguished by extremely rapid transcriptional induction, as mRNA levels increase approximately 20-fold within the first 5 minutes of DNA damage (Courcelle et al., 2001). Intriguingly, our experiments indicate that RecN has a very short half-life. Why should the cell invest so heavily in production of this short-lived protein? Studies in *B. subtillus* suggest that RecN localizes to repair complexes following DNA damage and may help to recruit other repair proteins (Kidane et al., 2004). Furthermore, *in vitro* experiments using *B. subtillus* proteins show that RecN forms multimeric complexes on ssDNA that RecA can disassemble (Sanchez and Alonso, 2005).

These results led us to ask if persistence of RecN is harmful. Cells lacking RecN are extremely sensitive to agents such as bleomycin that cause double-stranded breaks (Kosa et al., 2004; Picksley et al., 1984). Therefore, we tested the effects of expressing plasmid-borne RecN or the stable version of RecN, RecN-DD, on bleomycin sensitivity. In a wild-type strain, induction of both RecN and RecN-DD increased bleomycin sensitivity relative to an empty plasmid (Figure 2.6A). However, this effect was more pronounced with RecN-DD. In *clpP*$^-$ or *clpX*$^-$ strains, cells expressing RecN and RecN-DD were equally sensitive to bleomycin (Figures 2.6B,C). Western blots showed that RecN-DD is about 4-fold more abundant than RecN in a wild-type strain, but protein levels are approximately equal in the protease deficient strains. Therefore, the cell might need RecN in a very specific quantity for a very limited time, and proteolysis helps to ensure this balance and timing.

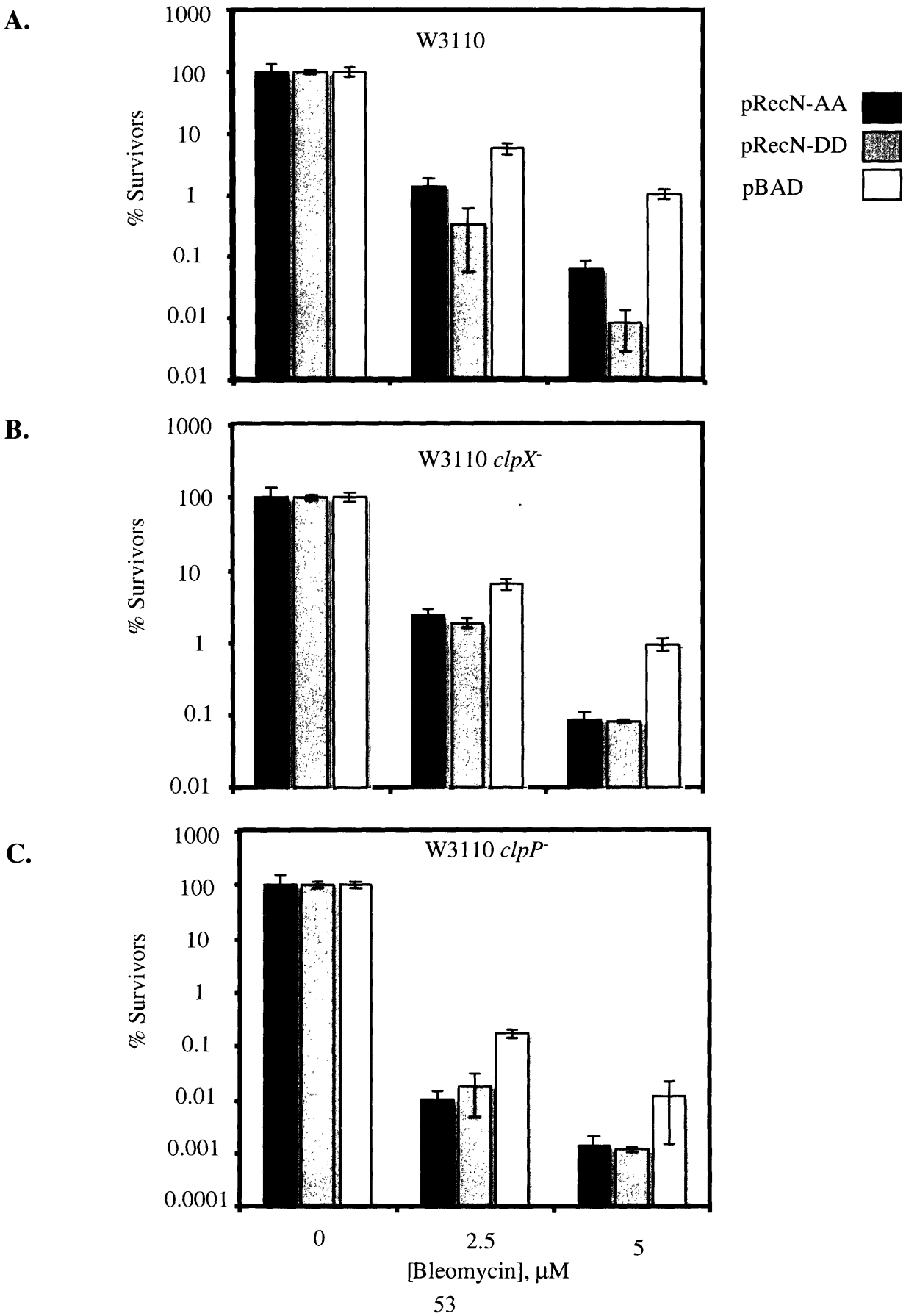

A.
B.
C.
W3110
W3110 clpX-
W3110 clpP-
% Survivors
1000
100
10
1
0.1
0.01
0.001
0.0001
pRecN-AA
pRecN-DD
pBAD
0
2.5
5
[Bleomycin], μM

Figure 2.6: RecN degradation affects cell survival

A. When exposed to the DNA damaging agent bleomycin, cells expressing plasmid-borne degradable recN (RecN-AA) had greater survival than those expressing non-degradable RecN (RecN-DD).

B, C. This effect was not seen in cells lacking ClpX or ClpP.

Discussion

E. coli must reshape its proteome to respond to environmental challenges. New protein synthesis and protein degradation are partners in this restructuring. Our results indicate that substrate selection by the ClpXP protease is a dynamic, environmentally responsive process. DNA damage results in increased capture of not only damage-induced substrates, but also of proteins with no known damage-dependent regulation. Interestingly, our results also reveal a damage-dependent, transcription-independent decrease in the capture of several known ClpXP substrates. Examination of substrates did not reveal a specific unifying feature or peptide motif to upregulate or downregulate degradation following DNA damage. Rather, we expect that unique factors may contribute to the observed changes in trapping for individual or small groups of proteins.

One feature that emerges from these trapping experiments is that stress-inducible proteins may be unusually labile to proteolysis. In *E. coli*, bulk proteolysis proceeds at a very modest 2-3% degraded per hour, and some experiments indicate that the majority of proteins have half-lives longer than a cell generation (Mosteller et al., 1980; Nath and Koch, 1971; Pine, 1970). Furthermore, a number of *E. coli* proteins are so stable that their degradation is virtually undetectable (Kirschbaum et al., 1975; Powell et al., 1973). In contrast, our results and previous results show that 8 of the approximately 30 proteins in the *E. coli* SOS regulon are captured or degraded by ClpXP (Frank et al., 1996; Neher et al., 2003b). Additionally, UmuC and SulA are known substrates for the Lon protease (Frank et al., 1996; Mizusawa and Gottesman, 1983). In several cases (RecN, LexA fragments, SulA, YebG), degradation is very rapid with protein half lives of only a few minutes.

Strong proteolytic regulation of the levels of stress-responsive proteins could allow the cell to maintain precise control over the duration of the stress response. Our experiments and others suggest that in many cases instability is directly encoded in these stress-responsive proteins by protease-recognition motifs. For example the C-terminal residues of SulA directly interact with Lon, and autocleavage of LexA reveals an encoded ClpX-recognition motif (Ishii and Amano, 2001; Ishii et al., 2000; Neher et al., 2003b). If stress-responsive proteins are naturally unstable, then their levels will fall rapidly after loss of the inducing signal ensuring a rapid return to a more normal physiological state.

A potential implication of this work is that instability may be a general feature of members of other stress-inducible regulons.

In addition to these damage-inducible, intrinsically recognized substrates, trapping also increased for a number of proteins with no known damage-dependent transcriptional induction. One Class III substrate, GyrB, is a good candidate for a damage-dependent change in accessibility to ClpXP. Two subunits of GyrB associate with two subunits of GyrA to make DNA gyrase, an enzyme that introduces negative supercoils into DNA. It is the cellular target of nalidixic acid, which causes DNA damage by trapping the DNA-gyrase complex after DNA cleavage and prior to DNA reunion (reviewed in Drlica and Zhao, 1997). Studies using oxolinic acid (a drug in same 4-quinolone family as nalidixic acid) suggest that following drug treatment, gyrase can be released to generate free DNA ends (Chen et al., 1996). However this release, like naldixic acid toxicity, is dependent on new protein synthesis (Chen et al., 1996; Deitz et al., 1966). These results suggest a possible model for the increase in GyrB trapping following nalidixic acid treatment. When DNA gyrase is bound to DNA, it may be inaccessible to ClpXP. However, following treatment the trapped gyrase complex could be released resulting in increased protease susceptibility.

In contrast, DNA damage may decrease ClpX access to some substrates resulting in a reduction in trapping. We suspect that this may explain the reduction in DPS and σ^S trapping. Degradation of both proteins is regulated by very specific stress signals. For σ^S, this regulation relies on the adaptor protein RssB (Muffler et al., 1996). RssB does not regulate DPS degradation, but the highly regulated nature of DPS degradation suggests that a similar, as yet unknown factor may (Stephani et al., 2003). Overexpression of the adaptor protein SspB decreases trapping of both DPS and σ^S, as assayed by 2-D gel electrophoresis (J. Flynn, unpublished). However, we did not observe a DNA-damage dependent change in SspB levels. This suggests the existence of a damage-inducible factor that blocks DPS and σ^S recognition in a SspB-like manner.

Importantly, trapping of approximately half of the identified substrates does not change dramatically. One known ClpXP substrate, Fnr, falls into Class V. Fnr, is a transcriptional regulator controlling the physiological switch between aerobic and anaerobic growth conditions (Khoroshilova et al., 1997). It is a ClpXP substrate both *in*

vivo and *in vitro* (P. Kiley/E. Merrett personal communication). These results indicate that some substrates are consistently degraded with high priority, despite a damage-dependent influx of new substrates.

Following environmental stress, a cell must use all of its regulatory tools to optimize survival. By utilizing quantitative proteomics to compare ClpXP substrate selection with and without DNA damage, our studies reveal the dynamic nature of protease substrate selection. Rather than identifying a single degradation signal we find that substrates and proteases use versatile strategies to ensure degradation of the right protein at the right time. The multiple changes that we observe suggest new strategies for proteolytic regulation.

Materials and Methods

Strains and plasmids

The strain used for trapping was generated by deleting the *leuB* gene as described (Datsenko and Wanner, 2000) from W3110 ΔSmpB-1 (Karzai et al., 1999). Additional protease mutants (*clpX*::Kan, *clpP*::Cm) were introduced by P1 transduction. A plasmid expressing the $ClpP^{trap}$ was derived from pJF105 (Flynn et al., 2003). The Myc affinity tag was replaced with a streptavidin affinity tag by digesting pJF105 with SpeI and HinDIII and inserting an oligo cassette encoding the streptavidin tag. The final C-terminal appended tag is: DSILTHRNRSHHHHHGGENLYFQGAYTSWSHPHFEK. For expression of epitope-tagged substrates, Invitrogen pBAD plasmids were used. N-terminally epitope tagged constructs were amplified from genomic DNA and cloned in to pBAD His A to give a native C-terminus. N-terminally tagged substrates were cloned into the following sites: YebG: EcoRI and BglII, RecN: SacI and HinDIII, DinD: Bgl II and KpnI, UvrA: Xho I and HindIII, arc-st11 control: XhoI and KpnI. The C-terminal AA to DD variant of RecN was generated using Quickchange.

In vivo trapping/metabolic labelling

EZ rich defined media lacking leucine (Teknova) containing 100 μg/mL ampicillin was used for trapping. For heavy and light media, respectively, $U^{13}C$ leucine (Cambridge Isotopes) or light leucine (Sigma) was added at a final concentration of 0.8 mM. Overnight cultures grown in light defined media were pelleted, washed, resuspended in the appropriate media and used 1:1000 to inoculate trapping cultures. Cells were grown to an OD_{600} of 0.2 at 30°C, and nalidixic acid (Sigma) was added to 50 μM to the light culture. At OD_{600} 0.4, cells were induced with 0.2 mM IPTG and grown for an additional 3 hours. Cells were harvested by centrifugation, resuspended in 3 mL/gram buffer N1 (50 mM sodium phosphate pH 8, 300 mM NaCl, 10% glycerol, 5 mM Imidizole), mixed and lysed by French press. To mix an equal number of cells, OD_{600} prior to harvesting, OD_{600} of resuspended cells and weight of cell pellet for heavy and light cultures were measured. These measurements were in good agreement, and so the OD_{600} of resuspended cells was used. Lysate was cleared by centrifugation, and .8 mL/L starting culture Ni-NTA beads (Quiagen) were added to the supernatent. After

incubation at 4°C with rocking for 2 hours, beads were poured in a column and washed with 400 mL N1, 200 mL N1 with 20 mM imidizole, and eluted with 6 mL N1 with 500 mM imidizole. Eluate was added to 4 mL streptactin superflow beads (IBA) and incubated at 4°C with rocking for 4 hours. Beads were poured in a column and washed with 200 mL S1 (50 mM sodium phosphate pH 8, 1 M NaCl, 10% glycerol). The beads were batch eluted using S2 (50 mM sodium phosphate pH 8, 300 mM NaCl, 10% glycerol, 5mM biotin), and eluate was concentrate using an Amicon ultra spin column (Millapore). For *in vivo* trapping without metabolic labeling, samples were prepared essentially as described, except that nalidixic acid was added prior to induction of the $ClpP^{trap}$ (Flynn et al., 2003a).

Mass Spectrometry

Samples for quantitative mass spectrometry were separated by 12.5% SDS-PAGE and trypsinized out of the gel using trypsin gold (Promega) according to the manufacture's protocol. Quantitative mass spectrometry was performed essentially as described (Everley et al., 2004). For non-quantitative mass spectrometry, sample preparation and identification are as described (Flynn et al., 2003).

***In vivo* degradation and Western blotting**

For *In vivo* protein degradation using plasmids, proteins synthesis was induced with .01% arabinose 30 minutes prior to the start of the timecourse. To test the effects of DNA damage, nalidixic acid was added to 50 μM for 30 minutes prior to the start of the timecourse. Protein synthesis in exponentially growing W3110 and W3110 *clpX*::Kan cells was stopped with 100 mg/mL chloramphenicol. Timepoints were sampled on ice cold TCA for a final concentration of 5%. Samples were pelleted, washed with 100% acetone and resuspended in SDS loading buffer. Following separation by SDS-PAGE, samples were transferred to PVDF (Millapore), and Western blots were performed according to the manufacture's protocol for ECF substrate (Amersham). Degradation of N-terminally epitope tagged substrates (RecN, YebG, DinD, Arc-st11) was followed using a-express antibody (Invitrogen). Degradation of C-terminally epitope tagged substrates (UvrA, Arc-st11) was followed using α-Myc antibody. Antibodies for DinI,

DPS, SulA, and MinD were kindly provided by Daniel Camerini-Otero, Regina Hengge, Mike Maurizi and Lawrence Rothfield, respectively.

Bleomycin sensitivity

Bleomycin was obtained from Sigma, and assays were performed essentially as described (Kosa et al., 2004). Induction of plasmid borne RecN, RecN-DD or the empty pBAD-His-A plasmid was achieved by addition of .1% arabinose at OD_{600} 0.1. Assays were started with the addition of Bleomycin at OD_{600} 0.3. All assays were done at least in triplicate.

Table 1: SILAC ratios of captured ClpXP substrates

gene	class	Peptides	H/L ratio	STDEV	Array	function
asnA	1	2	0.04	0.02	2.8 (1)	Asparagine synthetase A
rplM	3	2	0.07	0.02	no	50S ribosomal subunit L13
ydbK	3	2	0.08	0.01	no	Probable oxidoreductase
hslV	3	2	0.09	0.05	no	protease subunit
recN	1	7	0.10	0.02	20 (2)	DS break repair
fabG	3	2	0.11	0.03	no	3-Ketoacyl-ACP reductase
deaD	1	9	0.11	0.06	1.92 (1)	RNA helicase
murA	3	2	0.12	0.04	no	peptidoglycan synthesis
cysk	3	2	0.13	0.06	no	Cysteine synthase
rplE	3	4	0.14	0.01	no	50S ribosomal subunit L5
uvrA	1	4	0.14	0.04	7.0 (1)	Excision nuclease subunit A
cysJ	3	3	0.14	0.03	no	Beta-subunit cystine synthesis
rpsG	3	2	0.15	0.02	no	30S ribosomal subunit protein S7
rplD	3	2	0.17	0.01	no	50S ribosomal subunit protein L4
pta	3	2	0.17	0.05	no	Conversion of acetate to acetyl-CoA
gyrb	3	8	0.18	0.03	no	DNA gyrase, subunit B
rpoD	1	2	0.19	0.03	2 (2)	sigma 70
rfaE	3	3	0.19	0.01	no	Inner core lipopolysaccharide synthesis
rplJ	3	3	0.20	0.02	no	50S ribosomal subunit L10
rho	3	3	0.21	0.04	no	Transcription termination factor
glyA	3	2	0.22	0.02	no	Serine hydroxymethyltransferase
lepA	3	5	0.26	0.03	no	GTPase like
bioC	3	2	0.28	0.01	no	Biotin biosynthesis
carA	3	2	0.29	0.02	no	arginine biosynthesis
rapA	1	2	0.30	0.01	2 (2)	RNA polymerase recycling
yfgB	1	3	0.30	0.05	2.1 (2)	unknown
sucC	3	3	0.30	0.09	no	Succinyl-CoA synthetase beta-subunit
rplB	3	3	0.32	0.04	no	50S ribosomal subunit protein L2
sthA	3	2	0.33	0.04	no	pyridine nucleotide transhydrogenase
rplO	1	2	0.34	0.01	1.84 (1)	50S ribosomal subunit protein L15
tig	5	2	0.37	0.01	no	trigger factor
dcrB	5	4	0.37	0.05	no	phage absorbtion factor
yefI	5	2	0.38	0.19	no	unknown,glycosyltransferase like
acnB	5	5	0.38	0.07	no	Aconitase
yibN	5	3	0.38	0.05	no	unknown
rpsA	5	4	0.40	0.05	no	30S ribosomal subunit protein S1
rpoA	5	3	0.40	0.05	2.6 (1)	RNA Pol A subunit
yciW	5	3	0.41	0.00	no	unknown, oxidoreductase-like
yaeT	5	3	0.42	0.02	1.7 (1)	unknown
clpx	5	3	0.45	0.05	no	Protease subunit, ATPase
oxaA	5	2	0.46	0.04	no	helps insert proteins into membrane
metk	5	2	0.47	0.03	no	Methionine adenosyltransferase
fur	5	2	0.49	0.04	no	Ferric uptake regulation
prs	5	2	0.49	0.06	no	Phosphoribosylpyrophosphate synthetase
fnr	5	2	0.50	0.03	no	Fumarate-nitrate-reductase
pnp	5	3	0.52	0.10	no	Polynucleotide phosphorylase
hslU	5	3	0.53	0.06	1.7 (2)	Protease subunit, ATPase
rfbc	5	2	0.53	0.04	no	dTDP-4-deoxyrhamnose-3,5-epimerase
lpdA	5	4	0.53	0.07	no	Lipoamide dehydrogenase
sdhA	5	2	0.54	0.02	no	Succinate dehydrogenase subunit

gene	class	Peptides	H/L ratio	STDEV	Array	function
bfr	5	3	0.55	0.04	no	iron storage
fusA	5	6	0.55	0.07	no	translation elongation factor
aceF	5	2	0.57	0.02	1,95 (1)	Pyruvate dehydrogenase
infB	5	6	0.58	0.12	no	initiation factor
ffh	5	2	0.62	0.12	no	4.5S-RNP protein, SRP
yrbD	5	2	0.63	0.02	no	unknown
thrS	5	4	0.66	0.01	no	threonyl-tRNA synthetase
sucA	5	4	0.66	0.04	no	alpha keto gluterate dehydrogenase
yefG	5	3	0.67	0.05	no	possible glycosyltransferase
rne	5	4	0.69	0.02	no	mRNA trunover, processing
yjjk	5	7	0.72	0.08	no	unknown,, ABC transporter binding?
mreB	5	4	0.72	0.07	no	chromosome movement/segregation?
nuoG	5	2	0.72	0.00	no	NADH:ubiquinone oxidoreductase
dnaJ	5	3	0.73	0.12	no	chaperone
yaeQ	5	2	0.74	0.01	no	unknown
yqaB	5	2	0.79	0.09	no	putative phosphatase
atpB	5	5	0.79	0.07	no	membrane bound ATPase
ribD	5	2	0.83	0.19	no	riboflavin biosynthesis
exbb	5	3	0.83	0.04	no	iron uptake
ndh	5	13	0.86	0.10	1.7 (1)	NADH dehydrogenase
purD	5	2	0.93	0.01	1.62 (1)	Phosphoribosylglycinamide synthetase
purA	5	2	0.94	0.01	1.8 (1)	Adenylosuccinate synthetase
secD	5	2	0.95	0.08	no	membrane component of protein export
eno	5	2	1.01	0.07	no	glycolytic enzyme
ahpF	5	2	1.02	0.05	no	Alkyl hydroperoxide
hisB	5	4	1.06	0.09	no	IGP dehydratase, histidinol phosphatase
znuA	5	6	1.06	0.05	no	ABC zinc transporter
clpP	5	8	1.14	0.12	no	protease
yrbH	5	5	1.14	0.09	no	Arabinose 5-phosphate isomerase
arcA	5	4	1.14	0.11	no	2-component redox response regulator
yhbJ	5	4	1.29	0.17	no	unknown
dacA	5	4	1.43	0.08	no	Peptidoglycan synthesis
xerd	5	3	1.50	0.07	no	recombinase
guaA	5	5	1.53	0.14	1.8 (2)	GMP syntahase
cyoA	5	3	1.74	0.11	1.8 (1)	Cytochrome oxidase
acrB	5	3	1.78	0.13	no	drug eflux pump
znuc	5	2	1.85	0.27	no	ABC transporter for zinc
cysD	5	3	2.01	0.24	no	Sulfate adenylyltransferase
metQ	5	6	2.10	0.30	no	D-methionine transport
ydgA	5	2	2.22	0.47	no	unknown
rsd	5	2	2.53	0.07	1.7 (2)	regulates sigma 70
purM	4	3	3.22	0.12	no	Phosphoribosylaminoimidazole synthetase
pflB	4	2	3.34	0.38	no	Pyruvate formate-lyase
minD	2	2	3.63	0.35	.45 (2)	membrane ATPase activated minC
pykA	4	4	3.78	0.37	no	pyruvate kinase
cysA	4	6	4.14	0.48	no	Sulfate permease
dps	4	5	4.30	0.28	no	stationary phase DNA binding
cysC	4	2	4.40	0.25	no	Adenylylsulfate kinase
adhE	4	2	4.82	0.53	no	alcohol dehydrogenase
yfbG	4	2	5.46	3.26	no	associated w/ ssra/smpb comples
ftsA	4	2	5.48	0.50	no	septation at Z ring
oppF	4	3	6.48	1.61	no	component of peptide transporter

gene	class	Peptides	H/L ratio	STDEV	Array	Function
ydam	4	3	7.04	1.37	no	unknown
rpoS	4	14	7.48	1.10	no	Stationary phase sigma
putA	4	2	8.07	1.25	no	proline dehydrogenase
cysN	4	2	8.58	1.82	no	ATP sulfurylase
gldA	4	2	8.77	1.59	no	glycerol dehydrogenase
metE	4	3	12.32	5.14	no	methyltransferase
adhP	4	2	13.29	0.92	no	alcohol dehydrogenase
gadA	4	3	15.69	8.28	no	glutamate decarboxylase
slp	4	2	16.16	7.69	no	carbon starvatin outer membrane
ycbW	4	2	22.79	2.48	no	unknown
glgA	2	3	24.25	6.00	.39 (2)	glycogen biosynthesis
dacC	4	2	24.38	1.01	no	Peptidoglycan synthesis
argM	4	8	26.19	20.30	no	amino acid catabolism

Table 1: SILAC rations of captured substrates. The Peptides column indicates the number of unique peptides used in determining the SILAC ratios. H/L is the ratio of heavy (untreated) to light (treated) peptides for each sample. The array column gives information about transcriptional changes from two published sources: Courcelle et al. (1) and Quillard et al. (2).

Table S1: ClpP interactors from *clpX*$^-$, *clpA*$^-$ cells

gene	GI	P-interactor?*
atpA	399079	no
atpD	16131600	no
carB	16132067	no
crp	16131236	no
dnaK	16128008	yes
ftsZ	16128088	no
glmS	1790167	no
groL	16131968	yes
lacZ	114939	no
lon	16128424	no
ompA	49176370	no
recA	16130606	yes
rplP	16131192	no
rpoB	16131817	no
rpoC	16131818	yes
rpsJ	16131200	yes
slyD	16131228	no
trkA	16131169	no
tufA/B	1789737	yes
yeiE	49176192	no

* From Butland et al., Nature 433(7025):531-7

CHAPTER 3:

LATENT CLPX-RECOGNITION SIGNALS ENSURE LEXA DESTRUCTION AFTER DNA DAMAGE.

This chapter was originally published in *Genes and Development* **17**: 1084-9 (2003) as Neher, S.B., Flynn, J.M. Sauer, R.T., and Baker, T.A. J.M. Flynn originally identified LexA as one of the substrates captured by $ClpP^{trap}$ during normal growth. R.T. Sauer and T.A. Baker assisted in preparation of the manuscript.

Abstract

The DNA-damage response genes in bacteria are upregulated when LexA repressor undergoes autocatalytic cleavage stimulated by activated RecA protein. Intact LexA is stable to intracellular degradation but its auto-cleavage fragments are degraded rapidly. Here, both fragments of LexA are shown to be substrates for the ClpXP protease. ClpXP recognizes these fragments using sequence motifs that flank the auto-cleavage site but are dormant in intact LexA. Furthermore, we find that ClpXP degradation of the LexA-DNA binding fragment is important to cell survival after DNA-damage. These results demonstrate how one protein-processing event can activate latent protease-recognition signals, triggering a cascade of protein turnover in response to environmental stress.

INTRODUCTION

Protein degradation plays a critical role in allowing cells to adjust to changing conditions. Intracellular proteolysis is an essential component of many response pathways that permit bacteria to survive or recover from DNA damage, heat shock, or attack by reactive oxygen species (Gerth et al., 1998; Mizusawa and Gottesman, 1983; Robertson et al., 2002). In addition to stress-related regulatory functions, proteases serve to degrade damaged proteins as well as undamaged proteins that the cell no longer needs under a new set of environmental conditions. In each of these examples, one can ask the same question: what mechanisms allow intracellular proteases to degrade the appropriate substrates at the right time?

In bacteria, many intracellular proteases (e.g., ClpXP, ClpAP, and HslUV) divide the tasks of substrate recognition and proteolysis between a hexameric AAA+ ATPase and a multi-subunit peptidase with an internal degradation chamber (reviewed in Gottesman, 1996). The ATPase (ClpX, ClpA or HslU) recognizes specific substrates, unfolds these proteins, and translocates the denatured polypeptide into the peptidase (ClpP or HslV) chamber for degradation (Kim et al., 2000). In many instances, the ATPase components recognize substrates via specific peptide motifs, often at their N- or C-termini (Flynn et al., 2003a; Gonciarz-Swiatek et al., 1999; Levchenko et al., 1995). For example, the ssrA degradation tag, an 11-residue C-terminal peptide sequence, targets proteins for degradation by ClpXP and ClpAP (Gottesman et al., 1998). For some substrates, such as the stationary-phase sigma factor (σ^S), accessory proteins are needed for efficient degradation, allowing regulation of proteolysis through the synthesis or modification of these factors (Becker et al., 1999; Zhou et al., 2001). Here we provide evidence for an alternative mechanism of regulated degradation: We find that complete degradation of the LexA repressor requires an initiating cleavage event that is regulated in response to DNA damage.

The SOS regulatory system in E. coli controls the cellular response to DNA damage (reviewed in Freidberg et al., 1995). Under normal conditions, LexA repressor dimers negatively regulate the expression of genes involved in DNA repair, replication, and cell division. After DNA damage, single-stranded DNA is exposed and is bound by

the RecA protein. This event activates RecA, which then stimulates the auto-cleavage of LexA at a site between its N-terminal DNA binding domain and its C-terminal dimerization domain (Little, 1984; Phizicky and Roberts, 1981). The resulting N- and C-terminal fragments are then degraded rapidly (Little, 1983a). The C-terminal fragment is stabilized approximately 10-fold in Lon-defective cells (Little, 1983b), but the protease that degrades the N-terminal fragment has not previously been identified.

In recent proteomic experiments, we identified tryptic fragments from E. coli proteins that were trapped in vivo within a proteolytically inactive ClpP variant ($ClpP^{trap}$) and found that some peptides originated from LexA (Flynn et al., 2003). Western blots of the undigested trapped proteins indicated that the two auto-cleavage fragments of LexA represented the dominant captured forms. This result suggested that these autocleavage fragments might be ClpXP substrates. Here, we show that ClpXP degrades the autocleavage fragments of LexA, but not full-length LexA, both *in vivo* and *in vitro*. Recognition of these fragments for destruction occurs via peptide signals created or exposed by the initial auto-cleavage event. The use of these latent recognition signals allows specific recognition and degradation of the LexA fragments by ClpXP at the biologically appropriate time-after LexA has undergone RecA-stimulated self-cleavage in response to DNA damage.

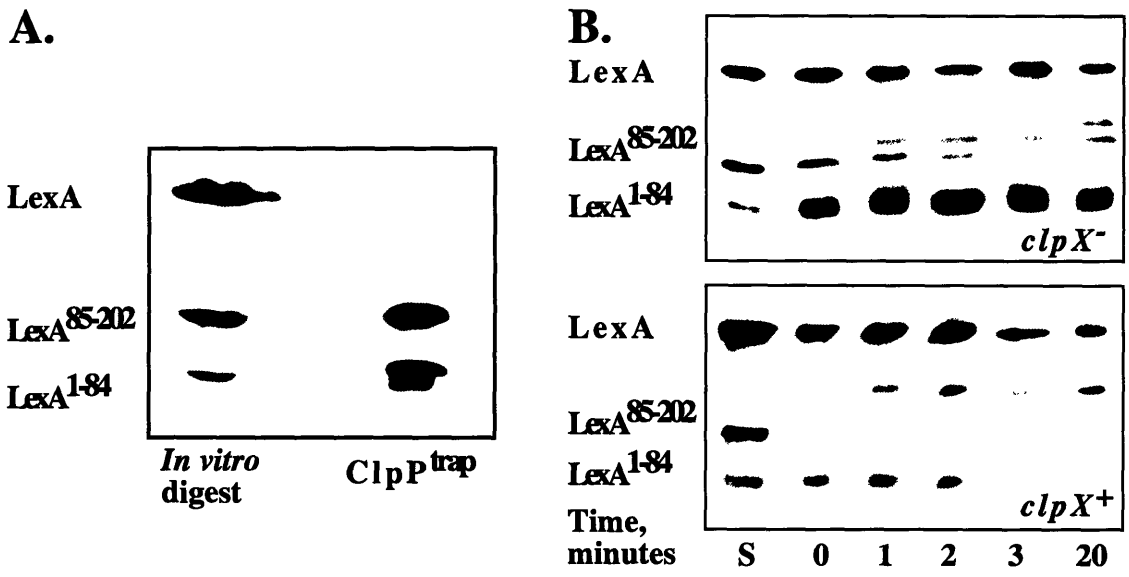
RESULTS AND DISCUSSION

The LexA auto-cleavage fragments are ClpXP substrates.

Capture experiments using $ClpP^{trap}$ were performed in E. coli exposed to the DNA-damaging agent nalidixic acid. The $ClpP^{trap}$ is an inactive (S97A) affinity tagged variant of ClpP that, when expressed in cells, allows the capture and purification of physiological ClpXP substrates (Flynn et al., 2003). A western blot of the trapped material revealed two bands that cross reacted with anti-LexA antibodies and had the same electrophoretic mobilities as the $LexA^{1-84}$ and $LexA^{85-202}$ fragments generated by autocleavage of purified LexA (Figure 3.1A). No full-length LexA was detected in the trapped material although we observed approximately 20 ng of each cleavage fragment and could detect as little as 1 ng of LexA protein. Furthermore, when purified LexA was added to a sample of the trapped material and prepared for western blotting, this protein was efficiently detected, indicating that full-length LexA could have survived this procedure. Trapping required ClpX as the LexA fragments were not detected in material isolated from *clpX*$^-$ cells (Flynn et al., 2003). We conclude that ClpX recognizes the two auto-cleavage fragments but not full-length LexA.

To determine the stability of the various forms of LexA *in vivo*, we induced the DNA-damage response with nalidixic acid, blocked protein synthesis, and measured the half-lives of intact LexA and the two cleavage fragments. The N-terminal $LexA^{1-84}$ fragment was rapidly degraded in a wild-type strain (half-life 2-5 min) but was not degraded to any detectable extent in an otherwise isogenic *clpX*$^-$ strain (Figure 3.1B). The C-terminal $LexA^{85-202}$ fragment was also unstable in wild-type cells (half-life ~1 min) but was only stabilized modestly in the *clpX*$^-$ cells (half life ~2 min), presumably because Lon protease also contributes to its degradation. Full-length LexA appeared to be reasonably stable to degradation in the wild-type and *clpX*$^-$ strains, although it was susceptible to continued auto-cleavage (Figure 3.1B).

To determine if the LexA fragments were indeed ClpXP substrates, we assayed for degradation *in vitro*. Purified LexA, $LexA^{1-84}$ and $LexA^{85-202}$ were incubated with ClpXP and an ATP regenerating system, and degradation was assayed by SDS-PAGE


A.
LexA
LexA85-202
LexA1-84
In vitro digest
ClpPtrap
B.
LexA
LexA85-202
LexA1-84
clpX-
LexA
LexA85-202
LexA1-84
clpX+
Time, minutes
S 0 1 2 3 20

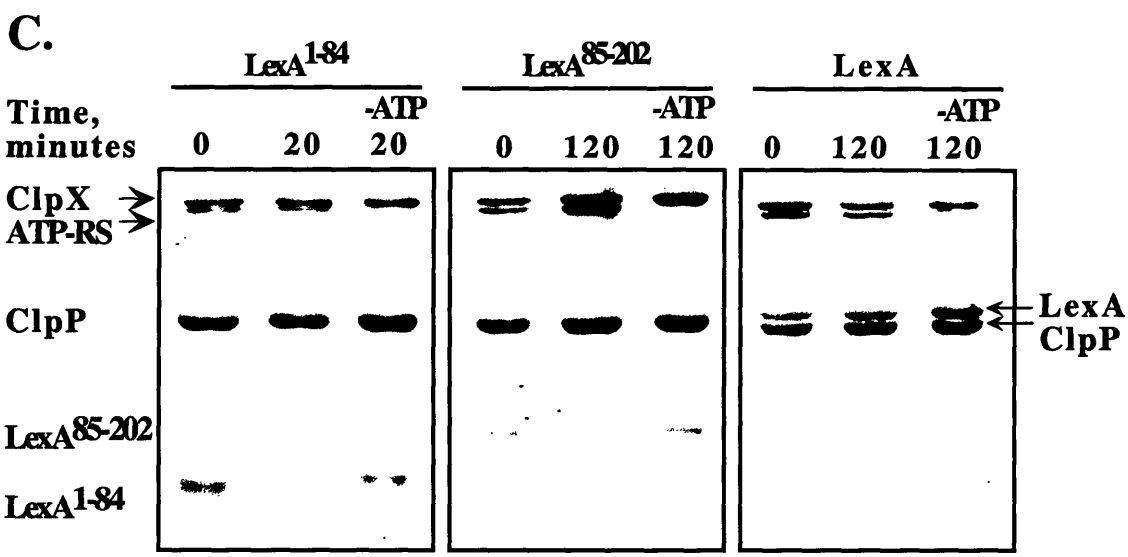

C.
LexA1-84
LexA85-202
LexA
Time, minutes
-ATP
0 20 20
0 120 120
0 120 120
ClpX
ATP-RS
ClpP
LexA85-202
LexA1-84
LexA
ClpP

Figure 3.1. LexA auto-cleavage fragments are captured and degraded by ClpXP.
A. Western blot using anti-LexA antibodies of the LexA species captured by the $ClpP^{trap}$ and generated by auto-cleavage *in vitro*.
B. The proteolytic stability of LexA, $LexA^{1-84}$ and $LexA^{85-202}$ *in vivo* was determined in *clpX*$^+$ (MC4100) and *clpX*$^-$ (SG22101) strains bearing plasmid pJL42. DNA damage was induced using nalidixic acid, and stability was measured after stopping protein synthesis. LexA was detected using anti-LexA antibody. LexA, $LexA^{1-84}$ and $LexA^{85-202}$ were identified by comparison with autocleaved LexA (lane S). It is difficult to see the continued disappearence of LexA, as expected due to auto-processing because the western blots are mildly overexposed to allow observation of the low levels of $LexA^{85-202}$.
C. Stability of purified LexA, $LexA^{1-84}$ and $LexA^{85-202}$ was measured using purified substrate, ClpX and ClpP. The band marked ATP-RS is creatine kinase, which has been added as part of the ATP-regenerating system. More complete time courses are presented in figures 3.2 and 3.4.

(Figure 3.1C). ClpXP degraded both fragments but not LexA in ATP-dependent reactions. Hence, both auto-cleavage fragments of LexA are ClpXP substrates, whereas the full-length protein is not.

The new C-terminal sequence of LexA$^{1-84}$ targets it to ClpXP

RecA-stimulated auto-cleavage of LexA creates a Val82-Ala83-Ala84-COOH sequence at the end of the N-terminal fragment, which is very similar to the Leu-Ala-Ala-COOH sequence at the end of the ssrA degradation tag (Keiler et al., 1996; Tu et al., 1995). These residues of the ssrA tag are the principal determinants of recognition by ClpX (Flynn et al., 2001). To test the importance of the C-terminal residues of LexA$^{1-84}$ for ClpXP degradation, we constructed a variant (LexA^{1-84DD}) with aspartic acids replacing both alanines. In ClpXP degradation assays in vitro (Figure 3.2A), LexA^{1-84DD} was not degraded to any detectable extent, whereas LexA$^{1-84}$ was degraded with a half-life of approximately 10 minutes. Thus, these alanines play a major role in targeting the N-terminal LexA fragment for degradation by ClpXP.

To test whether these alanines need to be at the C-terminus for recognition we constructed and purified a variant, LexA$^{1-87}$, extended by the next three residues (Gly^{85}-Glu^{86}-Pro^{87}) of intact LexA. ClpXP did not degrade this variant (Figure 3.2A), revealing that the Val^{82}-Ala^{83}-Ala^{84} sequence must be at the C-terminal end for efficient degradation. LexA$^{1-84}$ also inhibited degradation of another ClpXP substrate, GFP bearing an ssrA degradation tag, suggesting that LexA$^{1-84}$ and ssrA-tagged proteins compete for ClpX recognition or other processes involved in degradation (Figure 3.2B). By contrast, LexA^{1-84DD}, LexA$^{1-87}$, and full-length LexA failed to inhibit GFP-ssrA degradation. Together, these results demonstrate that LexA$^{1-84}$ carries a functional ClpX-recognition signal, whereas LexA^{1-84DD}, LexA$^{1-87}$, and the full-length protein lack an accessible or functional signal.

The Val^{82}-Ala^{83}-Ala^{84} sequence is within a folded region in the LexA crystal structure, and the side chain of Val^{82} is buried and packs against residues in the C-terminal domain (figure 3.3, Luo et al., 2001). As a consequence, it seems likely that auto-cleavage results in exposure and increased flexibility of the Val^{82}-Ala^{83}-Ala^{84} sequence, allowing unimpeded interactions with ClpX. However, as the LexA$^{1-87}$

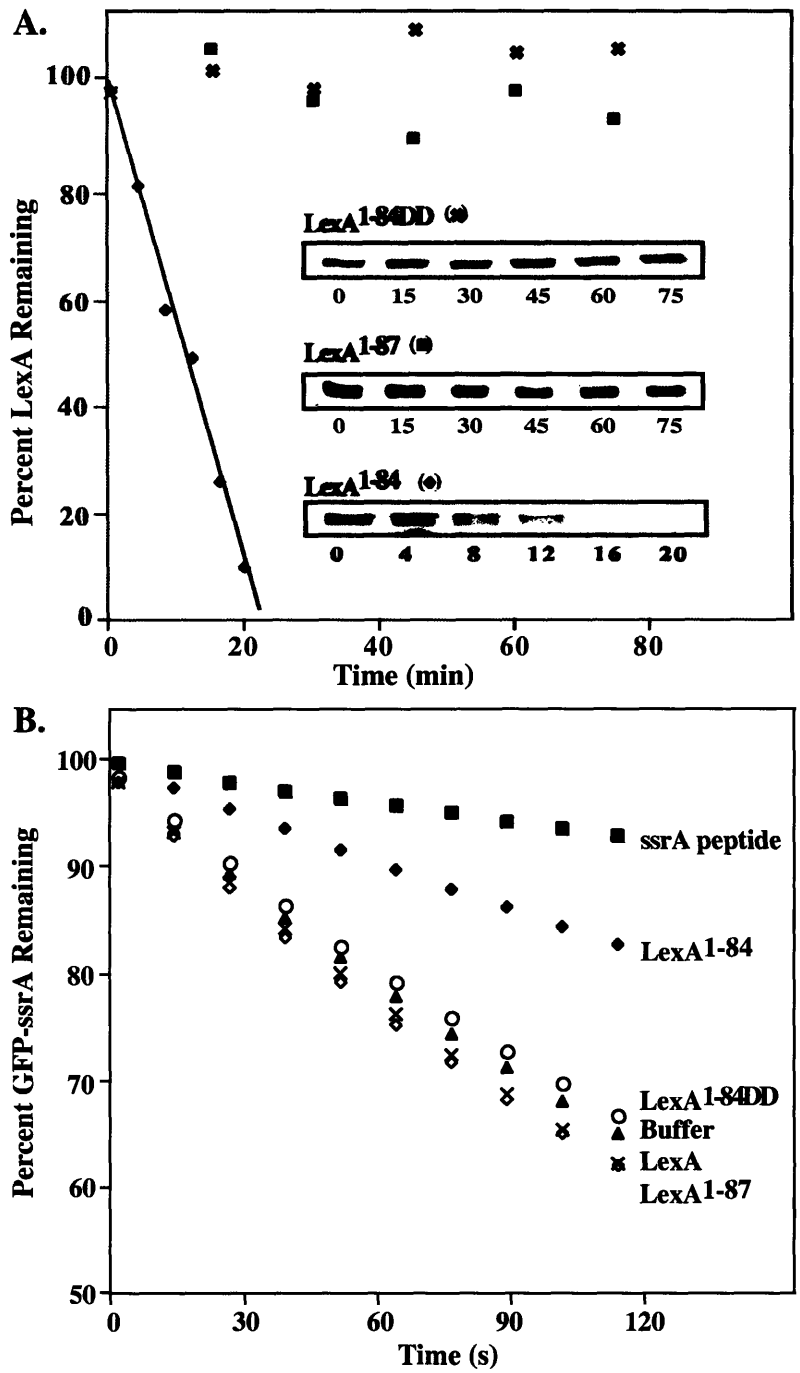


Figure 3.2. The new C-terminus of LexA$^{1\text{-}84}$ directs its degradation.

A. A time course of ClpXP degradation of LexA$^{1\text{-}84}$, LexA$^{1\text{-}87}$ and LexA$^{1\text{-}84DD}$ was quantified after SDS-PAGE. **B.** ClpXP degradation of GFP-ssrA was measured by loss of fluorescence at 511 nm in the presence of various forms of LexA at 20μM. An effective inhibitor of GFP-ssrA degradation, the ssrA peptide (20μM), is included for comparison.

fragment is not a ClpXP substrate, improved accessibility of ClpX to the Val^{82}-Ala^{83}-Ala^{84} sequence is not sufficient to explain how auto-cleavage activates proteolysis of this domain. Based on peptide studies, the α-COOH group of the ssrA degradation tag has been shown to be an important determinant for ClpX recognition (Kim et al., 2000). The resistance of $LexA^{1-87}$ to degradation strongly suggests that the a-COOH group of Ala^{84} is similarly important for recognition of $LexA^{1-84}$. Thus, our results indicate that auto-cleavage of the LexA Ala^{84}-Gly^{85} peptide bond directly creates an essential portion of the degradation signal for the resulting N-terminal fragment.

Sequences that target the C-terminal LexA fragment to ClpXP

Although most $LexA^{85-202}$ molecules seem to be degraded by Lon protease *in vivo* (Little, 1983b) our experiments show that $LexA^{85-202}$ is also degraded by ClpXP (Figures 3.1B, 3.1C). To search for peptide sequences within $LexA^{85-202}$ that might mediate ClpX recognition we tested which regions of this fragment bound to ClpX (Figure 3.4A). We prepared a covalent array of synthetic 12-residue peptides of the $LexA^{85-202}$ fragment, each sharing a 10-residue overlap with its sequence neighbors. The peptide filter was incubated with ClpX, washed, and bound ClpX was detected using an anti-ClpX antibody. Peptides corresponding to the first 26 amino acids of $LexA^{85-202}$ bound ClpX poorly. In contrast, a cluster of adjacent peptides containing sequences from 103-126 bound ClpX reasonably well. Hence, this region of the LexA sequence may contain a ClpX-recognition signal.

To investigate the function of this sequence, we constructed two fusion proteins consisting of LexA residues 85-103 or 85-126 attached to the Arc-st11 protein, a variant of the Arc repressor with a stabilizing C-terminal sequence (Milla et al., 1993). ClpXP degraded the $LexA^{85-126}$ fusion protein with a half-life (50±9 minutes) similar to the half-life for $LexA^{85-202}$ (60±3 minutes, Figure 3.4B). This result shows that sequence information between residues 85 and 126 of LexA is sufficient to target a protein to ClpXP for degradation. In contrast, the $LexA^{85-103}$ fusion protein was degraded substantially more slowly ($t_{1/2}$=184±24 minutes), indicating that the most important determinants for ClpXP degradation are located between residues 104 and 126, and not between residues 85 and 103.

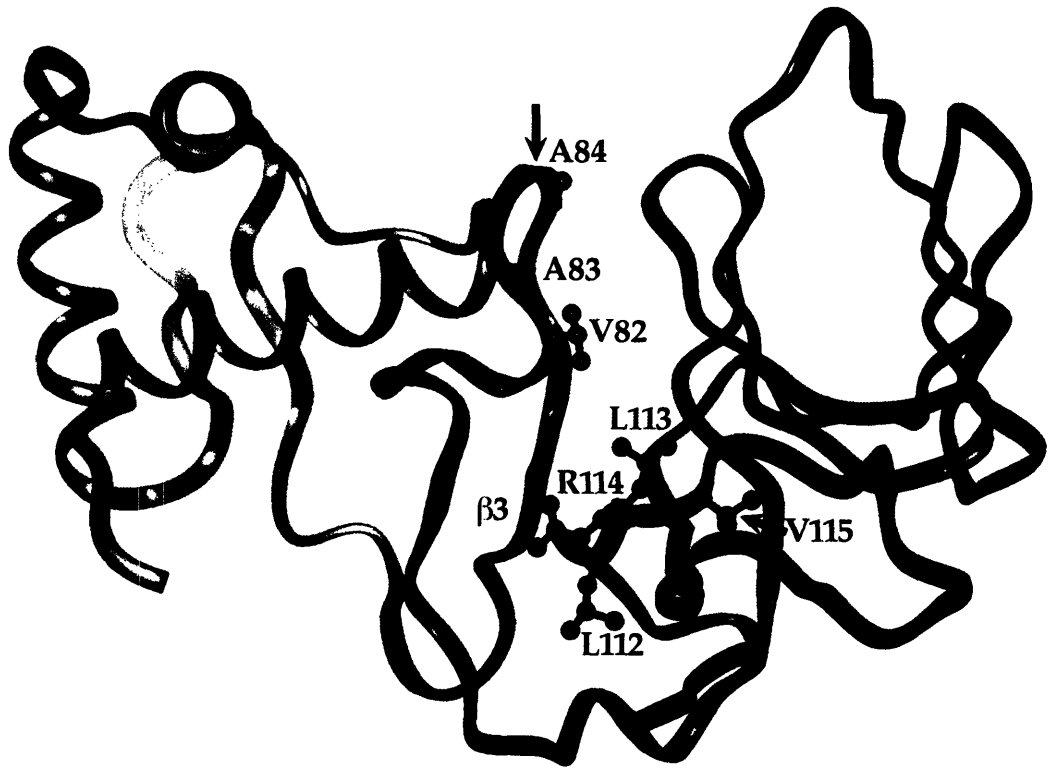


Figure 3.3. Location of latent ClpX-recognition signals in the structure of full-length LexA

The N-terminal domain is green, and the C-terminal domain is blue. Residues 82-84 are highlighted in light green and labeled. The proposed ClpX recognition site in the C-terminal domain is highlighted in light blue and labeled. The cleavage site is indicated by an arrow (Structure from Luo et al., 2001).

The 104-126 region contains a sequence (Leu^{112}-Leu^{113}-Arg^{114}-Val^{115}-Ser^{116}) with some similarity to a peptide motif proposed to function as a ClpX recognition sequence (Flynn et al., 2003). To test the importance of the LexA sequence motif, we constructed $LexA^{85-126}$ fusion proteins containing the Arg114→Asp or Val115→Asp mutations and found that both slowed ClpXP degradation ($t_{1/2}$= 118±22 and 122±30 minutes, respectively, Figure 3.4B). Taken together, these data support the conclusion that ClpX recognizes a region of the polypeptide chain about 30 residues distal from the new N-terminus of $LexA^{85-202}$. Other sequences, however, may also contribute to ClpX recognition of $LexA^{85-202}$.

Unlike $LexA^{1-84}$, the ClpX-targeting signal on the C-terminal domain is not directly adjacent to the site of auto-cleavage. To account for the fact that intact LexA, which contains the same peptide sequences as $LexA^{85-202}$, does not interact with ClpX, we propose that auto-cleavage disrupts the structure of the C-terminal domain in some fashion, helping to expose the 112-116 peptide signal. Although we do not know the structure of the isolated C-terminal domain, it is reasonable to propose that the 112-116 region will be more flexible and exposed after cleavage. In the structure of the full-length protein, a β-strand (β3) from the N-terminal domain forms an integral part of the C-terminal domain and contacts the proposed recognition signal. The loss of these contacts following cleavage may result in exposure of this signal to ClpXP (Figure 3.3).

ClpX helps cells survive DNA damage

To test the importance of ClpX in the overall cellular response to DNA damage, we assayed cell survival after exposure to increasing doses of UV irradiation (Figure 3.5A). These experiments showed that *clpX*$^-$ cells were more sensitive to UV irradiation than their wild-type counterparts, with the effect being most pronounced at the highest UV doses. When exposed to a UV dose of 200 J/m^2, *clpX*$^-$ cells had a roughly 10-fold lower survival frequency than wild-type cells. Because the N-terminal DNA-binding domain of LexA can function as a repressor on its own (Schmidt-Dorr et al., 1991), one interpretation of this result is that ClpXP degradation of this fragment may be required to allow maximal expression of one or more DNA-damage inducible gene products, thereby improving survival following near-lethal UV doses. Alternatively, the loss of ClpX could

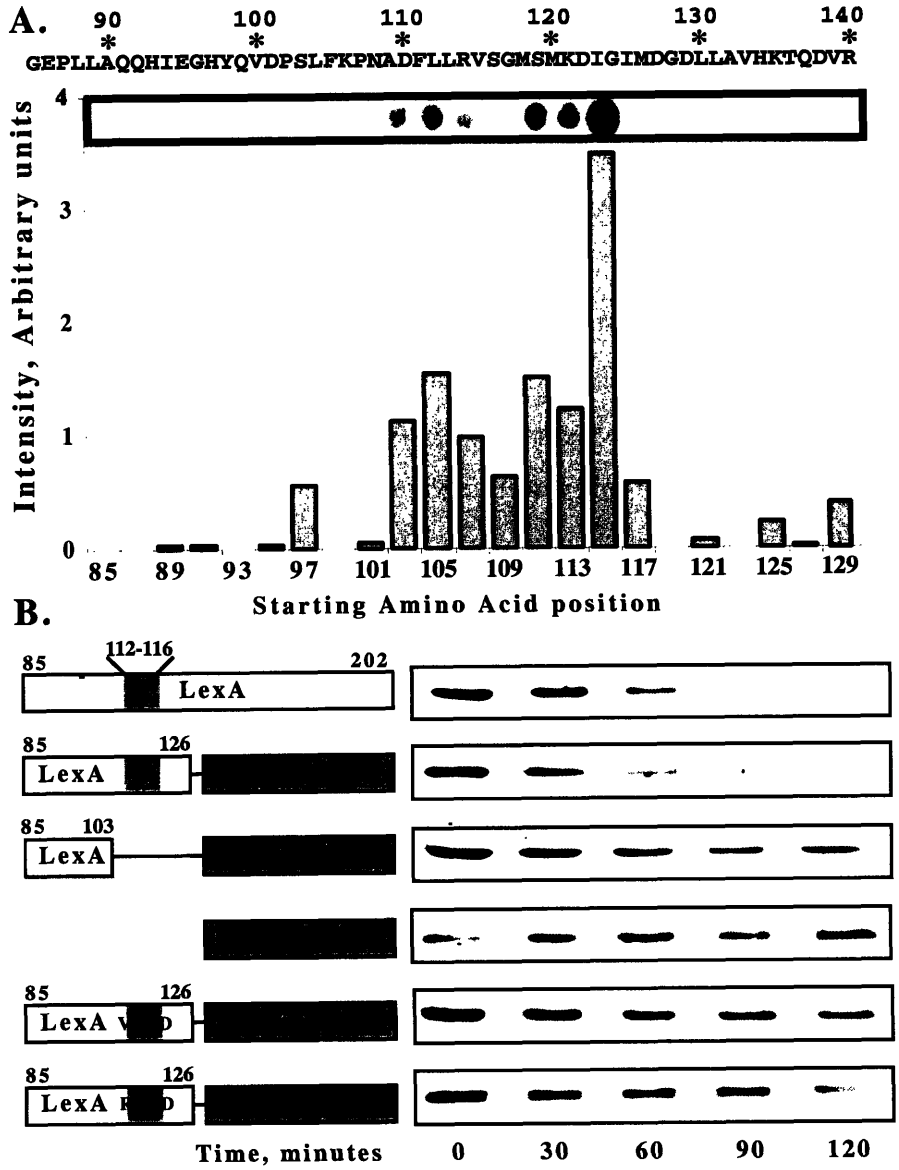


Figure 3.4. Identification of residues in LexA$^{85-202}$ important for ClpXP recognition.

A. An array of LexA peptides was incubated with purified ClpX protein, and bound ClpX was detected using anti-ClpX antibody. The chart shows the relative intensity of ClpX binding to individual sequences. The starting amino acid positions of the 12-residue peptides are listed below the chart. The signal intensity of the peptide beginning at residue 115 may be artificially high. In other experiments, we have observed high signals from different peptides with the same three initial residues (VSG).

B. Degradation of LexA$^{85-202}$ and LexA-Arc-st11 fusion proteins by ClpXP *in vitro*.

perturb the levels of proteins other than the LexA1-84 (e.g. UmuD', a known ClpXP substrate) thus affecting survival (Gonzalez et al., 2000).

To determine if excess levels of $LexA^{1-84}$ causes increased UV sensitivity, we tested the effect of expressing $LexA^{1-84}$ and $LexA^{1-84DD}$ on the percentage of cells surviving a fixed UV dose. We assayed survival of wild-type cells containing plasmid-born genes for either $LexA^{1-84}$ or $LexA^{1-84DD}$ under the control of the native LexA promoter (Figure 3.5B); western analysis revealed that these fragments were modestly overexpressed (2-4 fold, with $LexA^{1-84DD}$ accumulating to higher levels than $LexA^{1-84}$) compared to the chromosomal encoded LexA (data not shown). Cells expressing $LexA^{1-84DD}$ had approximately 10-fold lower survival than those with the $LexA^{1-84}$ producing plasmid. Furthermore, both strains were more sensitive to UV-irradiation than cells bearing the empty plasmid. Thus, accumulation of the LexA DNA-binding domain is deleterious to cell survival after DNA damage.

These data reveal that timely destruction of $LexA^{1-84}$ following damage-induced auto-cleavage is likely to play an important biological role. We suggest that failure to degrade the $LexA^{1-84}$ fragment following DNA damage results in the retention of some repressor activity and a consequent failure to fully induce one or more DNA-damage genes. This residual repressor activity, in turn, may account for our finding that ClpX improves bacterial survival following near-lethal UV doses. Our observation that cells expressing even modest levels of the nondegradable $LexA^{1-84DD}$ are more sensitive to UV irradiation than those expressing $LexA^{1-84}$ supports this theory, although we can't currently rule out more indirect explanations. In preliminary work, we did not detect reproducible differences in expression of the LexA-controlled *sulA*, *recA* and *uvrA* genes following DNA-damage in wild-type versus *clpX*$^-$ defective cells (data not shown), but there are more than 20 genes under LexA control and some may be more sensitive than others to repression mediated by the $LexA^{1-84}$ fragment.

Changes in environmental conditions often result in the modification of regulatory proteins by phosphorylation or proteolytic cleavage. Similarly, protein-binding partners often change during the progression of a biochemical pathway. The results presented here show that the ClpXP degrades the two LexA cleavage fragments, but not the intact protein. By mapping recognition signals in $LexA^{1-84}$ and $LexA^{85-202}$ we show that auto-

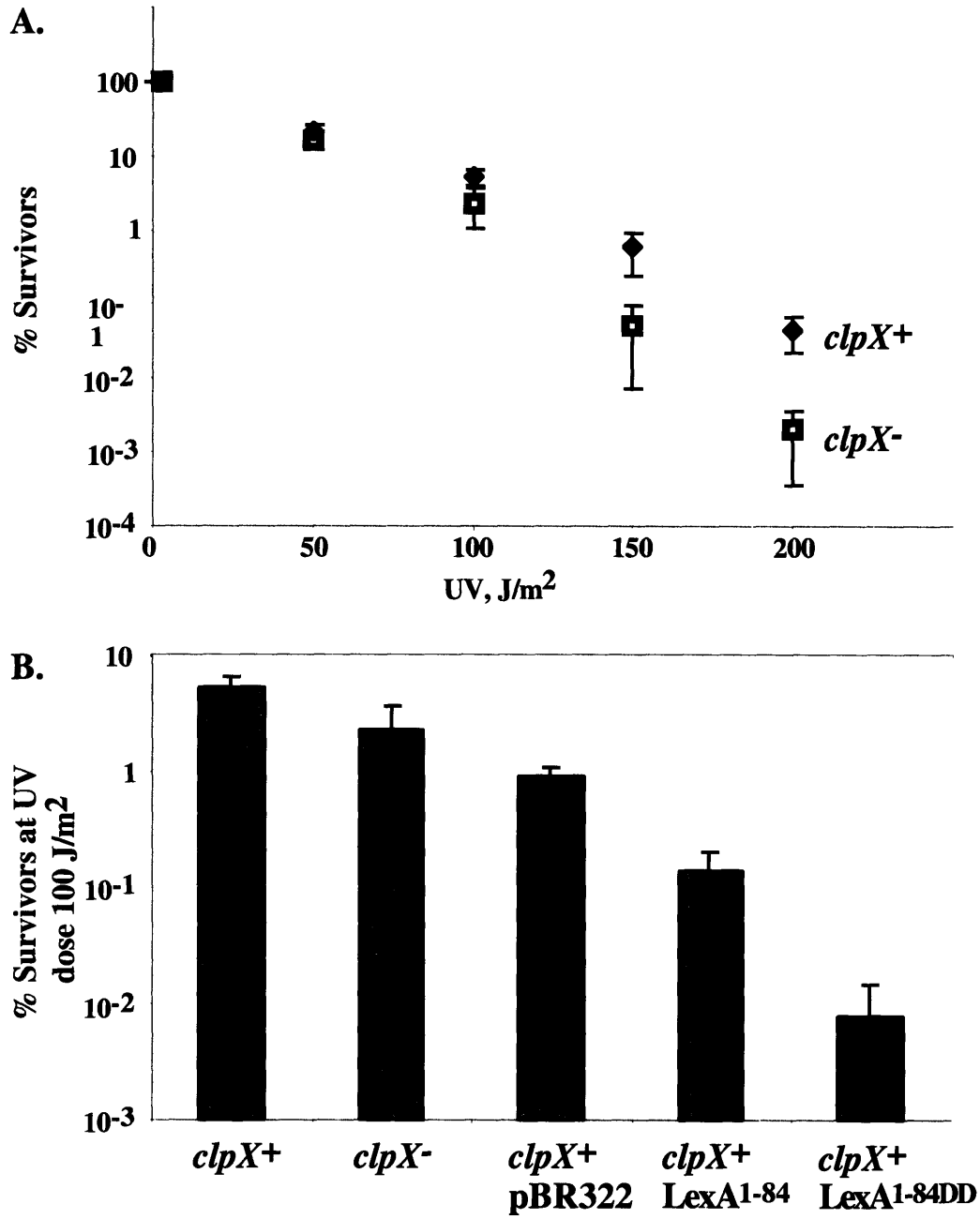


Figure 3.5. Cells accumulating LexA$^{1-84}$ are UV-sensitive.

A. Survival after UV irradiation of *clpX*$^+$ (MC4100) and *clpX*$^-$ (SG22101) cells. The percentage of surviving cells is plotted against UV dose. Each point is the average (±SD) of three trials.

B. Survival of MC4100 cells, SG22101 cells and MC4100 cells containing the empty parent vector or plasmids directing expression of LexA$^{1-84}$ or LexA^{1-84DD} was compared at a UV dose of 100 J/m^2.

cleavage activates otherwise dormant protease recognition signals in both fragments. Our analysis of the mechanisms used to target LexA to ClpXP thus highlights how changes in protein primary or tertiary structure can be coupled to the exposure of recognition signals for a destructive protease, thereby triggering a cascade of protein turnover in response to environmental change.

LexA is a member of a protein family that includes several phage repressors that also undergo RecA-mediated auto-cleavage (Eguchi et al., 1988; Little, 1984; Sauer et al., 1982). It will be interesting to see if ClpXP plays a role in degrading the auto-cleavage fragments of these proteins. The fragments of unrelated proteins that undergo proteolytic processing events may also be targeted for processive degradation by a similar mechanism. For example, RseA is an anti-sigma factor that is sequentially cleaved by two membrane proteases in response to periplasmic stress (Alba et al., 2002). The N-terminal cytoplasmic domain of RseA released by cleavage is captured by $ClpXP^{trap}$ (Flynn et al., 2003) and has been proposed to contain a Val-Ala-Ala-COOH sequence identical to the signal that targets $LexA^{1-84}$ for ClpXP degradation (Alba et al., 2002). Thus, we suspect that dormant degradation signals like those we have characterized in LexA will be used to couple the destruction of other proteins to changes that occur in the intracellular or extracellular environment.

Materials and Methods

Strains and Plasmids

Strains MC4100 and SG22101 (MC4100 *clpX::kan*) were used in UV survival assays. The pBR322 based plasmid pJWL42 contains wild-type *lexA* under the control of its native promoter (Markham et al., 1981). Plasmid pJWL228 was a construct for overexpression of LexA and consisted of wild type *lexA* under the control of the T7 promoter (Shepley and Little, 1996). Plasmids pSBN15 and pSBN24 were used for overexpression of $LexA^{1-84}$ and $LexA^{1-87}$ respectively, and were derivatives of pJWL228. Stop codons were inserted in the coding sequence after LexA residue 84 or 87, respectively, using a Quickchange kit (Stratagene). Plasmid pSBN16 was derived from pJWL228 for overexpression of the N-terminal fragment of LexA with aspartates

replacing the two terminal alanines ($LexA^{1-84DD}$) using Quickchange. The plasmid for the expression of $LexA^{85-202}$ (pSBN17) was constructed by PCR amplification of the sequence coding for amino acids 85-202 from pJWL228. The resulting PCR product was digested with BamHI and NdeI and ligated into BamHI/NdeI digested pET11a. Plasmid pSBN19 was created for the expression of a fusion of LexA amino acids 85-126 to Arc-st11 ($LexA^{85-126}$-Arc). It was made by amplifying the region of LexA coding for amino acids 85-126, digesting with NdeI and NheI, and ligating into NdeI/NheI digested pET11a-arc-st11 (Flynn et al., 2003a). The resulting N-terminal sequence was *MGEPLLAQQHIEGHYQVDPSLFKPNADFLLRVSGMSMKDIGIMAS***MGK** (LexA in italics, Arc in bold). Plasmid pSBN20 consisted of LexA residues 85-103 fused to Arc-st11 using an oligonucleotide cassette. The N-terminal sequence of this fusion was: *MGEPLLAQQHIEGHYQVDPS***MGK**. Plasmids pSBN21 and pSBN22 were R114D and V115D variants, respectively, of the LexA portion of pSBN19 and were constructed using the Quickchange kit. Plasmids pSBN22 and pSBN23 are variants of pJL42 for the expression of $LexA^{1-84}$ and $LexA^{1-84DD}$, respectively, under the control of the native promoter and were constructed as per pSBN15 and pSBN16. All constructs were verified by DNA sequencing.

Proteins

Purification of ClpP (Kim et al., 2000), GFP-ssrA (Yakhnin et al., 1998), ClpX (Flynn et al., 2003) and Arc-st11 (Robinson and Sauer, 1996b) have been described. LexA, $LexA^{1-84}$ and $LexA^{1-84DD}$ were purified from over-producing strains essentially as described for LexA (Little et al., 1994). $LexA^{85-202}$ was similarly purified except that a superdex 75 column was added as a final step (Little et al., 1994). After purification, LexA and the LexA fragments were dialyzed extensively into LexA storage buffer (20 mM KH_2PO_4/K_2HPO_4 pH 7.2, 2 mM EDTA, 80 mM NaCl, 5% glycerol). Molecular weights for $LexA^{1-84}$, $LexA^{1-84DD}$, $LexA^{85-103}$-Arc and $LexA^{85-126}$-Arc were confirmed by mass spectrometry.

Trapping/Detection of LexA

Trapping was carried out essentially as described using strain JF162 except that cultures were treated with 50 ug/mL of nalidixic acid for 2 hours before expression of the $ClpP^{trap}$ was induced. ClpP cross-reacts with the LexA antibody and therefore was removed before western blotting. Samples were dialyzed against 8 M urea for 6 hours, then incubated with Ni-NTA beads (Quiagen) with rocking for 1 hour. Supernatant was removed, dialyzed against LexA storage buffer, concentrated using a centricon YM-3 concentrator (Microcon) and used for western blotting (see below). In a control experiment, this treatment did not affect our ability to detect full length LexA, indicating that the protein is stable under these conditions.

Peptide array

A LexA peptide array was prepared by the MIT biopolymers facility using an Abimed instrument. The LexA sequence was spotted as twelve residue peptides, with each successive peptide offset by two residues. Peptides contained two additional C-terminal β-alanine residues and were attached to a cellulose filter via polyethylene glycol linkage at their C-terminus. Peptides interacting with ClpX were detected as described (Flynn et al., 2003).

In vitro degradation

$ClpX_6$ (0.3 μm), $ClpP_{14}$ (0.8 μm), and an ATP regenerating system (4 mM ATP, 50 mg/mL creatine kinase and 2.5 mM creatine phosphate) were incubated in buffer NB (50 mM Tris-HCl, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT) at 30° for 2 minutes. Substrate (10 mm) was added, and samples were removed at specific times, added to SDS loading buffer, and frozen on liquid nitrogen. After heating at 100° C for 5 minutes, samples were analyzed by 15% (N-LexA, C-LexA, Arc fusions) or 12.5% (LexA) SDS-PAGE. Gels were visualized by staining with Sypro orange (Molecular Probes) and scanning on a Molecular Dynamics Fluorimager 595. Imagequant (Molecular dynamics) was used to quantify degradation.

To measure inhibition of GFP-ssrA degradation, $ClpX_6$ (0.3 μm), $ClpP_{14}$ (0.8 μm), and an ATP regenerating system were mixed in PD buffer (25 mM Hepes-KOH pH 7.6, 5

mM MgCl2, 5 mM KCl, 15 mM NaCl, .032% v/v Nonidet P-40, 10% v/v glycerol) and incubated for 2 minutes at 30° C. GPF-ssrA (0.5 μm) and inhibitor (20 μm) were then added, the mixture was transferred to a prewarmed cuvette, and fluorescence readings were begun immediately. Loss of GPF-fluorescence was monitored on a Fluoro-Max 2 fluorimeter (ISA, Inc. Jobin Yvon/Spex). The excitation and emission wavelengths were 467 and 511, respectively.

In vivo degradation

Samples were prepared from MC4100 and SG22101 cells containing plasmid pJL42. Cultures at OD_{600} 0.2 were treated with 50 mg/mL nalidixic acid (Sigma) and incubated with shaking at 37° C for 30 minutes, then chloramphenicol (Sigma) was added to 100 mg/mL to stop protein synthesis. For each timepoint, cells were harvested by centrifugation, resuspended in SDS loading buffer and rapidly frozen on liquid nitrogen. Cells were lysed by boiling, centrifuged in a microfuge at top speed for 5 minutes and the resulting extract was separated by 15% SDS-PAGE. Western blots were performed with an ECF western blotting kit in accordance with the manufactures guidelines (Amersham) using rabbit polyclonal LexA antibody at a 1:5000 dilution (John Little). Blots were imaged using a Molecular Dynamics Fluorimager 595. LexA fragments were prepared from purified LexA as described (Little et al., 1994).

UV survival assays

Cells were grown to an OD_{600} of 0.5 in LB, gently pelleted, washed once and then resuspended in an equal volume of 0.85% saline. Suspensions were irradiated at a UV dose of 1.5 $J/m^2/s$ for set times using a 15W G15T8 germicidal lamp (GE). The UV intensity was measured using a UVX radiometer (UVP Inc). Appropriate dilutions were plated on LB agar plates (or LB agar plus 100 mg/mL ampicillin, as appropriate) and colonies were counted after 24 hours. The decreased plating efficiency of cells carrying the pBR322 plasmid, compared to the non-plasmid containing cells, was attributed to need to maintain selection for the plasmid, based on parallel platings on media lacking antibiotic. Each assay was done at least in triplicate.

Acknowledgements

Supported by NIH grant AI-16892 and HHMI. T.A.B. is an employee of HHMI. We thank members of the Baker and Sauer labs for helpful discussions and careful reading, Graham Walker and members of the Walker lab for advice and use of equipment, and John Little for antibodies and plasmids.

CHAPTER 4:

DISTINCT PEPTIDE SIGNALS IN THE UMUD AND UMUD′ SUBUNITS OF THE UMUD/D′ HETERODIMER MEDIATE TETHERING AND SUBSTRATE-PROCESSING BY THE CLPXP PROTEASE

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R.T. Sauer and T.A. Baker assisted in preparation of the manuscript.

Abstract

The *E. coli* UmuD′ protein is a component of DNA polymerase V, an error-prone polymerase that carries out translesion synthesis on damaged DNA templates. The intracellular concentration of UmuD′ is strictly controlled by regulated transcription, by post-translational processing of UmuD to UmuD′, and by ClpXP degradation. UmuD′ is a substrate for the ClpXP protease, but must form a heterodimer with its unabbreviated precursor, UmuD, for efficient degradation to occur. Here, we show that UmuD functions as a UmuD′-delivery protein for ClpXP. UmuD can also deliver a UmuD partner for degradation. UmuD resembles SspB, a well-characterized substrate-delivery protein for ClpX, in that both proteins use related peptide motifs to bind to the N-terminal domain of ClpX, thereby tethering substrate complexes to ClpXP. The combined use of a weak substrate recognition signal and a delivery factor that tethers the substrate to the protease allows regulated proteolysis of UmuD/D′ in the cell. Dual recognition strategies of this type may be a relatively common feature of intracellular protein turnover.

Introduction

Regulation of protein levels by proteolysis is an integral part of stress responses in all cells. In *Escherichia coli*, for example, the ClpXP protease degrades transcription factors that control responses to starvation and DNA damage as well as specific proteins induced by these stresses (Damerau and St John, 1993; Weichart et al., 2003). ClpXP is composed of a ATP-dependent protein unfoldase, the $ClpX_6$ hexamer, and a double-ring serine protease, $ClpP_{14}$ (Gottesman et al., 1993; Maurizi et al., 1990b). ClpX selects substrates for degradation, unfolds them, and translocates the unfolded polypeptide into a chamber within ClpP, where degradation occurs (Kim et al., 2000; Singh et al., 2000; Wang et al., 1997). Importantly, ClpXP degrades different substrate proteins at different times, depending on growth or environmental conditions. Therefore, it is critical to understand the mechanisms that permit the proper substrates to be selected for degradation in a regulated and coordinated fashion.

One fundamental mode of substrate recognition involves the binding of a substrate-processing site on ClpX to a peptide degradation signal, which is often at or near the N- or C-terminus of the target protein (Flynn et al., 2003b; Gonciarz-Swiatek et al., 1999; Gottesman et al., 1998). Peptide degradation sequences may be constitutively recognized or only become accessible to ClpX after cleavage by another protease or following a conformational change (Marshall-Batty and Nakai, 2003; Neher et al., 2003a). After recognition of the peptide degradation signal by the ClpX processing site, ATP-dependent conformational changes in ClpX are thought to generate a transient "pulling" force that destabilizes the attached native protein (Kenniston et al., 2003a; Singh et al., 2000). Using repeated cycles of ATP hydrolysis, ClpX unfolds the protein substrate and translocates it into ClpP for degradation. Some peptide degradation signals are sufficient to cause virtually any attached protein to be efficiently proteolyzed by ClpXP. For example, addition of the ssrA tag—a peptide added cotranslationally to nascent polypeptides when bacterial ribosomes stall (Keiler et al., 1996; Tu et al., 1995)—will target even hyperstable proteins for ClpXP degradation (Burton et al., 2001a; Kim et al., 2000; Singh et al., 2000).

A second mode of substrate recognition by ClpX involves tethering sites that interact with substrate-delivery or adaptor proteins. These accessory molecules enhance the degradation of specific ClpXP substrates without themselves being degraded. For example, the response regulator RssB forms a complex with the starvation sigma factor, σ^S, and accelerates its degradation by ClpXP (Zhou et al., 2001). Likewise, SspB binds specifically to ssrA-tagged proteins, helping deliver them to ClpXP for degradation (Levchenko et al., 2000). Although σ^S and ssrA-tagged proteins carry ClpX-degradation signals (Gottesman et al., 1998; Studemann et al., 2003), RssB and SspB improve the efficiency of their degradation at low substrate concentrations by tethering them to ClpXP (Levchenko et al., 2000; Wah et al., 2003; Zhou et al., 2001). This mechanism has been most clearly demonstrated for SspB-mediated degradation of ssrA-tagged proteins. One part of the SspB protein binds to the ssrA-degradation tag, while another part interacts with a tethering site on ClpX (Wah et al., 2003). When ClpX, SspB, and an ssrA-tagged substrate are all present, a stable ternary delivery complex is efficiently formed at concentrations lower than those that would support stable binding of ClpX directly to the ssrA-tagged protein (Wah et al., 2002).

The DNA-damage inducible UmuD′ protein is an important ClpXP substrate *in vivo*. An essential subunit of the error-prone translesion DNA polymerase (polV), UmuD′ is synthesized as a precursor, UmuD. Following DNA damage, UmuD cleaves itself between residues 24 and 25 in a RecA-mediated reaction to generate UmuD′ (Burckhardt et al., 1988; Shinagawa et al., 1988). Both UmuD and UmuD′ form homodimers, but UmuD/D′ heterodimers form preferentially (Battista et al., 1990). Importantly, UmuD′ only appears to be degraded by ClpXP when it is bound to UmuD (Gonzalez et al., 2000). Although residues within the precursor region of UmuD (and thus unique to UmuD) are essential for UmuD′ degradation in UmuD/D′ heterodimers, the UmuD subunit is not degraded. Moreover, homodimers of UmuD have been reported to be resistant to ClpXP degradation (Gonzalez et al., 2000). Hence, in this *trans*-targeting reaction, the UmuD subunit of a UmuD/D′ heterodimer appears to provide sequence information essential for the ClpXP degradation of the UmuD′ subunit, even though neither subunit seems to be degraded on its own.

Here, we show that ClpXP degradation of the UmuD′ subunit of a UmuD/D′ heterodimer occurs in a manner similar to SspB-mediated degradation of ssrA-tagged substrates. A peptide motif in the precursor region of UmuD resembles a motif used by SspB to tether itself to the N-terminal domain of ClpX (Wah et al., 2003). This peptide sequence in UmuD has previously been shown to be important for degradation of UmuD′ (Gonzalez et al., 2000). We show that UmuD-dependent degradation of UmuD′ by ClpXP can be blocked by the SspB tethering peptide and that the SspB tethering-motif can replace the sequence in UmuD. Furthermore, we demonstrate that the N-terminal domain of ClpX, which mediates interactions with SspB (Dougan et al., 2003; Wojtyra et al., 2003), is also essential for efficient UmuD-dependent degradation of UmuD′. Thus, UmuD behaves like a ClpX delivery factor; it carries a peptide motif essential for tethering itself and its dimeric partner to ClpX. In fact, we find that UmuD can also deliver another UmuD subunit for ClpXP degradation. Additional peptide signals recognized by ClpX are present in the UmuD′ protein sequence, at least one of which appears to function as a primary degradation signal. The joint use of tethering peptides and low-affinity primary degradation signals permits combinatorial control in regulated protein turnover.

Results

UmuD binds ClpX in a similar manner to SspB

We initially noticed that a sequence near the N-terminus of UmuD ($L^{9}R^{10}E^{11}I^{12}$), which had been implicated previously in mediating interactions between UmuD and ClpX (Gonzalez et al., 2000), resembled a peptide motif near the C-terminus of SspB ($L^{161}R^{162}V^{163}V^{164}$) that tethers this delivery factor to ClpX (Wah et al., 2003). A peptide (XB for ClpX-Binding) carrying this SspB sequence binds ClpX and inhibits SspB-stimulated degradation (Wah et al., 2003). Moreover, variants of SspB with L161A or V164A mutations are defective in substrate delivery (Wah et al., 2003).

We investigated the significance of the similarity between the UmuD and SspB peptide sequences by testing the effect of the XB peptide on UmuD-supported ClpXP degradation of UmuD′. The XB peptide inhibited ClpXP degradation of ^{35}S-labeled UmuD′ in UmuD/D′ heterodimers (Fig. 4.1A), with half-maximal inhibition at an XB peptide concentration of approximately 50 μM. This inhibition was specific, as high concentrations of the XB peptide did not inhibit ClpXP degradation of Arc-ssrA, a substrate unrelated to UmuD/D′ (Fig. 4.1A). These data support a model in which the XB peptide competes with UmuD for binding to ClpX, thereby inhibiting UmuD′ degradation. We also found that a UmuD peptide carrying the *LREI* motif inhibited UmuD-dependent degradation of UmuD′, albeit about 10-fold less efficiently than the SspB XB peptide (data not shown).

Previous experiments have shown that changing the *LREI* sequence of UmuD to *AAAA* results in a variant that is ineffective in supporting degradation of UmuD′ (Gonzalez et al., 2000). We reasoned that replacing the UmuD sequence with the SspB sequence might improve the ability of UmuD to support ClpXP degradation of UmuD′ in heterodimers. This outcome was observed (Fig. 4.1B). A UmuD mutant (UmuD^{XB}) with $E^{11}I^{12}V^{13}$ replaced by $V^{11}V^{12}K^{13}$ (resulting in the same *LRVVK* sequence found at the C-terminus of *E. coli* SspB) supported ClpXP degradation of UmuD′. Importantly, UmuD^{XB} ($K_M \approx 15$ μM) was more effective than UmuD ($K_M \approx 32$ μM) at promoting ClpXP degradation of UmuD′ (Fig. 4.1B). These data suggest that the XB region

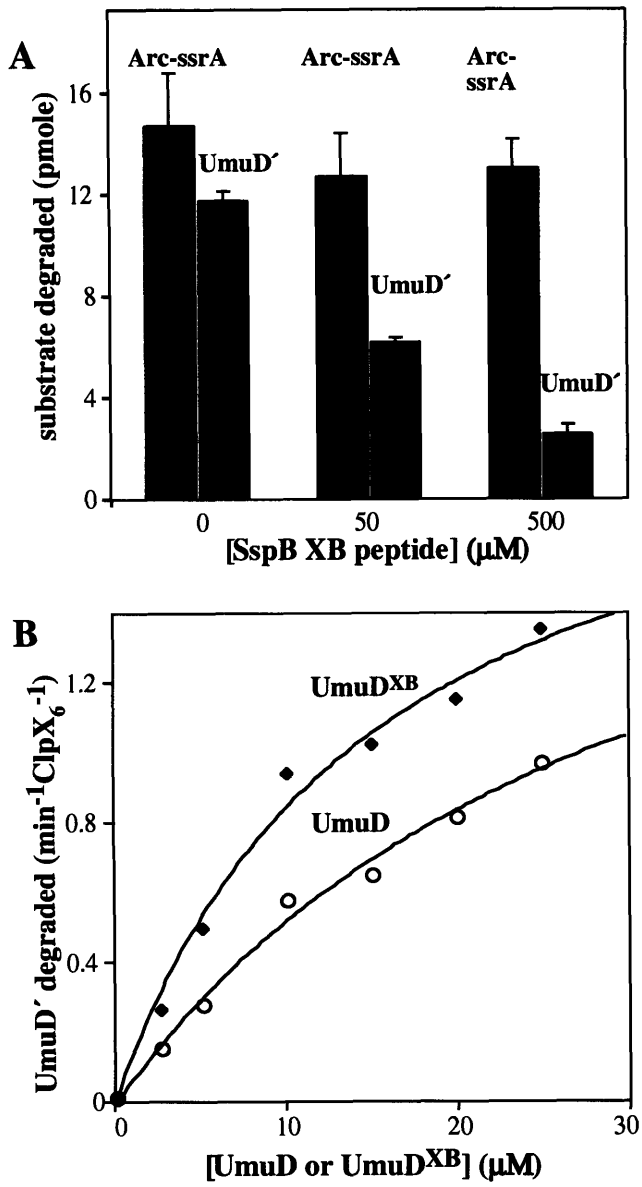


Figure 4.1. A. ClpXP degradation of ^{35}S-labeled UmuD′ in UmuD/D′ heterodimers (10 μM) or ^{35}S-labeled Arc-ssrA (10 μM) was measured after 30 min in the presence of 0, 50, or 500 μM SspB XB peptide. **B.** ClpXP degradation of ^{35}S-labeled UmuD′ (10 μM) was measured as a function of the concentration of UmuD (K_M = 31.8 ± 8.9 μM; V_{max} = 2.1 ± 0.3 min^{-1} ClpX$_6^{-1}$) or UmuD^{XB} (K_M = 15.4 ± 3.4 μM; V_{max} = 2.1 ± 0.4 min^{-1} ClpX$_6^{-1}$). Steady-state kinetic parameters were obtained by fits to the Michaelis-Menten equation.

from SspB and the *LREI* motif from UmuD serve equivalent functions in ClpXP-mediated degradation.

We next investigated whether the function of UmuD in degradation of UmuD′ was compromised when the N-terminal domain of ClpX was deleted in the $ClpX^{\Delta 1\text{-}46}$ variant. The N-terminal domain of ClpX binds the XB peptide of SspB (D. Wah, G. Hersch, & I. Levchenko, personal communication) and is essential for SspB stimulation but not for degradation of ssrA-tagged substrates (Dougan et al., 2003b). UmuD-supported degradation of UmuD′ by $ClpX^{\Delta 1\text{-}46}P$ was severely diminished (Fig. 2A). After a two-hour incubation of UmuD/D′ with $ClpX^{\Delta 1\text{-}46}P$, only minimal degradation of UmuD′ was observed under conditions where degradation by wild-type ClpXP was robust. As expected, Arc-ssrA was degraded efficiently both by $ClpX^{\Delta 1\text{-}46}P$ and ClpXP (Fig. 4.2B). These results show that UmuD-dependent delivery of UmuD′ to ClpXP requires the first 46 amino acids of ClpX.

UmuD is also a ClpXP substrate

Because UmuD has all the sequence information present in UmuD′ but has been reported to be resistant to ClpXP degradation (Gonzalez et al., 2000), we were interested in potential mechanisms by which ClpXP might discriminate between these proteins. However, control experiments indicated that ^{35}S-labeled $UmuD_2$ homodimers were degraded by ClpXP *in vitro* (Fig. 4.3A), in a reaction dependent upon ClpX and ATP (Fig. 4.3A; data not shown). Indeed, the steady-state kinetic parameters for ClpXP degradation of $UmuD_2$ ($K_M \approx 26$ μM; $V_{max} \approx 1$ min^{-1} $ClpX_6^{-1}$) indicate that $UmuD_2$ homodimers are degraded with an efficiency similar to the UmuD′ subunit of the UmuD/D′ heterodimer (Figs. 4.1B & 4.3B). We considered that the apparent degradation of $UmuD_2$ by ClpXP might actually result from degradation of UmuD/D′ molecules generated by autocleavage during the reaction. However, MS/MS analysis of the fragments resulting from ClpXP degradation of $UmuD_2$ revealed peptides overlapping the Cys-Gly peptide bond where UmuD is cleaved to generate UmuD′ (Fig. 4.3C). This result shows that unprocessed UmuD is a substrate for ClpXP degradation.

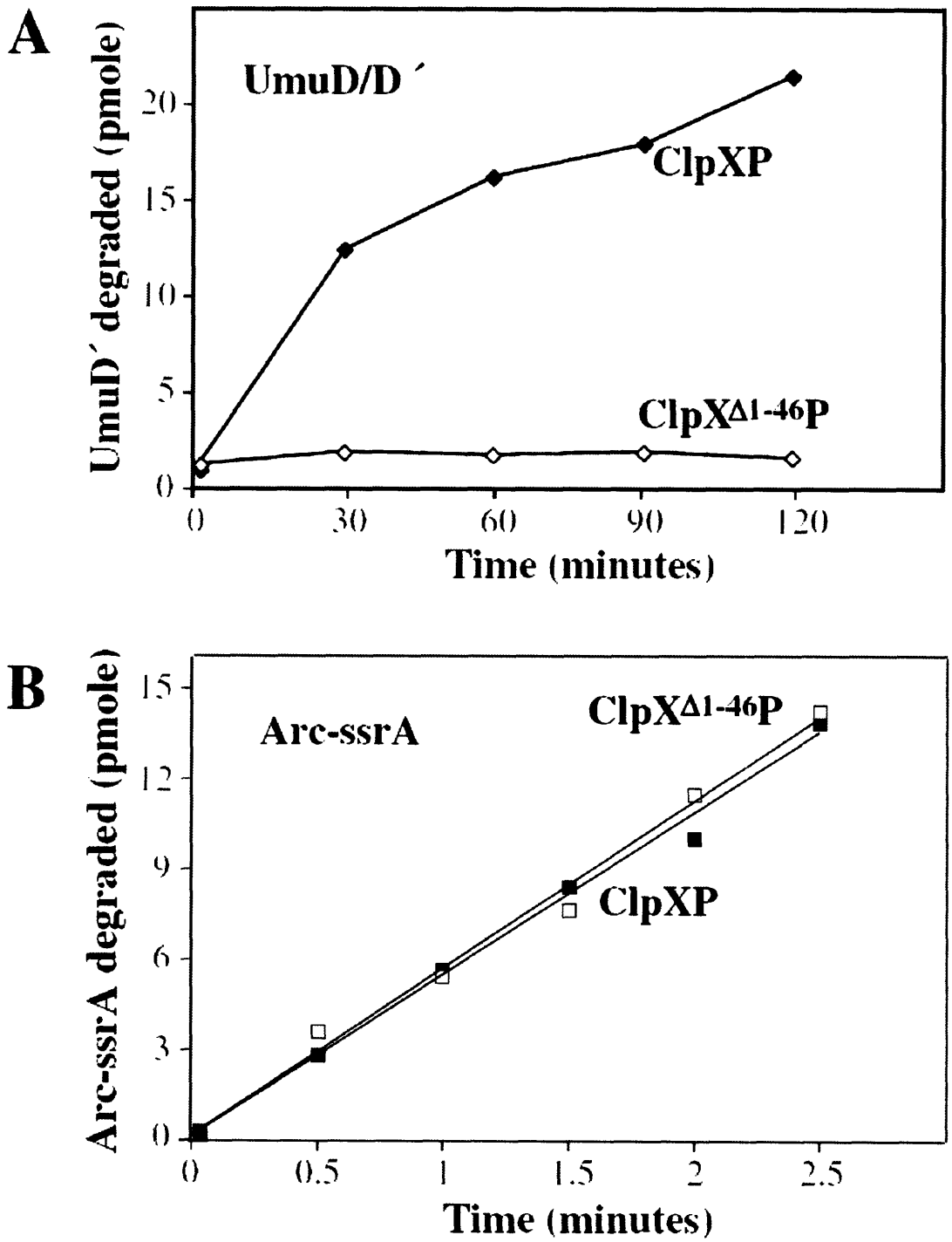


Figure 4.2. Degradation of ^{35}S-labeled UmuD′ in UmuD/D′ heterodimers (upper panel) or Arc-ssrA (lower panel) by ClpXP and ClpX$^{\Delta 1\text{-}46}$P. In all experiments, $ClpX_6$ or ClpX$^{\Delta 1\text{-}46}$ was present at 0.3 μM, $ClpP_{14}$ was present at 0.8 μM, and substrates were present at 10 μM.

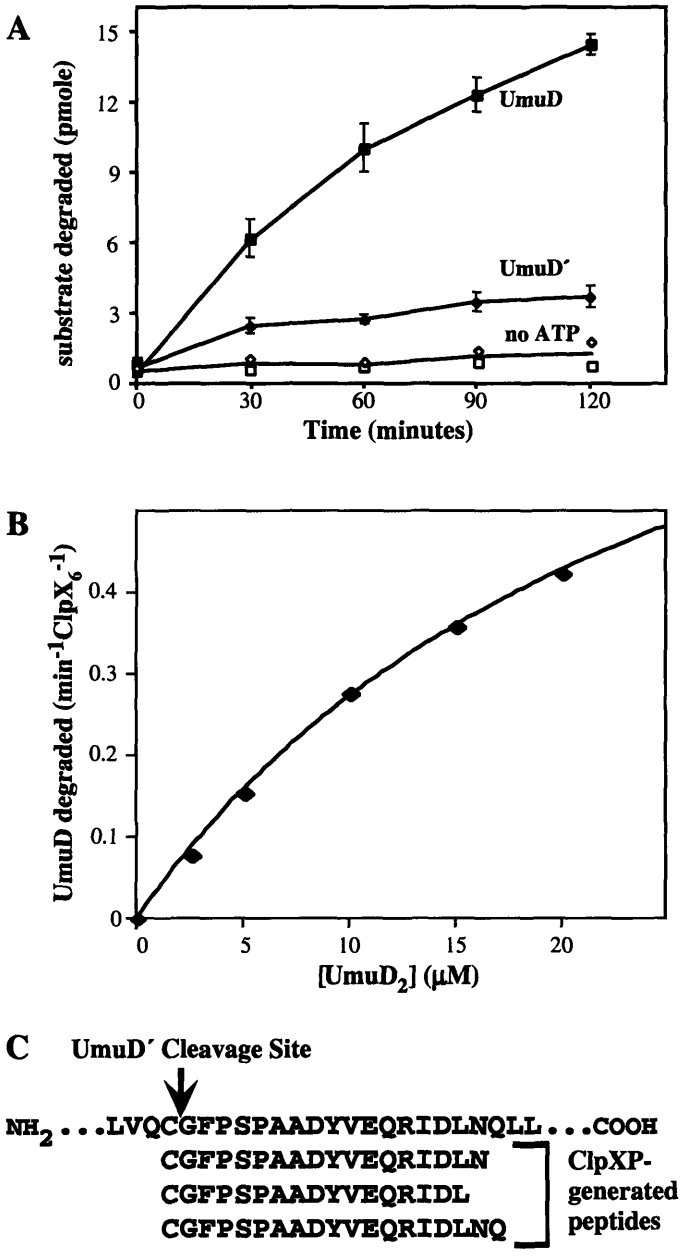


Figure 4.3. A. ClpXP degradation of 10 μM ^{35}S-labeled $UmuD_2$ or $UmuD'_2$.

B. Michaelis-Menten plot of ClpXP-mediated degradation of increasing concentrations of $UmuD_2$ ($K_M = 26.4 \pm 2.3$ μM; $V_{max} = 1.2 \pm 0.1$ min^{-1} $ClpX_6^{-1}$).

C. Sequences of peptides that overlap the site of autocleavage between Cys^{24} and Gly^{25} were identified by tandem mass-spectrometry following ClpXP-mediated degradation of $UmuD_2$.

Two-site model for ClpXP interaction

As UmuD in a $UmuD_2$ homodimer can be degraded by ClpXP, we revisited the question of ClpXP sensitivity of UmuD in a UmuD/D′ heterodimer. Previous work established that the UmuD subunit of the heterodimer is not degraded and that UmuD can in fact catalytically target excess UmuD′ for ClpXP degradation (Gonzalez et al., 2000). In agreement with these studies, we found that a four-fold excess of unlabeled UmuD′ almost completely inhibited ClpXP degradation of ^{35}S-labeled UmuD (Fig. 4.4A). Because $UmuD'_2$ homodimers are poor substrates for ClpXP and the unlabeled UmuD′ was efficiently degraded in this experiment (data not shown), the most likely mechanism of inhibition is that the addition of UmuD′ leads to a decrease in the population of $UmuD_2$ homodimers as UmuD/D′ heterodimers are formed. Thus, as expected from previous studies (Gonzalez et al., 2000), we conclude that only the UmuD′ subunit in a UmuD/D′ heterodimer is degraded; this degradation releases the UmuD subunit to form dimers with a new UmuD′ partner.

To explain why UmuD is degraded when present as a homodimer, but only UmuD′ is degraded within the heterodimer, we propose the following two-site recognition model. When ClpXP recognizes a UmuD/D′ heterodimer or a $UmuD_2$ homodimer, only one of the two subunits can be degraded efficiently because one subunit interacts with a "tethering" site on ClpX, whereas the second subunit is presented to the "substrate processing" site on ClpX. By this model, UmuD′ would be the only subunit degraded in a UmuD/D′ heterodimer because it lacks the sequence motif required to interact with the tethering site on ClpX. A cartoon representation of this model is shown in Fig. 4.4B. In a UmuD homodimer, by contrast, either subunit could bind to the tethering site or to the substrate-processing site and thus either subunit could be a substrate. However, for each round of binding of the homodimer to ClpXP, only the subunit bound to the "substrate processing" site will be degraded (see Discussion).

Sequence information in UmuD′ contributes to its recognition by ClpXP.

We also tested ClpXP degradation of ^{35}S-labeled $UmuD'_2$ homodimers and found that they were degraded by ClpXP, albeit slowly compared to $UmuD_2$ homodimers or

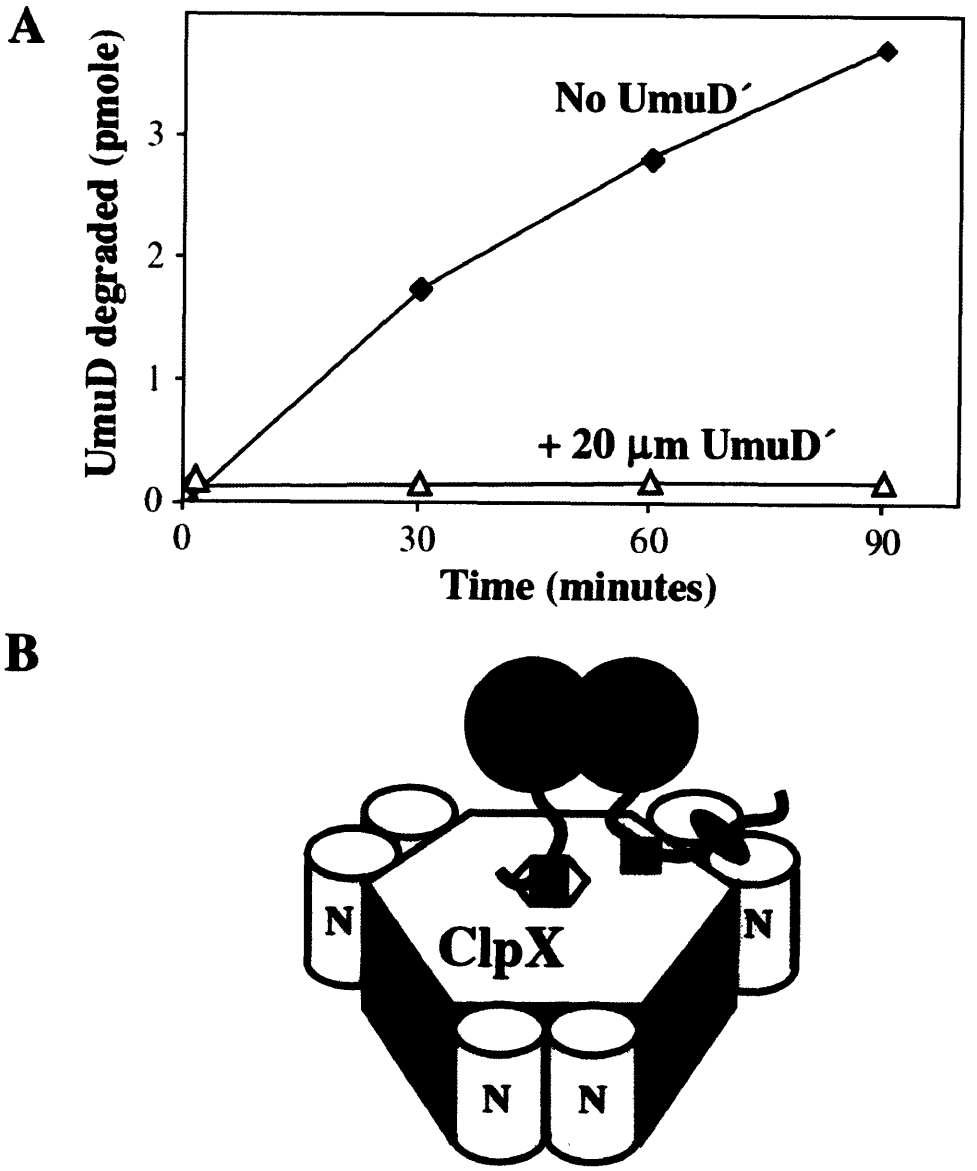


Figure 4.4. A. ClpXP degradation of ^{35}S-labeled UmuD (5 μM) is inhibited by increasing concentrations of UmuD′.

B. Cartoon representation of *trans*-targeting. A tethering motif (shown as an oval) on the UmuD subunit of the UmuD/D′ heterodimer binds to the N-terminal domain of ClpX, thereby leashing its UmuD′ partner to the enzyme and allowing a weak degradation tag (shown as a square) to interact with the central protein-processing pore.

UmuD′ in a UmuD/D′ heterodimer. Only a small fraction of the UmuD′ homodimer was converted to acid-soluble peptides in a two-hour incubation (Fig. 4.3A). However, this low level of degradation was consistently higher than that detected in reactions lacking ClpX or ATP (Fig. 4.3A), indicating it is in fact due to the activity of the ClpXP enzyme.

To search for potential degradation signals in UmuD or UmuD′ that might interact with the "substrate-processing" site of ClpX, we probed a peptide array for sequences that bind ClpX. This array consisted of a set of 12-residue UmuD peptides covalently linked to a nitrocellulose filter, with each peptide sharing a 10-residue overlap with its neighbors. ClpX-interacting regions were identified in far-western blotting using ClpX and an anti-ClpX antibody. Three regions present in both UmuD and UmuD′ (residues ~33-37, 41-51 and 85-109) interacted most strongly with ClpX (Fig. 4.5A). Based on the structure of UmuD′ (Ferentz et al., 2001), these sequences all contain residues exposed on the protein surface. None, however, showed strong similarity to other ClpX-targeting motifs that have been reported (Flynn et al., 2003).

We tested the importance of the most N-terminal of these ClpX-binding regions by constructing variants of UmuD and UmuD′ with an R37A mutation (numbering relative to the UmuD sequence). This arginine was chosen for mutagenesis because positively-charged amino acids appear to be important in ClpX recognition of many substrate degradation signals (Flynn et al., 2003). When present in the UmuD′ subunit of a UmuD/D′ heterodimer, the R37A mutation caused this subunit to be degraded substantially more slowly that its wild-type counterpart (compare lanes 1-3 and 4-6; Fig. 4.5B). In contrast, when the mutation was present on the UmuD subunit of a $UmuD^{R37A}$/D′ heterodimer, degradation of the UmuD′ subunit occurred as efficiently as with wild-type UmuD (lanes 7-9; Fig. 4.4B). Control experiments demonstrated that both mutant proteins retained the ability to form dimers (data not shown). Thus, although the R37A mutation fails to completely block degradation of UmuD′, these experiments reveal that sequence information within UmuD′ can influence the efficiency of its recognition/degradation by ClpXP. These data support the idea that UmuD′ (and UmuD) contain one or more weak primary degradation signals that are recognized by the substrate-processing site on ClpX and are therefore important for ClpXP degradation.

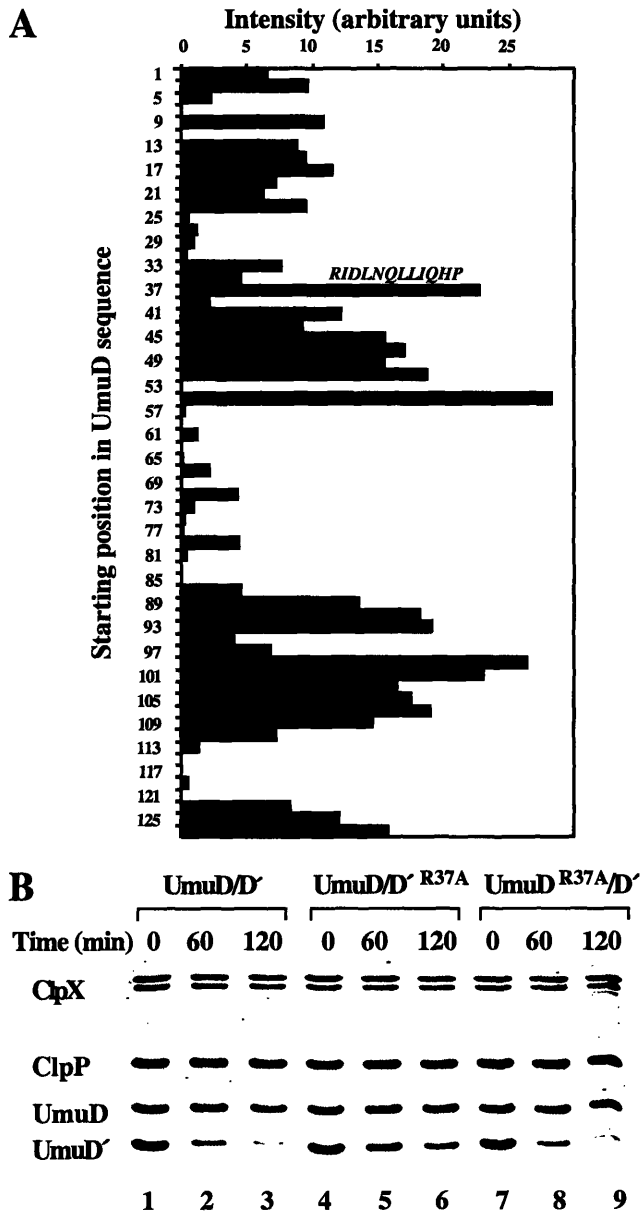


Figure 4.5. A. Overlapping 12-residue peptides from the UmuD sequence were arrayed by covalent attachment to a membrane, incubated with ClpX, washed, and bound ClpX was detected by far-western blotting using an anti-ClpX antibody and quantified by spot intensity. The sequence position of the N-terminal residue in the UmuD sequence for every other peptide is listed.

B. The R37A mutation reduces ClpXP degradation of the UmuD′ subunit of the heterodimer when it is present in the UmuD′ but not the UmuD subunit.

Discussion

The results presented here support a model in which the UmuD/D′ complex must interact with ClpX at distinct "tethering" and "substrate-processing" sites for efficient ClpXP degradation to occur. UmuD carries a specific peptide motif that interacts with ClpX at the tethering site, whereas its UmuD′ partner has one or more weak degradation signals recognized by the substrate-processing site. UmuD therefore functions in a manner analogous to SspB to deliver a bound protein-partner to ClpXP for degradation. In fact, UmuD and SspB carry related sequence motifs (*LREI* in UmuD, *LRVV* in SspB) that are important for tethering to ClpX, and both occur within inherently flexible regions of each protein. In each case, these tethering interactions would enhance degradation by increasing the effective concentration of the degradation signal(s) on the partner molecule relative to the substrate-processing site of ClpX. Because the ClpX N-terminal domain is required for both SspB- and UmuD-mediated delivery, we assume that the tethering site is located within the N-terminal domain. The substrate-processing site, by contrast, must be part of the AAA+ core of ClpX, as this portion of ClpX is fully active in the degradation of certain substrates (Singh et al., 2001).

It is important to note that delivery or *trans*-targeting for ClpXP degradation is not a general property of any oligomeric complex in which one subunit contains a ClpX degradation tag or a tethering motif. For example, ClpXP unfolds and degrades only the subunit(s) bearing a degradation tag in hetero-multimers containing tagged and untagged subunits (Burton et al., 2001a; Burton et al., 2001b). Similarly, SspB binds to but fails to stimulate ClpXP degradation of a substrate in which the ClpX-interaction residues of the ssrA degradation tag have been mutated (Levchenko et al., 2000). These observations emphasize the dual requirement for a degradation signal and a tethering sequence for *trans*-targeting. Bipartite peptide signals required for ClpXP degradation have also been documented for σ^S and CtrA (Ryan et al., 2002; Studemann et al., 2003), substrates for which delivery factors are known or suspected to be involved in ClpXP degradation. In these cases, one signal is likely to mediate interaction with the delivery factor and its tethering motif and the other with ClpX. In principle, a single protein could also interact with ClpX via a tethering motif and a degradation tag if these sequences were far enough

apart and positioned in a way that allowed simultaneous contacts with their respective interaction sites in ClpX. This model, for example, could explain why the determinants of ClpXP degradation of the λ O protein are complex and involve multiple peptide sequences (Gonciarz-Swiatek et al., 1999).

The experiments presented here demonstrate that the $UmuD_2$ and $UmuD'_2$ homodimers can be degraded by ClpXP. $UmuD_2$ homodimers are degraded by ClpXP with a K_m similar to that for degradation of UmuD/D′ heterodimers (Figs. 1B and 3B), whereas $UmuD'_2$ homodimers are much poorer substrates. By contrast, previous studies reported that the UmuD′ subunit of a UmuD/D′ heterodimer was the only form of the protein degraded by ClpXP, suggesting that ClpX recognition required a unique signal present only in the heterodimer (Gonzalez et al., 2000). Our results support a different model. Namely, that UmuD and UmuD′ contain low-affinity signals for ClpXP degradation, which are only recognized efficiently when the substrate is tethered to ClpX via a UmuD partner subunit. Peptide-binding studies and mutagenesis suggest that a sequence around Arg37 in UmuD may serve as one such degradation signal.

Importantly, our results are in complete agreement with the previous conclusion of Woodgate and colleagues that UmuD can catalytically target UmuD′ for degradation (Gonzalez et al., 2000). Consistent with this model, we find that excess UmuD′ inhibits UmuD degradation. This result supports our model that one subunit of the dimer must be "tethered" to ClpX for the other subunit to be efficiently recognized and degraded. Because the *LREI* tethering motif is absent from UmuD′, only UmuD can make the tethering interaction. With the additional assumption that a single subunit of UmuD cannot simultaneously contact the tethering and substrate processing sites on ClpX, this model explains why the UmuD′ molecule in the UmuD/D′ heterodimer is always the subunit degraded.

This hierarchy of UmuD and UmuD′ interactions with ClpX is undoubtedly important in regulating proteolysis in the cell. As noted previously (Gonzalez et al., 2000), the *trans*-targeting of UmuD′ to ClpXP by UmuD provides a mechanism to limit UmuD′ availability and therefore to reduce error-prone DNA synthesis, which is catalyzed by a complex of UmuC with a $UmuD'_2$ homodimer (Tang et al., 1998; Tang et al., 1999). Because UmuD/D′ heterodimers form preferentially (Battista et al., 1990),

UmuD′ will be degraded by ClpXP whenever UmuD is also present at a concentration sufficient to support heterodimer formation, with proteolysis then releasing the UmuD subunit to target additional molecules of UmuD′ for destruction. As a consequence, $UmuD'_2$ homodimers will accumulate only when the vast majority of UmuD has been converted via DNA-damage/RecA-mediated autocleavage to UmuD′. ClpXP control of the relative levels of UmuD and UmuD' may have additional importance because UmuD has been suggested to play a separate role in cell cycle control (Opperman et al., 1999). It is unclear whether ClpXP degradation of $UmuD_2$ homodimers plays any significant intracellular role, as Lon protease degrades these molecules efficiently (Gonzalez et al., 1998).

In principle, tethering sites could occur at many positions on ClpX as long as the binding of the delivery protein did not prevent substrate binding and/or processing. In this regard, it is interesting that SspB and UmuD appear to use a common tethering site. Both delivery proteins contain similar tethering motifs and have a common need for the N-terminal domain of ClpX for these interactions. Moreover, the tethering motif of SspB substitutes for that of UmuD and blocks the UmuD interaction when added *in trans* as a peptide. The use by multiple delivery proteins of a common tethering site on ClpX could permit an additional layer of cellular regulation. By competition for this site, the synthesis of a new delivery factor in response to environmental cues could alter the "prioritization" of substrates for ClpXP degradation.

Materials and Methods

Proteins and Peptides

Purifications for ClpP (Kim et al., 2000) and Arc (Robinson and Sauer, 1996) used established procedures. $ClpX^{\Delta 1-46}$ was a gift from Samia Siddiqui. *E. coli* BL21 transformed with pAG99 or pAG98 (Sutton et al., 2002) was used for the purification of $UmuD_2$ (Lee et al., 1994) or $UmuD'_2$ (Ferentz et al., 1997). To generate ^{35}S-labeled UmuD and UmuD′, cells were grown in M9 minimal media lacking methionine to an OD_{600} of 0.4 and induced with 0.4 mM IPTG for 80 min. Express ^{35}S-protein labeling mix (NEN) was added to 20 μCi/mL of culture and cells were grown for an additional 30 min before harvesting. ^{35}S-labeled UmuD and UmuD′ were then purified by the procedures for unlabeled proteins. Plasmids for the expression of $UmuD^{R37A}$, $UmuD^{XB}$ and $UmuD'^{R37A}$ were generated from pAG98 and pAG99 using the Stratagene quick-change kit, and the mutant proteins were purified like their wild-type counterparts. The SspB XB peptide had the sequence NH_2-CRGGRPALRVVK-COOH (Wah et al., 2003). A UmuD peptide with the sequence NH_2-WKPADLREIVT-COOH was synthesized for inhibition studies.

ClpX was purified from 10 L of WM53/pTB9 cells grown at 37 °C in 25.5 g/L Bacto tryptone, 15.5 g/L yeast extract, 4 g/L NaCl and 100 mg/L ampicillin in a Bio Flo IV fermenter (New Brunswick Scientific) to an OD_{600} of 8, shifted to 25 °C and induced with 0.25 mM IPTG. After 3 hours, cells were harvested, resuspended in 4 mL buffer A (50 mM Tris-HCL [pH 8.2 at 4 °C], 100 mM KCl, 1 mM $MgCl_2$, 5 mM DTT, 10% glycerol) per gram cell paste, and set III protease inhibitors (Calbiochem) were added to 0.17 μL/mL of suspension. Following lysis by French press at 10,000 psi, insoluble material was removed by centrifugation, $AmSO_4$ was added to 35% saturation, and precipitated material was collected and dissolved in buffer A to 10 mg/mL. The conductivity was matched to that of buffer PS_A (50 mM Tris-HCL [pH 8.2 at 4 °C], 0.5 M $AmSO_4$, 0.5 mM DTT, 10% glycerol) and the protein concentration was adjusted to 5 mg/mL. Following centrifugation, the supernatant was loaded onto a phenyl sepharose HR column (Amersham) at 3-4 mgs protein/mL resin. ClpX eluted approximately 80% through a linear gradient to buffer A and was precipitated with 35% $AmSO_4$, redissolved and desalted into buffer A using a HiPrep 26/10 column (Amersham). Protein was

loaded onto Q-Sepharose (3 mgs protein/mL resin) and eluted with a gradient to buffer A plus 300 mM KCl. Peak fractions containing ClpX were loaded onto a Bio-gel HTP hydroxyapatite (Biorad) column (4 mgs protein/mL resin) and eluted with a linear gradient to 260 mM K_2HPO_4/KH_2PO_4 (pH 7.2), 5 mM DTT, 10% glycerol. Peak fractions were pooled, precipitated with 35% $AmSO_4$, and redissolved and desalted into buffer A plus 20 mM $AmSO_4$ for storage.

Degradation Assays

Buffer NB (50 mM Tris-Cl [pH 8.0], 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT) was used for ClpXP degradation of UmuD/D′, $UmuD_2$ and $UmuD'_2$. PD buffer (Kim et al., 2000) was used for Arc-ssrA degradation. An ATP regeneration system (16 mM creatine phosphate, 0.32 mg/mL creatine kinase, 5 mM ATP) was included in all ClpXP degradation reactions. Degradation reactions were preformed at 30 °C and contained 0.3 μM $ClpX_6$, 0.8 μM $ClpP_{14}$ and the indicated concentration of substrate. When monitoring the release of acid-soluble peptides, reactions were stopped by adding TCA to 10%, samples were placed on ice for 20 min, and insoluble material was removed by centrifugation at 4 °C in a microcentrifuge (13,000 rpm). Radioactivity in the supernatant was assayed by scintillation counting. Proteolysis of UmuD and UmuD′ is reported as the number of pmoles degraded in a reaction volume of 2.4 μL. For degradation monitored by SDS-PAGE, reactions were stopped by adding SDS sample buffer and freezing in liquid nitrogen. Samples were electrophoresed on 15% polyacrylamide gels, stained using SYPRO© orange (Molecular Probes), and visualized using a Molecular Dynamics model 595 fluorimager.

For identification of UmuD degradation products by mass spectrometry, $UmuD_2$ (10 μM) was digested with ClpXP for 2 hours at 30 °C. The resulting peptides were separated by reverse-phase chromatography on a Vydac C18 Mass Spec HPLC column, using a 1 hour gradient from 5% to 95% buffer B (Buffer A is 5% acetonitrile, 0.1% formic acid; Buffer B is 90% acetonitrile, 0.1% formic acid, 10% isopropanol). Peptides were identified on a LCQ electrospray ion-trap mass spectrometer (Thermofinnigan). Sequence analysis was achieved by collision-induced fragmentation within the ion trap; peptides reported had a Sequest cross-correlation value of 2.5 or higher.

Peptide array

A peptide array containing UmuD peptide sequences was prepared by the MIT Biopolymers facility using an Abimed instrument. Each UmuD peptide sequence contained twelve residues and was offset by two residues from the succeeding peptide. Peptides interacting with ClpX were detected by indirect Western blotting using an anti-ClpX antibody as described (Flynn et al., 2003), and the intensity of the interaction was quantified using Imagequant (Molecular Dynamics).

Acknowledgements

We thank Graham Walker for advice and enthusiastic support throughout this project, Samia Siddiqui for $ClpX^{\Delta 1-46}$, Elizabeth Courtenay for mass spectrometry, and Samia Siddiqui, Igor Levchenko, Greg Hersch, and David Wah for sharing results prior to publication. This work was supported by NIH grants AI-16892 and GM-499224 and the Howard Hughes Medical Institute. TAB is an employee of HHMI.

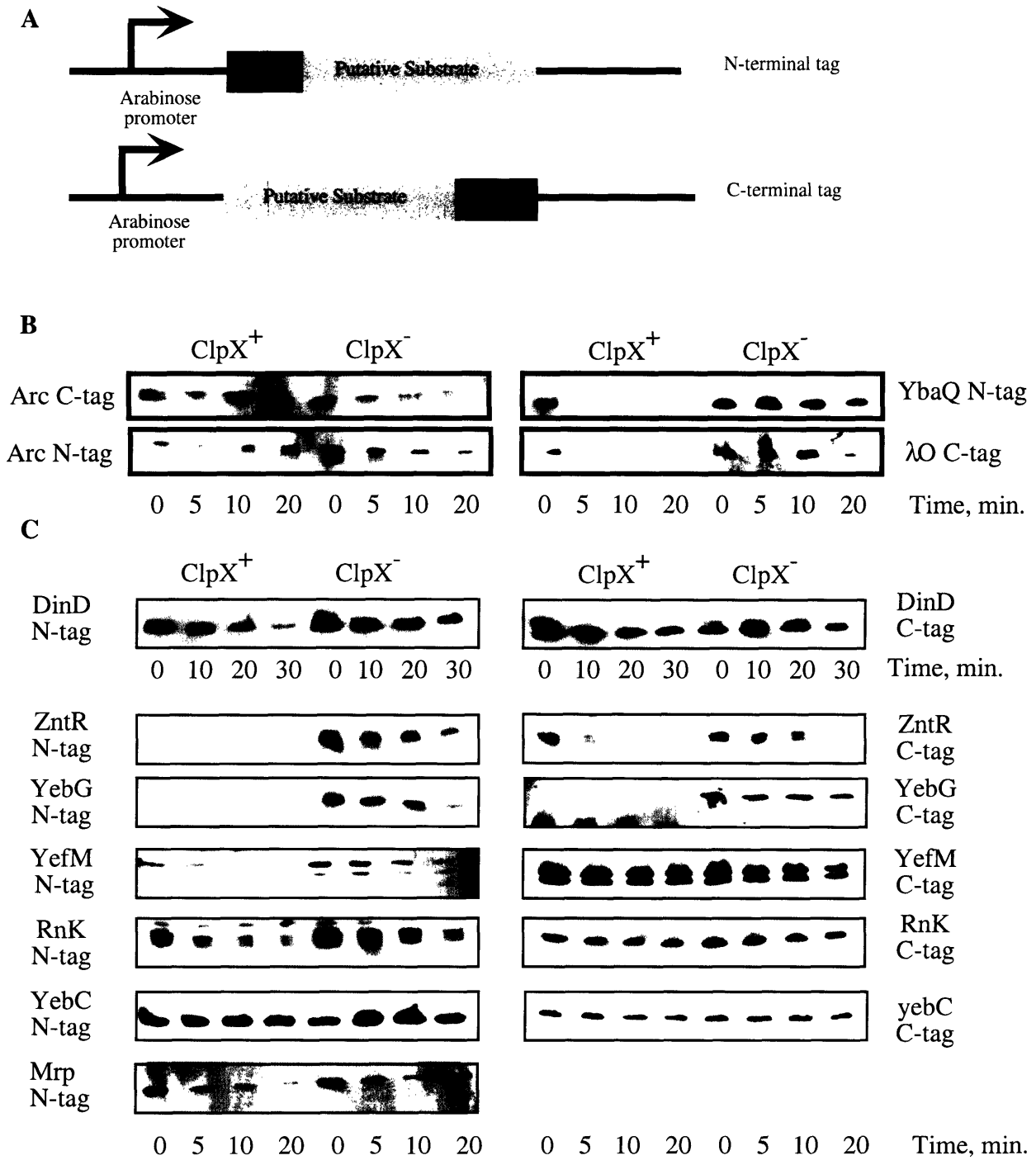
APPENDIX

1. Initial trapping trapping and characterization of ClpXP substrates after DNA damage

Identification of ClpXP substrates under a variety of environmental conditions will be necessary in order to realize the full spectrum of proteins degraded by ClpXP. Our first attempt to identify ClpXP substrates following DNA damage yielded a set of approximately 50 proteins not previously identified without the addition of a DNA damaging agent to the growth media (Flynn et al., 2003). Our ability to determine the damage-dependence of trapping was limited in that substrates were identified by mass spectrometry only once for each environmental condition. In some cases identification relied on only one peptide, making low-abundance substrates especially sensitive to sampling error. Therefore substrate trapping could not unequivocally be attributed to DNA damage, and we used a quantitative method of mass spectrometry, as described in chapter 1.

Despite its limitations, this first data set did yield about 50 potential new ClpXP substrates (Table A.1). Among these substrates are known damage-inducible proteins such as SulA, DinD, DinI and YebG. The substrates also included a number of proteins not known to be induced by DNA damage. To validate members of this list, we set out to determine the ClpX-dependence of *in vivo* degradation. Antibodies were available for some substrates, and others were tested using epitope tags as described below.

Because antibodies were not available for many of the trapped proteins, we developed a method to test the ClpX-dependence of *in vivo* degradation using epitope tags. This method took advantage of commercially available vectors (Invitrogen pBAD system) with a choice of N or C-terminal epitiope tags and an arabinose inducible promoter. An important control was to confirmed that neither the N or C-terminal epitope tag conferred ClpXP sensitivity onto an stable reporter protein, the Arc repressor (Figure A.1). Additionally, we tested degradation of both a substrate with a good C-terminal ClpX recognition signal (YbaQ) using an N-terminal epitope tag, and a substrate with a good N-terminal recognition signal (lambda O) using a C-terminal epitope tag. As expected, these substrates showed robust ClpX-dependent degradation, so addition of



Appendix A.1: Use of Epitope tags to test degradation of putative ClpXP substrates

A. Diagram of promoters and epitope tags. **B.** Addition of epitope tags do not target Arc-st11 for ClpXP degradation. Furthermore, epitope tags do not interfere with the degradation of known ClpXP substrates. **C.** Results from epitope tagging test substrates.

the epitope tags did not inhibit degradation of these test substrates (Figure A.1). We selected 11 potential substrates for *in vivo* testing. Table A.2 lists these substrates, details of their construction, optimal conditions for degradation and observed results.

In vivo degradation experiments revealed that about half the substrates showed ClpX-dependent degradation (Figure A.1). It is possible that proteins for which we did not observe degradation are still ClpXP substrates, however, conditions may not be optimal to observe their degradation. Some rapidly degraded substrates are YebG, a damage inducible protein of unknown function, YefM, part of a two-component toxin/antitoxin system, and ZntR, a MerR family transcriptional regulator. One potentially informative result was that degradation of some substrates, such as DinD, was more robust with the epitope tag at one termini than the other. This preference could indicate which termini contains a signal for ClpX, as an epitope tag on this termini might mask the ClpX-recognition signal. Degradation of others, such as YebG, occurred with either tag. These substrates may have recognition tags at both termini.

This study demonstrates the effectiveness of using epitope tags to test potential ClpXP substrates. It also reveals that about half of a semi-random assortment of trapped proteins show robust ClpX-dependent degradation *in vivo*. Furthermore, available clones and prior confirmation of ClpXP dependent degradation make these substrates attractive targets for future studies. Indeed, ClpXP degradation of one identified substrate, ZntR, is likely to be modulated by DNA or cofactor binding (M. Pruteanu, unpublished).

Table A.1: Proteins captured by the ClpP^{trap} only after DNA damage

This table includes only those proteins identified after treatment of cells with nalidixic acid. Gel slice refers to the region of the gel where peptides from each protein were identified, with 1 being the highest molecular weight slice and 5 being the lowest. Additionally the number of unique peptides used for identification of each peptide is listed.

Table A.1: Proteins Unique to DNA damaging conditions

gene	gi #	function	Gel slice	# peptides
15	137940	phage phi-80 protein 15	1,2,5	4,3,16
ackA	16130231	acetate kinase	1	1
acpP	15801211	acyl carrier protein	1	2
Atp-6	15640957	ATP synthase alpha subunit	1	2
AtpF	15804336	FO ATPase, subunit B	1	17
cdd	7449917	cytidine deaminase	2	1
CII	133360	phage phi-80 regulatory protein	1	4
dapD	15799848	lysine biosynthisis	1	2
dinD	1790076	DNA damage inducible D	1,3,5	1,2,2
dinI	16129024	RecA inhibitor damage ind.	3	1
eno	147479	CTP synthase (enolase)	1	2
gdn	16129970	6-phosphogluconate dehydrogenase	1	1
glpK	16131764	glycerol kinase	1,3,4,5	1,3,2,4
GreA	15803721	Trxn. Elongation factor	1	1
hemL	16128147	aminomutase	3,5	1,4
mioC	16131610	biotin biosynthesis	2	1
mopB	15804734	GroES chaperone	2,3	3,4
mrp	12230998	methionyl-tRNA syntase	1	2
N	132275	phage phi 80	3	3
nuoB	16130222	NADH dehydrogenase chainB	4	3
nuoE	15802832	NADH dehydrogenase	1	2
pepQ	16131693	proline dipeptidase	5	5
pfs	21239015	MTA/SAH nucleosidase	2	1
phoP	15801327	2 component Mg regulator	1	2
rblB	1503844	50S ribosomal subunit L2	5	2
recA	16130606	recombination/co-protease	3	2
rfbC	16129978	epimerase	1	2
rho	16131639	trxn termination factor	3,5	4,3
rimI	1361224	acetylates rib. Prot. S18 S5	1	2
rnK	16128593	regulator nucleotide interconv.	1	4
rplP	15803840	50S L16	1	1
rplF	15803832	ribosomal subunit	1	2
rplU	16131076	50S L21	3	2
rplY	15802741	50 S ribosomal L25	3	2
RpsG	1583344	ribosomal subunit	1	2
rpsJ	15803848	30S S10	3	6
rpsK	133720	ribosomal protein S11	3	2
rpsL	15803855	30 S S12	2	1
rpsO	133794	ribosomal protein S15	3	3
sdhA	1786942	succinate dehydrogenase	5	1
secB	16131480	protein export	3	1
sodA	16131748	superoxide dismutase	1	4
speB	16130838	agmatinase polyamine synthesis	5	2
sulA	15800817	cell division inhibitor	1,2,3	12,4,4
trxA	16131637	thioredoxin reductase	3	6
ybeD	16128614	unknown	3	2
ydhD	16129612	unknown	3	1
yebC	16129817	in operon with RuvC	5	9
yebG	16129801	unknown/damage inducible	3	3
yefM	16129958	unknown function	3	5
yheA	16131216	unknown	3	1
yhgI	1176270	unknown	1	4
zntR	16131171	zinc responsive trxn. Reg.	2	2

Materials and Methods:

Substrate trapping and identification by mass spec were essentially as described, except that cultures were treated with 50 μM nalidixic acid for 30 minutes before induction of the trap (Flynn et al., 2003).

Test of in vivo degradation

Substrates were amplified from genomic DNA by PCR and separately cloned into pBAD HisA and pBAD His-mycA (Invitrogen). pBAD His-myc A was altered by substitution of an AvrII site for the NcoI site using Quickchange kit as per the manufacturer's instructions (Invitrogen). *In vivo* degradation was tested in W3110 and W3110 *clpX*::Kan strains by growing cells to an OD_{600} of .2 and inducing substrate production with the indicated amount of arabinose. Cells were allowed to double to OD_{600} .4, and protein synthesis was stopped with 100μg/mL chloramphenicol. Samples were frozen on liquid nitrogen, separated by SDS-PAGE and transferred to PVDF. Membranes were probed with Anti-express antibody (N-terminal tag) or Anti-Myc antibody (C-terminal tag), developed with ECF and scanned on a Molecular dynamics 595 Fluorimager.

Table A.2: Characteristics of test substrates

	C-tag				N-tag			
gene	Cloned w/	$T_{1/2}$	ClpX dependent?	Optimal Arabinose	Cloned w/	$T_{1/2}$	ClpX Dependent?	Optimal Arabinose
sulA	AvrII EcoRI	5 min	No	0.0500%	EcoRI BglII	fast	?	>.2%
dinD	AvrII KpnI	20 min	Perhaps	0.0010%	BglII KpnI	15min	yes	0.010%
secB	AvrII EcoRI	no deg	No	0.0010%	EcoRI BglII	no deg	no	0.001%
yefM	AvrII EcoRI	5 min	Perhaps	0.0001%	EcoRI BglII	10 min	yes	0.010%
sodA	AvrII EcoRI	no deg	No	0.0100%	EcoRI BglII	no deg	no	0.200%
yebC	AvrII EcoRI	10 min	Perhaps	0.0010%	EcoRI BglII	10 min	perhaps	0.010%
yebG	AvrII EcoRI	<5min	Yes	0.1000%	EcoRI BglII	<5 min	yes	0.200%
rnk	AvrII EcoRI	no deg	No	0.0010%	EcoRI BglII	5 min	yes	0.001%
rfbC	AvrII EcoRI	no deg	No	0.0100%	EcoRI BglII	fast	?	0.200%
mrp	AvrII EcoRI	nd	Nd	nd	EcoRI BglII	10 min	perhaps	0.010%
zntR	AvrII EcoRI	<5 min	Yes	0.1000%	EcoRI BglII	<5 min	yes	0.200%

Table A.2: Results from test substrates selected for confirmation of *in vivo* ClpX-dependent degradation. The table lists restriction enzymes used for cloning, the approximate substrate half life, the ClpX-dependence of degradation and optimal concentration of arabinose used for induction for both N and C tagged substrates.

2. Preliminary characterization of DinD

Despite many decades of intense study, nearly 17% of the genes in the *E. coli* genome have no assigned function (Serres et al., 2004). Although their function is unknown, the majority of these genes are likely to be biologically relevant. Studies of the loneliest of the unknowns, the so-called "orphan genes" (whose ORF have no similarity to known proteins) indicate that over 90% of these genes are expressed (Alimi et al., 2000; Tao et al., 1999). Some of these genes may remain uncharacterized due to either redundancy or loss that results in a subtle or conditional phenotype. Despite potential difficulties, investigators continue to elucidate the functions of unknown genes to good effect. One relevant example is *dinI*, originally identified computationally as a LexA-controlled gene and shown ten years later to be a modulator of RecA function (Lewis et al., 1994; Lusetti et al., 2004; Yasuda et al., 1998).

About 13% of the proteins trapped by ClpP have no known function. One protein of unknown function, DinD, was among the trapped substrates selected for *in vivo* confirmation of degradation (see Appendix 1). DinD proved to be a ClpXP substrate both *in vivo* and *in vitro* (figure A.2). In order to fully understand the biological role of DinD, and determine the significance ClpXP-mediated degradation to this role, we set out to further characterized DinD's function.

Although the focus of several previous studies, DinD's precise function has proved to be elusive. Initially named pscA-68, DinD was identified as a cold sensitive mutant in chromosome segregation that resulted in filamentous cells with large, central nucleoid masses (Kudo et al., 1977). A later study searching for damage inducible loci showed that DinD was a member of the LexA regulon (Kenyon and Walker, 1980). These two studies converged when pcsA-68 was shown to be a specific mutation (V239M) in the DinD gene (Ohmori et al., 1995). The cold sensitive cell division phenotype appeared to be specific to the V239M allele, as neither loss nor overexpression of DinD caused any apparent defect in cell growth (Ohmori et al., 1995). When expressed from a high copy number plasmid, DinI, RecA and DinG suppressed the DinD V239M cold sensitive phenotype (Yasuda et al., 1996). Additionally, DinD was identified as a host factor that increased the frequency of Tn10 and IS903 transposition

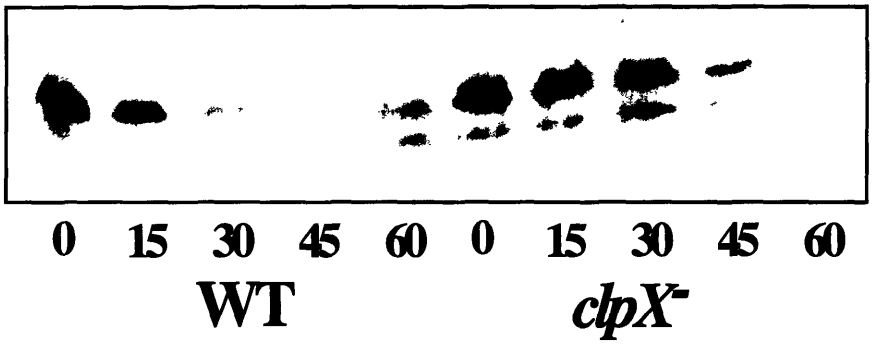
(Derbyshire, personal communication). Although all these studies provide important clues about DinD's function, none proposes a concrete role for DinD in the cell.

When taken together, these data suggest that DinD may have a role in the processing or repair of recombination junctions. By this model, the DinD V239M mutation may have altered affinity for recombination intermediates, preventing product release and resulting in the filamentous cell morphology. This cell morphology is reminiscent of certain alleles of *recA* and of deletion of *recG*. These alleles of *recA* were identified on the basis of their toxicity and improper chromosome segregation (Campbell and Davis, 1999). One example mutant, E96D, bound DNA more tightly and dissociated more slowly than the wild type, resulting in reduced recovery of recombination products. A similar morphological defect is also observed in UV exposed cells lacking *recG*, a helicase involved in the rescue of blocked replication forks (Ishioka et al., 1997). Disruption of RecG results in a defect in homologous recombination (Lloyd and Buckman, 1991). The *dinD* V239M mutation, like the *recA* and *recG* defects, may block the efficient resolution of replication repair intermediates required for normal chromosome segregation.

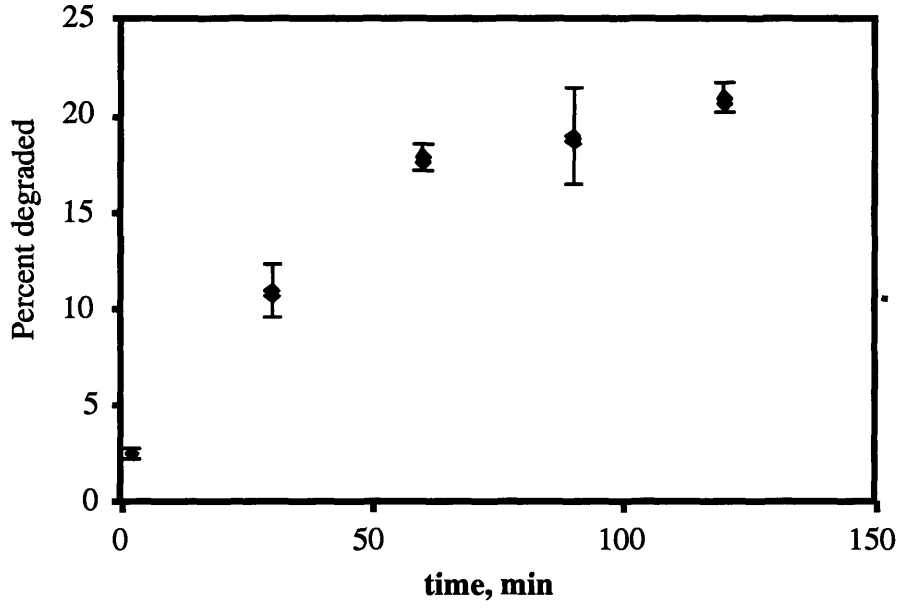
Identification of *dinD* in a screen for transposition-modifying host factor provides further support for this model (K. Derbyshire. personal communication). *dinD* was identified in a screen based on transposon mutagenesis for host factors that modulate the frequency of transposition. A mutation in the promoter region of DinD resulted in an increase in IS903 and Tn10 transposition. This mutation is suspected to increase DinD levels. A host factor that behaved similarly to *dinD* in this screen was *radA* (*sms*). *RadA* was identified on the basis of its sensitivity to DNA damaging agents, and genetic experiments suggest that it is involved in the processing of branched DNA structures (Beam et al., 2002; Diver et al., 1982). These results suggest that DinD may interact with recombination junctions, and a change in its cellular level can affect the resolution of these junctions and progression of transposition.

An expected property of a protein that interacts with recombination intermediates is the ability to bind DNA. Filter binding and gel shift experiments showed that DinD bound sequence-nonspecifically to double stranded DNA with a K_D of 20 ± 3 nM (Figure A.3). This binding was not dependant on magnesium. DinD also bound single stranded

A.



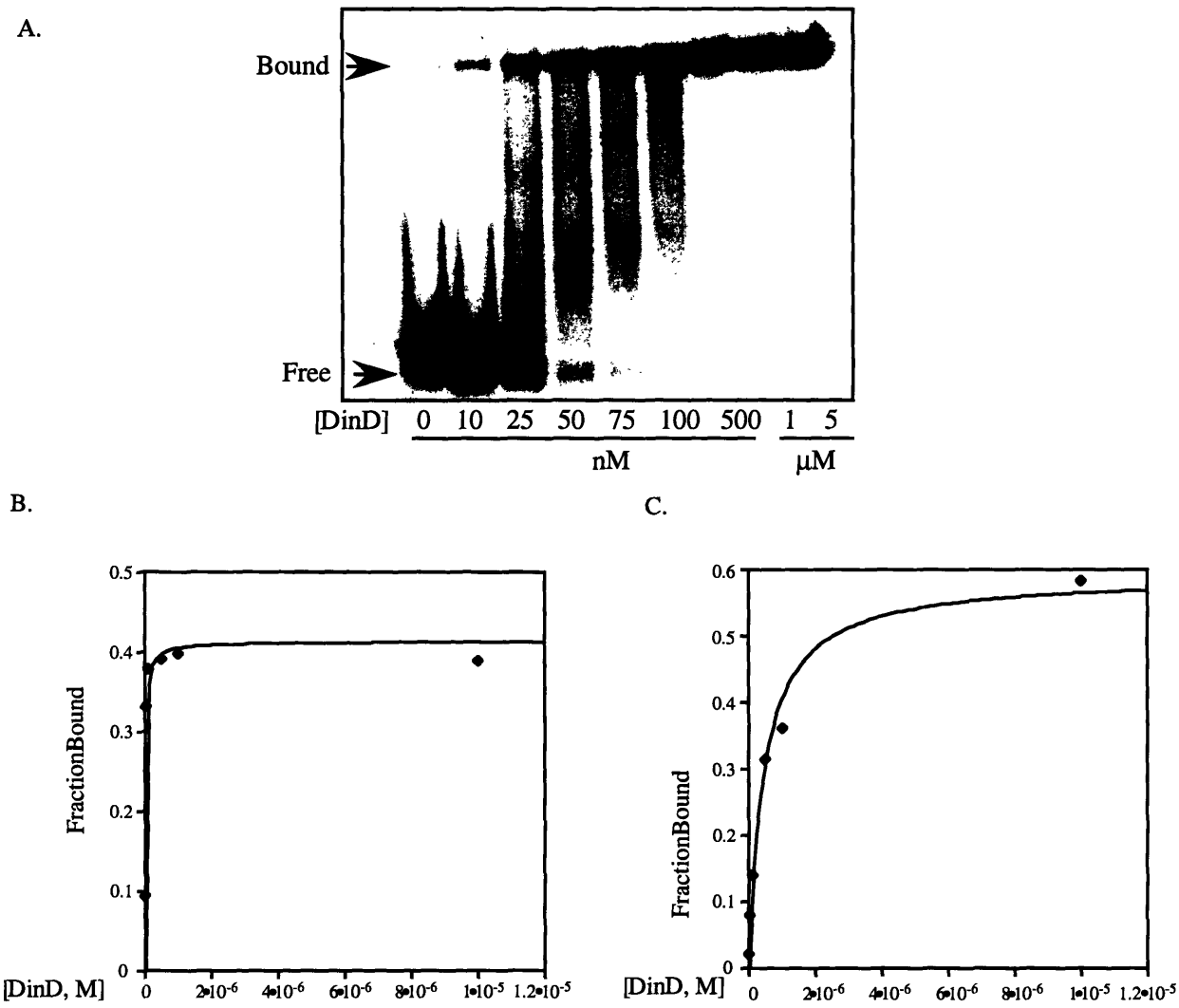
B



Appendix A.2: DinD degradation

A. DinD degradation *in vivo* was followed by western blot after stopping protein synthesis with chloramphenical.

B. ClpXP mediated degradation of labelled DinD [10 μM] was followed *in vitro* by release of TCA soluable counts.



Appendix A.3: DinD is a DNA-binding protein

A. DinD binds to double-stranded DNA as shown by gel shift assay.

B. DinD binds to double-stranded DNA with a K_D of 20 nM.

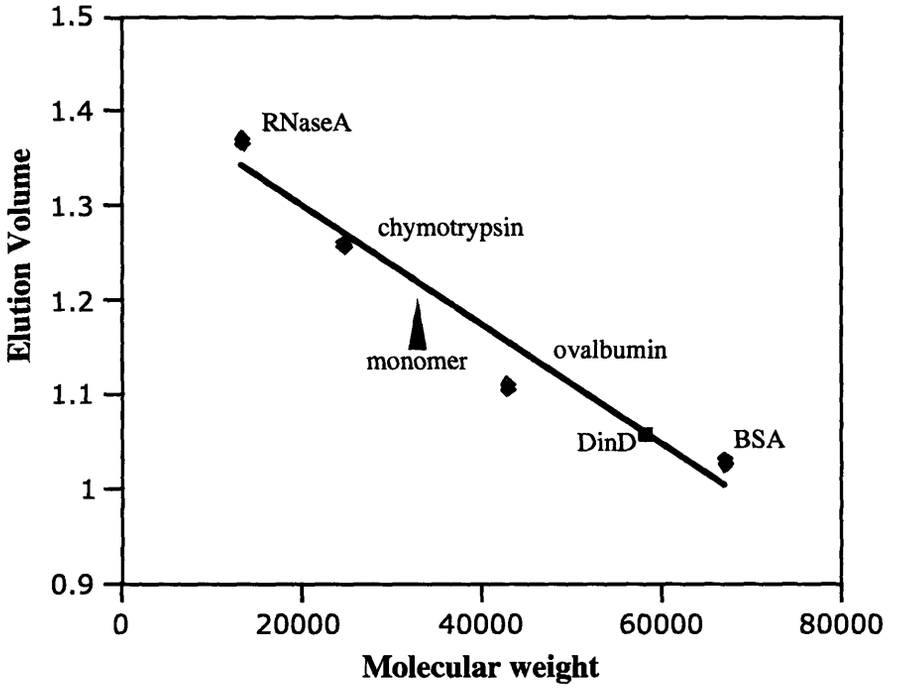
C. DinD binds to single-stranded DNA with a K_D of 45 μM.

DNA with a K_D of 45 μM and could bind a double stranded segment as small as 22 base pairs. A smeary pattern observed from gel shifts, as well as the propensity of the DinD to stick in the wells, suggested that multiple molecules of DinD might bind to each molecule of DNA. Intrigued by the general DNA binding characteristics of DinD, we set out to determine its oligomeric state and cellular protein level. Analytical ultracentrifugation suggested that DinD was a dimer in solution. This result was in good agreement with results obtained from gel filtration (figure A.4). We raised antibodies against DinD and found that it was present at about 1000 copies per cell after DNA damage, and was difficult to detect prior to DNA damage. DinD is limited in comparison to HU or DPS (60,000 and 180,000 copies per cell, respectively) suggesting that the DinD might be reserved for binding to more specialized structures (Ali Azam et al., 1999).

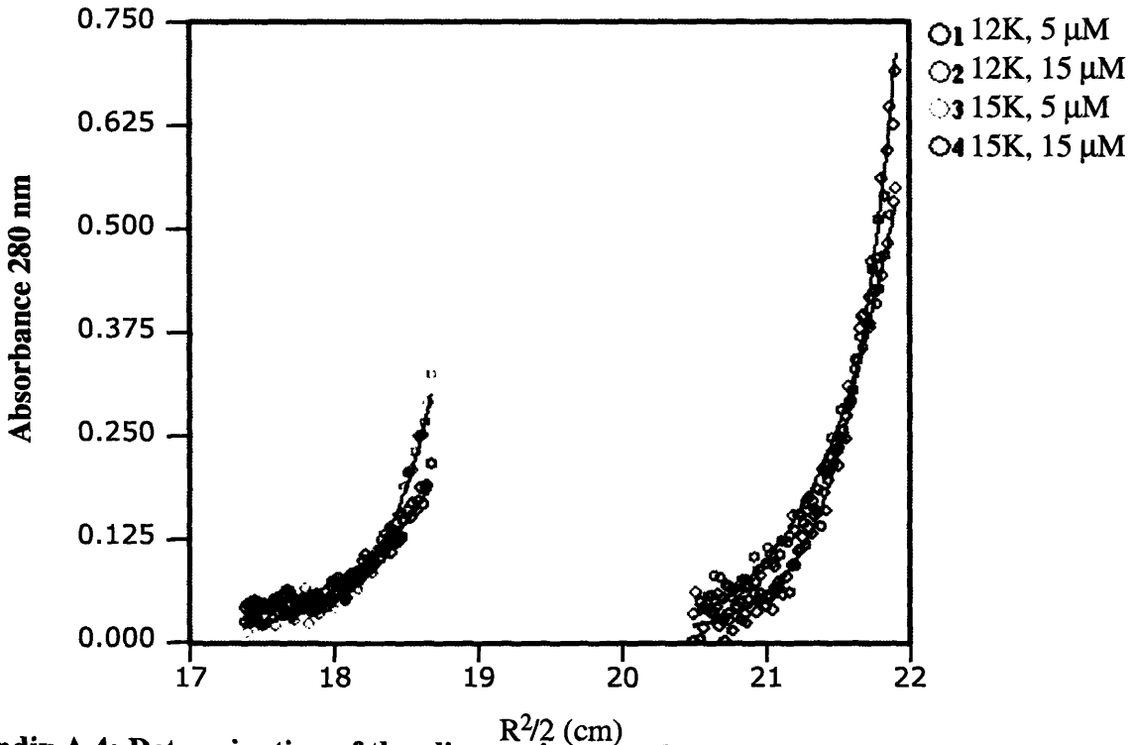
Although I believe a role for DinD in interacting with recombination intermediates is most consistent with the majority of the data, some of our own observations as well as some from the literature should be addressed. First, DinD must have a non-essential role in the response to DNA damage, as its deletion does not result in increased sensitivity to DNA damage (Kenyon and Walker, 1980; Ohmori et al., 1995). Additionally, the effect on transposition might be specific to certain transposases, as our observations suggest that phage Mu can form plaques with similar frequency in a DinD deletion and wild-type host. However, we do not know the precise nature of the mutant identified in the Derbyshire screen.

Another point to consider is that a region of DinD reported as a multicopy suppressor of the cold-sensitive phenotype is not required for DNA binding. Ohmari et al. report that a region of DinD from the BsshII site to the RsaI site can function as a multicopy suppressor (Ohmori et al., 1995). However, our Southwestern blots using partially trypsinized DinD showed that the first 76 amino acids (which included this region) were not necessary for DNA binding. One might predict that a wild type DNA binding region would suppress the effect of the mutant region when present in multiple copies. A possible explanation for the activity of the region reported by Ohmari is that it could be the dimerization region. If the active form of DinD is a dimer, then a fragment

A.



B.



Appendix A.4: Determination of the oligomeric state of DinD

A. Results from Superdex 75 gel filtration suggest that DinD is a dimer in solution. The expected elution volumes for a monomer is shown.

B. Analysis of ultracentrifugation from two concentrations and speeds returned a reduced mass expected for a dimer.

that blocks dimerization with a full-length molecule could suppress its toxic activity. Our results do suggest, though, that the V239M mutation is in the DNA binding region.

Several experiments are necessary to further characterize DinD. First, it would be useful to determine the affinity of the DinD V239M mutant for DNA. These studies should be extended to a variety of DNA molecules representing recombination intermediates. If DinD or the mutant had an increased affinity for any of these structures it would provide clues to a molecular mechanism. A plasmid for production of the V239M protein was constructed as part of this work. Genetic information could also be very useful in determining a more precise function for DinD. One possibility would be to combine the DinD deletion with a series of deletions of genes with known roles in recombination. These double mutants could be tested for sensitivity to DNA damaging agents, with the hopes of finding a protein of known function with which DinD is redundant.

Methods

Purification of DinD and production of antibodies

DinD was cloned into pET15b with an N-terminal His-tag and expressed from ER2256 cells. Cells were resuspended in buffer N1 (50 mM NaPhos pH8, 200mM NaCl, 10% glycerol) and lysed by two passes in a French press. The lysate was cleared by centrifugation and applied to Ni-NTA beads. Beads were washed with 200 mL buffer1, 100 mL buffer N1 + 20 mM imidizole and eluted with buffer N1 + 500 mM imidizole. Peak fractions were dialyzed into S1 (200 mmNacl, 50 mM NaPhos pH 6.8, 10% glycerol) and applied to a MonoS column. The column was washed extensively with buffer S1 and then eluted with a linear gradient over 20 mL to buffer S2 (500 mM Nacl, 50 mM NaPhos pH 6.8, 10% glycerol). Peak fractions were dialyzed into storage buffer (200 mmNacl, 50 mM NaPhos pH 8, 10% glycerol) and stored at -80°. DinD was easy to work with, also bound to HAP and could be purified in native form if necessary. Antibodies against the purified DinD protein were produced from rabbits by Covance. To generate 35S-labeled DinD, cells were grown in M9 minimal media lacking methionine to an OD600 of 0.4 and induced with 0.4 mM isopropyl -D-thiogalactoside for 80 min.

Express ^{35}S protein-labeling mix (NEN) was added to 20 μCi/ml (1 Ci = 37 GBq) of culture, and cells were grown for an additional 30 min before harvesting. ^{35}S-labeled DinD was then purified by the procedures for unlabeled protein.

Production of a DinD deletion: The DinD gene was replaced with a Kanamycin cassette as described (Datsenko and Wanner, 2000). The deletion was confirmed by PCR of genomic DNA and western blot.

Filter binding, gel shift and far Western analysis

A variety of PCR products and oligo cassettes available in the lab were annealed and kinased according to standard protocols. A 280 BP yefM PCR product (filter binding) and a 90 bp oligo cassette composed of TB1472 and TB1473 (band shifts) were used for results shown here. For filter binding, DinD and DNA were preincubated in 50 mM HEPES pH 7.6, 100 mM KCl, 10% glycerol for 10 minutes, then applied to a filter binding apparatus containing 4 mL of the same buffer and rapidly filtered onto nitrocellulose filters prepared by prewashing with .5M KOH. Gels shifts were most likely to be well shifts, but the best results were obtained using 2% Metaphor Agarose gels in .5x TBE at 4°.

Determination of oligomeric state

Gel filtration was performed on a SMART system using a Superdex 75 column in 50 mM HEPES pH 7.6, 100 mM KCl, 10% glycerol. DinD's elution volume was compared to a standard calibration mix. For analytical ultracentrifugation, protein samples were centrifuged in an Optima XL-A centrifuge (Beckman-Coulter, Fullerton, California) using a 60 Ti rotor. DinD at 5, 15, and 24 μM in storage buffer was centrifuged at 4° at 8,000, 15,000, and 20,000 rpm. A reduced mass was calculated using SEDNTERP (http://www.rasmb.bbri.org/) and fit to the transport equation using Sedfit (http://www.analyticalultracentrifugation.com).

In vitro and *in vivo* degradation were carried out as described in chapter 2 and appendix 1.

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