A Genetic Screen for Isolating "Lariat" Peptide Inhibitors of Protein Function

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SUMMARY

Functional genomic analyses provide information that allows hypotheses to be formulated on protein function. These hypotheses, however, need to be validated using reverse genetic approaches, which are difficult to perform on a large scale and in diploid organisms. We developed a genetic screen for isolating "lariat" peptides that function as *trans* dominant inhibitors of protein function. A lariat consists of a lactone-cyclized peptide with a covalently attached transcription activation domain, which allows combinatorial lariat libraries to be screened for protein interactions using the yeast two-hybrid assay. We isolated lariats against the bacterial repressor protein LexA. LexA regulates bacterial SOS response and LexA mutants that cannot undergo autoproteolysis make bacteria more sensitive to, and inhibit resistance against, cytotoxic reagents. We showed that an anti-LexA lariat blocked LexA autoproteolysis and potentiated the antimicrobial activity of mitomycin C.

INTRODUCTION

Ideal reagents for reverse analysis of protein function in diploid organisms have a dominant mode of action, are easily and rapidly generated against any given target, inhibit protein interactions and activities, and block specific interactions with a protein while leaving other interactions unperturbed. Further, to demonstrate the therapeutic potential of inhibiting targets with small molecule drugs, reverse analysis must also be performed with reagents that directly inhibit their target rather than blocking steps in target transcription or translation. Intracellular inhibitors with these characteristics can be rapidly obtained by genetically screening conformationally constrained, scaffolded peptides (peptide aptamers) from combinatorial peptide aptamer libraries using the yeast two-hybrid (Y2H) assay (Geyer and Brent, 2000). Constrained peptides are preferred as they generally bind tighter (Geyer and Brent, 2000) and are more stable (Davidson and Sauer, 1994) than linear peptides. The scaffold protein enhances solubility and allows a transcription activation domain to be fused to the peptide aptamer, which is essential for the Y2H assay. Combinatorial libraries of peptide aptamers should in principle contain members that bind any protein target.

There are several limitations with displaying peptides on the surface of scaffold proteins. First, some scaffold proteins are not stable enough to display and constrain a variety of peptides on their surface. For the green fluorescent protein scaffold, as many as one-third of peptides destabilize its structure (Woodman et al., 2005; Abedi et al., 1998). Second, some scaffold proteins are not stably expressed at high levels in a variety of cell types. For the thioredoxin (TrxA) scaffold, it has been shown that many TrxA peptide aptamers are not stable in human cell culture (Woodman et al., 2005). Third, scaffold proteins may possess intrinsic biological activity that is independent of the constrained peptide, which makes it difficult to decipher peptide aptamer activity (Woodman et al., 2005). Fourth, the conformation of the displayed peptide may be dictated by the location where the ends of the loop are inserted into the scaffold protein. Many peptides lose activity when they are transferred to other peptide scaffolds (Klevenz et al., 2002). Fifth, the size of many scaffold proteins limits their use as drugs or drug leads as they are usually not membrane permeable and they are susceptible to degradation by proteases. Further, the size of the scaffold prevents their chemical synthesis, makes their structure difficult to solve, and limits their access to sites on proteins such as active site crevices.

An alternative method of displaying peptides without many of the above limitations involves constraining peptides by cyclization. There are many examples of natural and synthetic cyclic peptide inhibitors (Horswill and Benkovic, 2005). Recently, methods have been developed to express genetically encoded cyclic peptides using engineered inteins (Scott et al., 1999). Unlike scaffold-constrained peptides, cyclic peptides are displayed independently of a scaffold protein, are resistant to exoproteases, and are amenable to chemical synthesis, structural studies, and membrane transport. Combinatorial libraries of cyclic peptides have been screened using forward and reverse approaches to isolate cyclic peptides that inhibit cellular processes (Kinsella et al., 2002; Nilsson et al., 2005; Cheng et al., 2007; Naumann et al., 2008; Kritzer et al., 2009) and disrupt protein interactions (Horswill et al., 2004; Tavassoli and Benkovic, 2005; Tavassoli et al., 2008), respectively. It is not possible, however, to isolate cyclic peptides that interact with specific proteins using genetic systems such as the Y2H assay, since there is no N or C terminus to fuse a transcription activation

domain. As a result, it has been difficult to obtain cyclic peptides for the reverse analysis of protein function.

Here, we describe a novel genetic assay to isolate lariat peptides that interact with a target protein using the Y2H interaction trap (Gyuris et al., 1993). A lariat peptide consists of a cyclic peptide or "noose" region with a covalently attached transcription activation domain. We generated lariats that were compatible with the Y2H assay by blocking the intein-producing cyclic peptide reaction (Scott et al., 1999) at an intermediate step, which produces a lariat that contains a transcription activation domain covalently attached through an amide bond to a lactone-cyclized peptide. Lariat peptides can be used to study the function or validate the therapeutic potential of protein targets.

We demonstrate the feasibility of this approach by generating a lariat inhibitor of the bacterial repressor protein LexA. LexA is a putative antimicrobial target, which when inhibited should potentiate the activity of cytotoxic antibiotics. When LexA is bound by activated RecA it undergoes autoproteolysis and no longer represses genes in its regulon (Lin and Little, 1988). LexA mutants that block autoproteolysis (Walker, 1984) make bacteria more sensitive to stress induced by compounds such as the DNA damaging reagent mitomycin C (MMC) (Lin and Little, 1988) and they decrease antibiotic resistance (Cirz et al., 2005; Miller et al., 2004). LexA inhibitors that block autoproteolysis would increase the sensitivity of bacteria to cytotoxic reagents and since LexA is not present in humans it would have no effect on host DNA damage repair systems.

RESULTS

Construction and Screening of Combinatorial Lariat Peptide Libraries

To generate the lariat, we blocked the asparagine cyclization step in the intein-producing cyclic peptide reaction (Scott et al., 1999) (Figure 1A) by mutating asparagine at position I_{C-1} to alanine (Figure 1B). We created a combinatorial library of lariats with the noose region containing the amino acid sequence SX_7EY, where X represents amino acids encoded by the NNK codon. Glutamate (E) and tyrosine (Y) amino acids in the noose region were included to facilitate cyclization (Scott et al., 2001; Naumann et al., 2005). We constructed a lariat peptide expression plasmid library (pIL-XX) in the MATa yeast strain EY93. To estimate the number of library members that correctly produced lariats in yeast, we sequenced randomly chosen library members and showed that ~31% had a correct sequence (see Figure S1 available online). We also analyzed processing of 20 library members with the correct sequence and showed that ~70% of these library members produced a lariat product by western analysis (Figure S1). In total, ~25% of the library is capable of producing a lariat. We mated a library of approximately five million lariats in the MATa strain EY93 to the MATα strain EY111, which contains the LexA target plasmid and Y2H reporter genes. We screened 2×10^8 MATa/α yeast cells that contain the lariat library, the LexA target, and Y2H reporter genes using the Y2H interaction trap (Figure 1C) (Finley and Brent, 1994) and isolated 14 positive interactions based on the activation of the LEU2, ADE2, and LacZ reporter genes. Sequencing of the 14 positive plasmids revealed 12 plasmids with the L2 sequence and 2 plasmids with the L1 sequence (Figure 1D). We chose the L2 lariat for further anal-

ysis as it contained more charged amino acids, which we hypothesized would enhance its solubility.

Characterization of Anti-LexA Lariats

To confirm the importance of the lariat structure for the L2 lariat-LexA interaction, we cloned the noose region of the L2 lariat into the following modified intein expression plasmids (Figure 2): (1) L2 inactive intein plasmid (pIN-L2), which expresses a lariat precursor that does not undergo any steps in the intein-mediated cyclization reaction; (2) L2 linear plasmid (pLIN-L2), which expresses an L2 peptide that is not cyclized by a lactone bond; (3) L2 active intein plasmid (pACT-L2), which expresses a cyclic L2 peptide; and (4) L2 control lariat plasmids, which express amino acids from the noose region of the L2 lariat in a scrambled (pIL-L2-scrambled) or inverted (pIL-L2-inverted) order. We confirmed expression of these L2 constructs using western analysis with an antibody against the N-terminal hemagglutinin (HA) tag (Figure 3A). The L2 lariat plasmid produced unprocessed (~32 kDa) and lariat (~18 kDa) HA-tagged products; L2 inactive intein and L2 linear peptide plasmids produced only unprocessed and linear peptide (~18 kDa) HA-tagged products, respectively; and the L2 active intein plasmid produced unprocessed and I_C domain (~17 kDa) HA-tagged products. In the EY93 yeast strain, L2 active intein processing was slow relative to the L2 lariat and the unprocessed product migrated slightly slower in a SDS-PAGE gel. Similar differences in active L2 intein and L2 lariat processing were also observed in E. coli, where after IPTG induction ~65% of the L2 lariat was correctly processed, whereas only ~19% of the active L2 intein underwent processing to the lariat intermediate (Figure S2).

We did not detect any interactions between LexA and the L2 inactive intein, the L2 active intein, and the L2 linear peptide or between L2 scrambled, L2 inverted, and CPGC noose peptide (pIN01) lariat controls in Y2H assays when the LEU2, ADE2, and LacZ reporter genes were assayed together (Figure 3B). Only the L2 lariat interacted with LexA in the Y2H assay when all reporters were used, which highlighted the importance of the lactone constraint in this interaction. We detected a weak interaction between the L2 inactive intein and a very weak interaction with the L2 active intein when only the LEU2 reporter gene was used (Figure S3). The dependence of the interaction on the lactone constraint was not unique to this anti-LexA lariat as we have also shown that lactone cyclization was important for lariats that were isolated against two other target proteins (Figure S4).

We performed an alanine scan to determine amino acids important for the interaction of the L2 lariat with LexA (Figure 3C). All of the alanine mutants except for L2 E9A did not interact with LexA. Glutamic acid at position 9 has been proposed to facilitate production of cyclic peptides from an active intein (Scott et al., 2001; Naumann et al., 2005). The E9A mutation is still active in the Y2H assay, suggesting that E9 does not contribute to the L2 lariat interaction with LexA and is not required to produce the lariat. Interestingly, we found that the L2 E9A mutation resulted in better lariat stability (Figure S5).

We also analyzed the ability of the L2 lariat to interact with two LexA mutants, S119A and K156A. LexA exists in either a noncleavable (NC) or cleavable (C) conformation, where the C conformation accounts for <0.1% of the LexA population at pH 7 (Roland et al., 1992). Luo et al. (2001) proposed that RecA
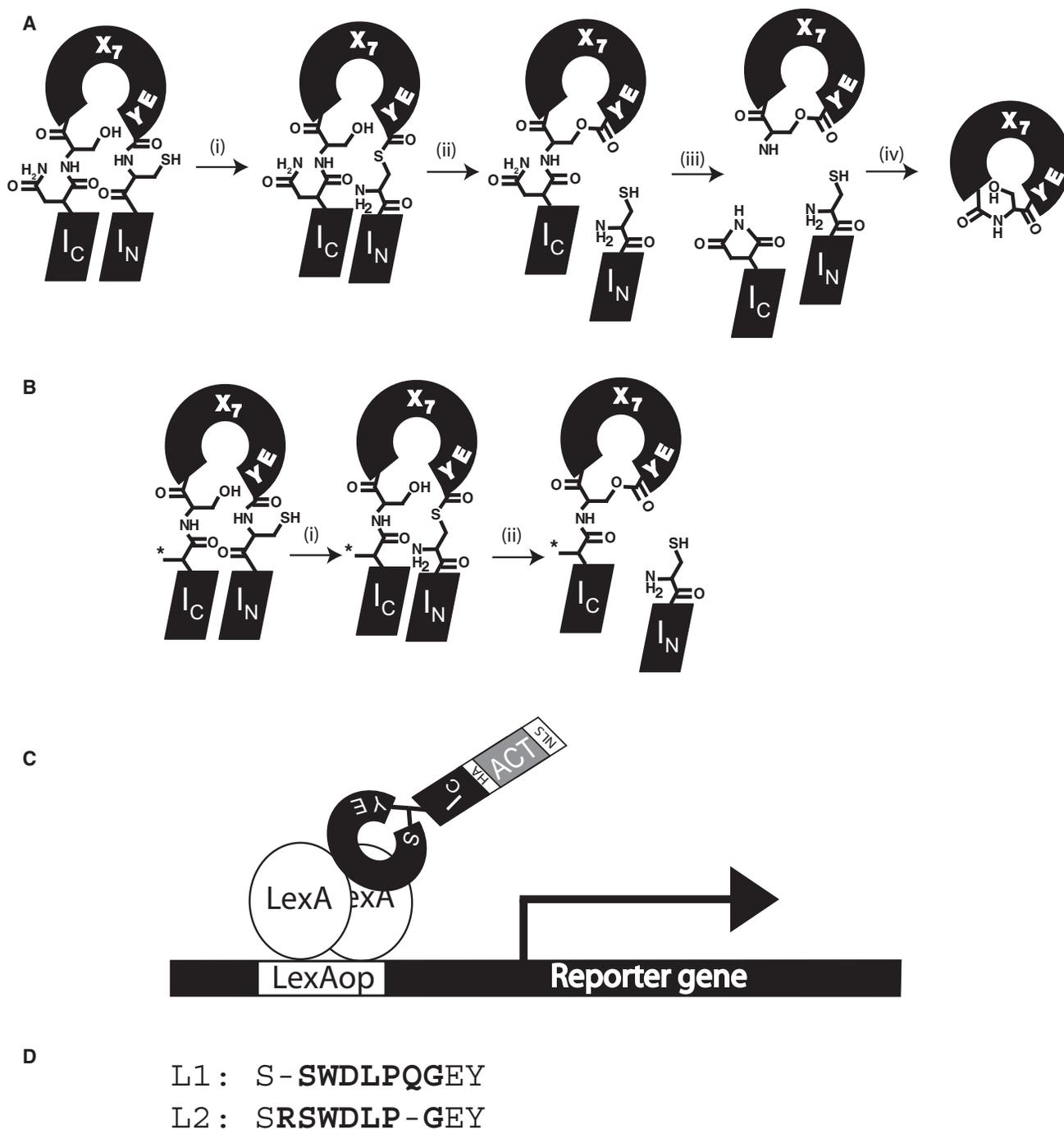
Figure 1. Production and Isolation of Anti-LexA Lariats

(A) Intein-mediated peptide cyclization. (i) Unprocessed intein undergoes an N-to-S acyl shift using the I_{N+1} cysteine at the peptide-I_N junction. (ii) Transesterification reaction involving I_{C+1} serine at the I_C-peptide junction and the thioester formed in step (i), which releases the I_N domain and produces the lariat intermediate. (iii) I_{C-1} asparagine undergoes a side chain cyclization, which releases the I_C domain and generates a lactone-cyclized peptide. (iv) Lactone-cyclized peptide undergoes a thermodynamically favored O to N acyl shift to produce a lactam-cyclized peptide.

(B) Lariat-producing intein. The lariat is produced by mutating the asparagine at position I_{C-1} to alanine (*), which inhibits asparagine cyclization and stops the reaction at the lariat intermediate.

(C) The LexA-lariat Y2H assay. The LexA operator (LexAop) is upstream of stably integrated Y2H reporter genes. Plasmid pEG202 expresses LexA, which binds to the LexAop. Plasmid pIL-XX expresses members of the lariat library that contain and I_C domain fused to an N terminus HA tag, a transcription activation domain (ACT), and a nuclear localization sequence (NLS). X represents amino acids coded by the NNK codon. E and Y represent constant glutamate and tyrosine amino acids. Lariats that interact with LexA bring the transcription activation domain near the transcription start sites of the reporter genes and activate transcription.

(D) Amino acid sequences of the noose region from two anti-LexA lariat peptides (L1 and L2). Amino acids from the combinatorial region are bolded and dashes are used to align common amino acids in L1 and L2.
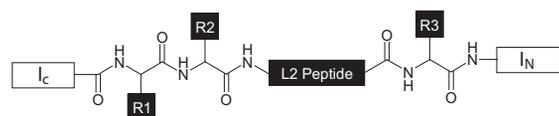
L2 Constructs	I_C	R1 I_{C-1}	R2 I_{C+1}	L2 Peptide	R3 I_{N+1}	I_N
Active	I_C	Asn	Ser	RSWDLPGEY	Cys	I_N
Inactive	I_C	Ala	Ser	RSWDLPGEY	Ala	I_N
Linear	I_C	Ala	Ser	RSWDLPGEY	Stop	I_N
Lariat	I_C	Ala	Ser	RSWDLPGEY	Cys	I_N
Scrambled	I_C	Ala	Ser	DPGLRSWEY	Cys	I_N
Inverted	I_C	Ala	Ser	GPLDWSREY	Cys	I_N
pIN01	I_C	Ser	Ser	CPGCEY	Ala	I_N

Figure 2. Sequences of L2 Active, L2 Inactive, L2 Linear, L2 Lariat, L2 Scrambled, L2 Inverted, and pIN01 Inteins

The L2 active intein contains Asn at position I_{C-1} and Cys at position I_{N+1}, which are necessary to produce the lactam-cyclized peptide. The L2 lariat contains an asparagine to alanine mutation at position I_{C-1}, which blocks the asparagine side chain cyclization reaction. The L2 inactive intein contains the same mutations as the lariat and a cysteine to alanine mutation at position I_{N+1}. The cysteine to alanine mutation at I_{N+1} blocks the N to S acyl shift. The L2 linear peptide contains two stop codons at the I_{N+1} position. L2 scrambled and L2 inverted are the same as the L2 lariat except that the L2 peptide sequence is rearranged or inverted, respectively. pIN01 intein is an inactive intein with a CGPC peptide noose.

promotes LexA autoproteolysis by shifting the equilibrium toward the C conformation. LexA S119A mutant has a change in the active site serine nucleophile that prevents cleavage (Slilaty and Little, 1987), but does not alter the NC conformation (Luo et al., 2001). LexA K156A mutant has a change in the active site lysine general base that prevents cleavage and shifts the equilibrium toward the C conformation (Luo et al., 2001). The L2 lariat interacted with LexA and LexA K156A but not with LexA S119A (Figure 3D), which suggested that the L2 lariat interacted near the LexA autoproteolysis active site.

We used surface plasmon resonance (SPR) to determine the dissociation constant (K_D) between LexA and a synthetic L2 lariat peptide (Figure 3E). An L2 lariat peptide was synthesized with an alanine and biotin moiety on the N terminus "tail," which replaces the I_C domain in the L2 lariat. The synthetic L2 lariat interacted with LexA with a K_D of 37 μM. In contrast, we were unable to detect binding between LexA and the synthetic biotinylated linear L2 peptide at concentrations up to 125 μM (Figure S6). The in vitro binding constant may differ from the in vivo binding constant since the L2 lariat may interact with a specific chromosome-associated conformation of LexA. In *E. coli*, ~20% of LexA is free in solution with the majority of LexA associated with DNA (Sassanfar and Roberts, 1990). Further, structural modeling and biochemical studies suggest that LexA undergoes a conformational change when it binds its operator (Butala et al., 2007; Chattopadhyaya and Pal, 2004; Groban et al., 2005). It is conceivable that the K_D of the L2 lariat could be decreased further by affinity maturation using a more stringent Y2H screen. Previously, Colas et al. (2000) used random PCR mutagenesis and a single operator LexA Y2H *LacZ* reporter to enhance the affinity of an anti-Cdk2 aptamer from 100 nM to 5 nM.

The *lexA* promoter contains several tandem LexA operators (Little et al., 1981; Brent and Ptashne, 1981) and LexA expression is autoregulated by a feedback mechanism (Little and Harper,

1979). To investigate whether L2 lariat was associated the *lexA* promoter, we used a Ni^{2+}-nitriloacetic acid (Ni^{2+}-NTA) chromatin precipitation and quantitative PCR assay with His-tagged L2 lariat and Ni^{2+}-NTA affinity chromatography (Tamimi et al., 2004). The L2 lariat showed an ~5-fold greater association with the *lexA* promoter compared to the L2 scrambled lariat (Figure 3F).

We used LC-ESI-TOF mass spectrometry (MS) to determine whether the L2 lariat was stably expressed from a His tag bacterial expression plasmid (pETIL-L2) in BL21 CodonPlus (BL21-CP) *E. coli*. After purification of the L2 lariat using a Ni^{2+}-NTA column, we observed two products near the calculated molecular weight of the L2 lariat. The lighter product (8651 Da) corresponded to the L2 lariat, which represented 26% of the total product, and the heavier product (8669 Da) corresponded to a hydrolyzed lariat product, which represented 74% of the total product (Figure 4A). Previous MS studies on the cyclic peptide-producing intein reaction have reported the lariat intermediate as present mainly in the hydrolyzed form (Scott et al., 1999, 2001). Together these results suggest that hydrolysis of the lactone bond may be caused by high temperatures and acidic conditions used in the MS analysis. To determine the amount of lariat present prior to MS analysis, we forced the cleavage of the lariat lactone using $Na^{18}OH$ (Hagelin, 2005), digested the lariat with trypsin, and analyzed the molecular weight of fragments using LC-ESI-TOF MS (Figure 4B). Lariat lactones cleaved by $Na^{18}OH$ were 2 Da heavier than lactones cleaved prior to $Na^{18}OH$ treatment. We observed incorporation of ^{18}O into two trypsin fragments that resulted either from hydrolysis of the lactone bond or from an α-H elimination that generated dehydroalanine, which was followed by a Michael addition (Figure S7). The fraction of ^{18}O incorporated in these fragments indicated that 46% of the lariat was cyclized prior to MS analysis (Figure S8). This value represented a low estimate of the amount of L2 lariat present in *E. coli* as some of the lariat may have been hydrolyzed during Ni^{2+}-NTA affinity chromatography and reverse-phase HPLC purification and lyophilization prior to being hydrolyzed by $Na^{18}OH$. These data, combined with the fact that many lactone-cyclized peptides exist in nature (Grünewald and Marahiel, 2006), support the existence of the lariat structure in vivo.

Despite the observation that a significant portion of the lariat was hydrolyzed, we were still able to isolate lariats that activated the reporter genes in the Y2H assay. Since activation of Y2H reporters depends on a variety of parameters including protein expression levels, interaction strength, and the threshold for reporter activation, it is difficult to predict whether this level of cyclization would be sufficient for other targets. Other applications may require pure lariat populations and in these cases it may be possible to generate more stable lariats as we have shown that the lariat fraction can be enhanced by mutations to the lariat noose region. For example, the L2 E9A mutant was present as ~61% lariat in *E. coli* compared to L2 lariat, which was present at ~26% lariat. These values represent a low estimate of the amount of lariat present since we did not correct them for the lariat that was hydrolyzed during the MS analysis (Figure S5).

Activity of Anti-LexA L2 Lariat

We monitored the ability of the L2 lariat to inhibit MMC-induced depletion of LexA at the *lexA* promoter using chromatin immunoprecipitation (ChIP) and quantitative PCR (Wade et al., 2005)

A

B

C

D

E

$$K_d = 37 +/- 11\ \mu M$$

F
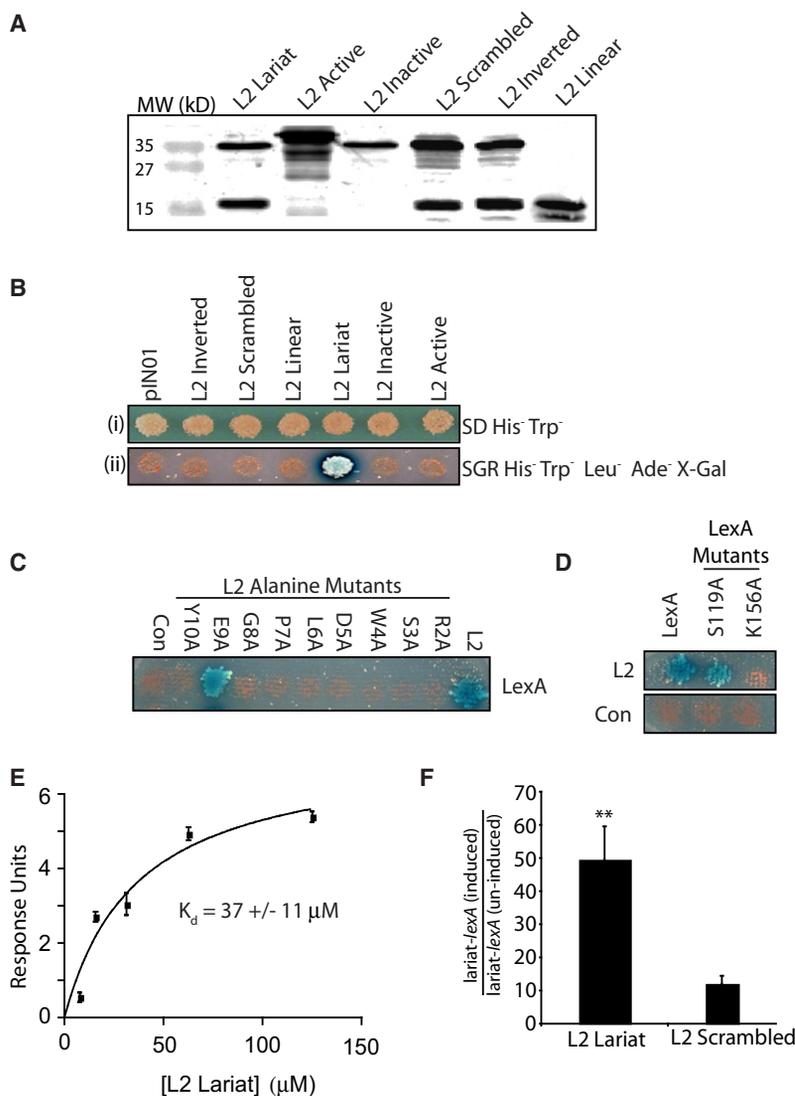
Figure 3. Analysis of the L2 Lariat

(A) Western analysis of L2 lariat and intein processing in EY93 using an anti-HA antibody. The HA-tagged L2 inactive intein is ~32 kDa. The L2 lariat, L2 scrambled lariat, and L2 inverted lariat produced two products, an HA-tagged unprocessed lariat intein and an HA-tagged lariat, which are ~32 kDa and ~18 kDa, respectively. The HA-tagged L2 linear peptide is ~18 kDa. The L2 active intein produced two products, an HA-tagged unprocessed intein and an HA-tagged I_C domain, which are ~32 kDa and ~17 kDa, respectively. In SDS-PAGE, the unprocessed active intein runs at a slightly higher molecular weight. MW represents molecular weight markers (kDa).

(B) Y2H analysis of the interaction of LexA with L2 lariat and intein constructs. pIN01 is an inactive lariat expression plasmid that expresses an inactive intein with a CGPC peptide noose. (i) Yeast growth on nonselective synthetic dextrose (SD) His⁻ and Trp⁻ media. (ii) Yeast growth on SGR His⁻, Trp⁻, Leu⁻, Ade⁻, and X-Gal media, which selects for the activation of *LEU2*, *ADE2*, and *LacZ* Y2H reporter genes.

(C) Y2H analysis of the interaction of LexA with L2 lariat and L2 lariat alanine mutants. Yeast were grown on SGR His⁻, Trp⁻, Leu⁻, Ade⁻, and X-Gal media that selects for the activation of *LEU2*, *ADE2*, and *LacZ* Y2H reporter genes. Con represents L2 scrambled lariat.

(D) Y2H analysis of the interaction of L2 lariat and LexA and LexA mutants. Yeast were grown on SGR His⁻, Trp⁻, Leu⁻, Ade⁻, and X-Gal media that selects for the activation of *LEU2*, *ADE2*, and *LacZ* Y2H reporter genes. Con represents L2 scrambled lariat.

(E) SPR analysis of L2 Lariat-LexA interaction. Maximum SPR response units were calculated for L2 lariat concentrations ranging from 7.8 μM to 125 μM. The binding isotherm was fit to a one site binding model to calculate the K_D ± standard error using Prism4.0 (GraphPad) statistical software. Representative SPR sensograms are shown in Figure S6. Error bars represent the standard deviation from three independent experiments.

(F) Analysis of the association of the L2 and L2 scrambled lariats with the *lexA* promoter using Ni^{2+}-NTA chromatin precipitation assay. Precipitated *lexA* promoter DNA was quantified by real-time PCR and normalized to the corresponding input DNA. Histograms show the ratio of Ni^{2+}-NTA precipitated *lexA* promoter DNA (lariat-*lexA* complex) between IPTG-induced and uninduced His-tagged L2 and L2 scrambled lariats. Error bars represent the standard deviation from three independent experiments. **$p < 0.01$ between L2 lariat and L2 scrambled lariat.

(Figure 5A). MMC is a potent inducer of bacterial SOS response that causes the release of LexA from its promoter (Lin and Little, 1988). We observed that MMC treatment depleted 94% of LexA from the *lexA* promoter in cells expressing the L2 scrambled lariat control, whereas only 14% of LexA was depleted in cells expressing the L2 lariat. Expression of L2 active intein, L2 inactive intein, and L2 linear peptide resulted in 73%, 86%, and 88% of LexA being depleted from *lexA* promoter after MMC treatment, respectively.

We monitored the ability of the L2 lariat to block MMC-induced LexA autoproteolysis using western analysis with an anti-LexA antibody. MMC activates RecA coprotease activity and induces cleavage of LexA (Lin and Little, 1988). We monitored degradation of LexA after exposure to MMC (Yasuda et al., 1998) in the presence and absence of L2 lariat, L2 scrambled lariat, L2 linear

peptide, L2 inactive intein, and L2 active intein (Figure 5B). We observed that LexA was significantly degraded (98%) after 1 hr treatment with MMC in cells expressing L2 scrambled control. In contrast, the L2 lariat completely blocked MMC-induced LexA degradation. L2 linear peptide, L2 active intein, and L2 inactive intein partially blocked MMC-induced LexA proteolysis with 87%, 65%, and 67% of LexA remaining, respectively.

We tested the ability of L2 lariat to inhibit *E. coli* growth in the presence of MMC using the survival assay described by Lin and Little (1988). We expressed L2 scrambled lariat, L2 lariat, L2 linear peptide, L2 inactive intein, and L2 active intein in BL21-CP cells; exposed the bacteria to MMC in 0.85% NaCl for 1 hr; and assayed their survival (Figure 6). Expression of the L2 lariat enhanced the activity of MMC and reduced BL21-CP cell viability to ~30% relative to the L2 scrambled lariat. Expression
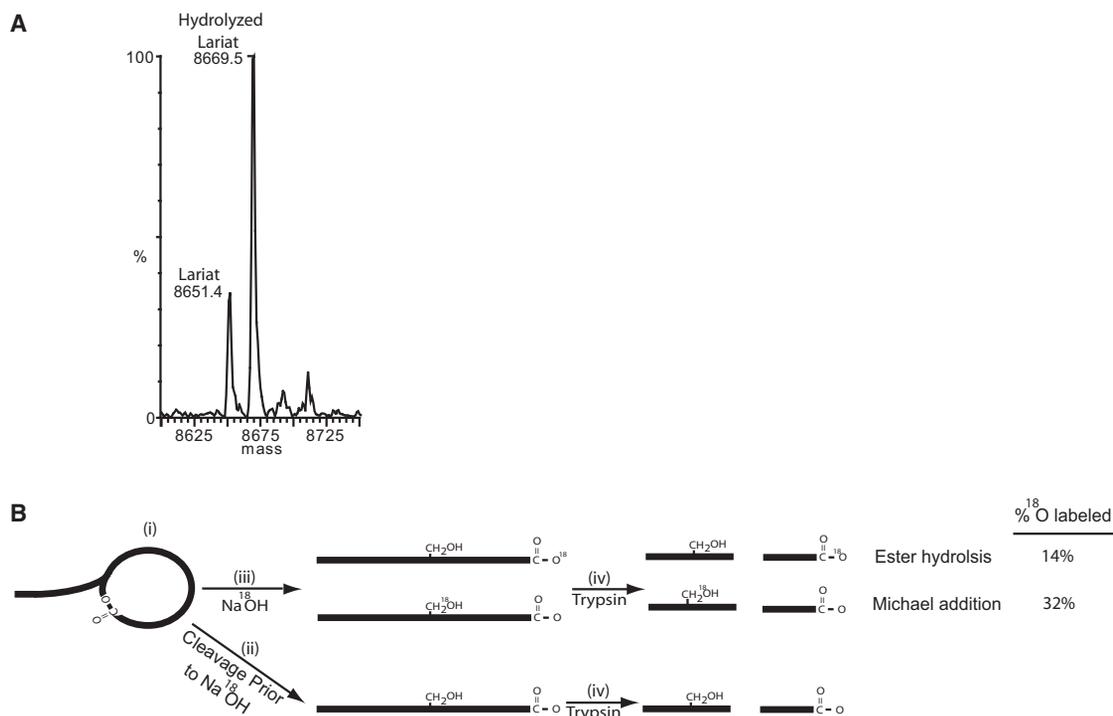
Figure 4. Stability of L2 Lariat

(A) ESI-TOF MS analysis of His tag purified L2 lariat produced in BL21-C P. L2 lariat (8651.7 calc; 8651.4 obs) and hydrolyzed L2 lariat (8669.7 calc; 8669.5 obs). (B) Analysis of the amount of lariat present prior to MS analysis. (i) Lariat cyclized through a lactone bond. (ii) Lariat cleaved prior to Na^{18}OH treatment. (iii) Products from the Na^{18}OH-induced cleavage of lactone bond. (iv) Trypsin digest of the cleaved lariat to confirm the location of ^{18}O incorporation. The percentage of each fragment containing ^{18}O is shown and corresponds to the amount of lariat cyclized through a lactone bond prior to MS analysis (Figure S8).

of L2 linear peptide, L2 active intein, and L2 inactive intein enhanced the activity of MMC by ∼68%, 65%, and 53%, respectively, relative to the L2 scrambled lariat. A possible reason for the low activity of the L2 active intein in the activity assays was the low processing of L2 active intein in *E. coli* relative to the L2 lariat (Figure S2). The L2 lariat is identical to the L2 active intein except for an asparagine to alanine mutation at position I_{C-1}. Asparagine at position I_{C-1} decreased production of the lariat intermediate from the active construct; however, it is required for converting the lariat to a lactam-cyclized peptide, which suggested that it may be difficult to engineer the active construct to improve its processing efficiency.

In summary, the L2 lariat had the highest activity in *lexA* promoter ChIP, LexA proteolysis, and survival assays. L2 inactive and active constructs showed similar activity in these assays, which was lower than the L2 lariat and corresponded with the lower affinity of the L2 inactive intein in the Y2H assay. Since the L2 active construct does not process well, its activity is most likely due to the unprocessed product, which is similar to the L2 inactive product. The L2 linear construct showed the lowest activity, which corresponds with its inability to activate reporter genes in the Y2H assay and to show an interaction with LexA in the SPR assay.

DISCUSSION

We developed a new strategy for displaying cyclic peptides with a covalently attached linear peptide. The N terminus of the lariat can be used to fuse protein moieties such as a transcription activation domain, localization sequence, fluorescent protein, membrane permeable peptide, etc. Lariats can be produced in prokaryotic and eukaryotic environments and the noose region can be used to display combinatorial peptide libraries. Lariats are constrained by a lactone bond, allowing a variety of peptide sequences to be displayed. Lactone bonds are stable under reducing conditions, which allows lariat libraries to be screened using intracellular assays such as the Y2H assay. This is in contrast to disulfide bond-constrained peptide libraries, which are not stable under reducing conditions and must be screened using in vitro selection strategies such as phage display (McLafferty et al., 1993).

Lariats have advantages over intein-produced lactam-cyclized peptides for analyzing intracellular protein function. First, for *Ssp DnaE* intein-based constructs, the lariat is produced at higher levels than its corresponding L2 cyclic peptide. Second, lariat libraries can be screened using the Y2H assay to isolate lariats against a target protein. Combinatorial libraries of cyclic peptides generated by intein-mediated cyclization have been successfully screened to isolate cyclic peptides that inhibit protein interactions and activities (Naumann et al., 2008; Horswill et al., 2004; Tavassoli and Benkovic, 2005; Tavassoli et al., 2008; Kritzer et al., 2009) and cell phenotypes (Kinsella et al., 2002; Nilsson et al., 2005). These selection strategies, however, do not allow cyclic peptides to be targeted to a specific protein. Here, we have shown that synthetic lariat peptides, which still retain the same binding activity as the lariat, can be generated
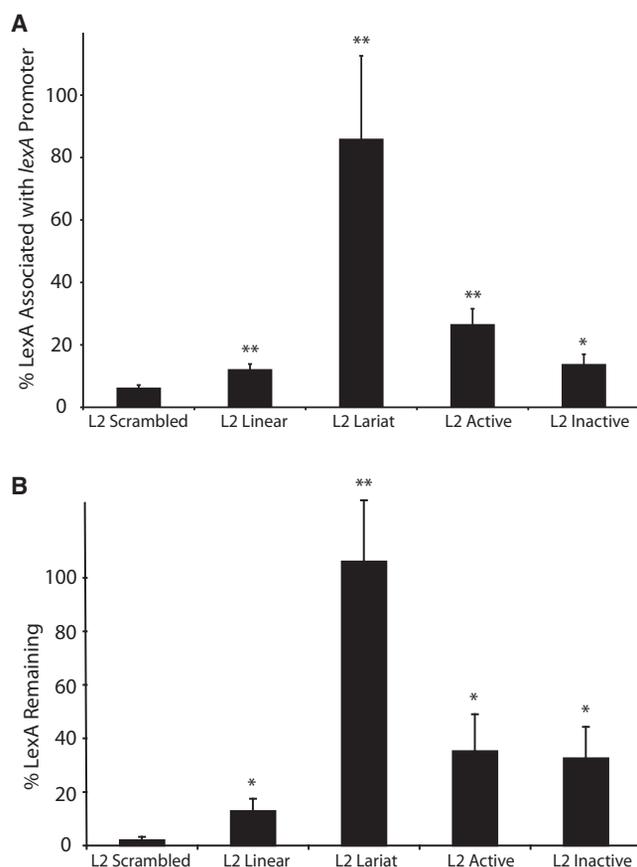
Figure 5. L2 Lariat Inhibition of LexA Autoproteolysis

(A) ChIP analysis of the influence of L2 lariat and intein constructs on MMC-induced depletion of LexA at the *lexA* promoter. The percentage of LexA remaining bound to the *lexA* promoter after MMC treatment was measured as a ratio of the occupancy units of MMC-treated to untreated samples multiplied by 100. Error bars represent the standard deviation from three independent experiments. **p < 0.01 and *p < 0.05 between L2 lariat and intein constructs and the L2 scrambled lariat.

(B) Inhibition of MMC-induced LexA cleavage by L2 lariat. BL21-CP cells expressing L2 lariat and intein constructs were treated with MMC and chloramphenicol. Cell extracts were analyzed by western analysis using an anti-LexA antibody. The percentage of LexA that does not undergo proteolysis after 1 hr treatment with MMC is reported. Error bars represent the standard deviation from three independent experiments. **p < 0.01 and *p < 0.05 between L2 lariat and intein constructs and the L2 scrambled lariat.

based on the noose sequence. Alternatively, Kinsella et al. (2002) showed that cyclic peptides that inhibit IL-4 signaling in B cells still retain their activity when they are expressed in a mutated intein construct that does not produce cyclic peptides. Therefore, it is conceivable that in cases where the lariat cyclic peptide structure is not dependent on the geometry of the lactone bond that it should be possible to generate peptides, which retain the activity of the lariat peptide, that are cyclized through an amide backbone bond.

In summary, we have presented a new method to isolate lariat peptides against a given target protein using the Y2H system. We used lariat peptide Y2H assay to generate lariat inhibitors of LexA and validate LexA as a therapeutic target for potentiating the antimicrobial effects of reagents that activate the SOS
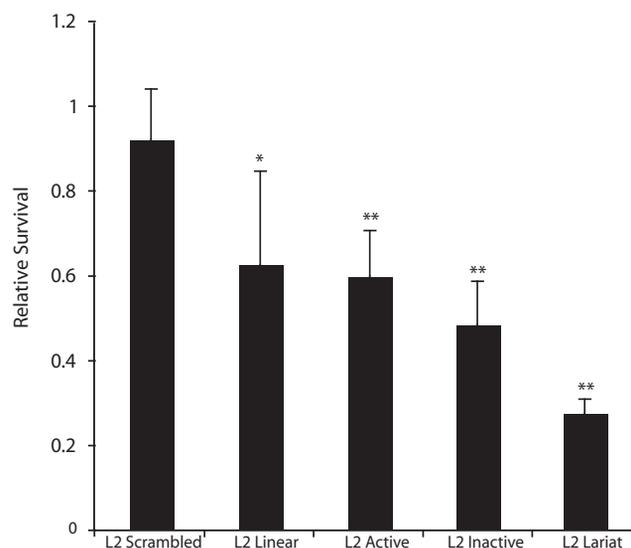
Figure 6. Survival Assay for MMC-Treated BL21-CP Cells Transformed with L2 Lariat and Intein Constructs

Normalized relative cell survival was calculated by dividing the number of cfu after 30 min MMC treatment by the number of cfu at the zero hour time point. Error bars represent the standard deviation of five independent experiments. **p < 0.01 and *p < 0.05 between L2 lariat and intein constructs and the L2 scrambled lariat.

response pathway. The lariat technology provides a rapid high throughput assay for isolating peptide inhibitors that can be used for the reverse analysis of protein function or as drugs or pseudo-drugs for validating therapeutic targets.

SIGNIFICANCE

Lariat peptides possess desired traits for characterizing the function and therapeutic potential of proteins. Lariats consist of a lactone-cyclized peptide with a covalently attached transcription activation domain. The transcription activation domain allows combinatorial lariat libraries to be screened against protein targets using the yeast two-hybrid assay (Y2H). Combinatorial lariat libraries should in principle contain members that bind any protein target. Lariat peptides are constrained by a lactone bond, which allows them to be used in reducing intracellular environments. Lariats function as *trans* dominant inhibitors, which, unlike genetic approaches that inactivate genes by deletion or creation of loss-of-function mutations, can be easily used to inhibit protein function in diploid organisms. Lariats inhibit their protein target directly and have the potential to block specific interactions with a protein while leaving other interactions unperturbed. This is in contrast to *trans* dominant agents like antisense RNA/DNA, ribozymes, and RNAi that block transcription and translation of their target. Thus, results obtained using lariat inhibitors can be used directly to evaluate the therapeutic potential of inhibiting targets with small molecule drugs. Lariats can be easily and rapidly generated against any given target compatible with the Y2H assay and thus are easier to generate than

antibodies or dominant-negative proteins. The transcription activation domain of lariats can be replaced by other peptide and chemical moieties such as affinity tags, fluorescent molecules, localization sequences, etc., which give them advantages over "head-to-tail" cyclized peptides, which have no free end to attach moieties. Together, these properties make lariats an ideal reagent for assessing whether small molecules inhibitors can be generated against a protein target. Further, the small size of lariats makes them amenable to chemical synthesis and allows their structure to be easily solved, which makes them useful as potential drugs or drug leads.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for a detailed description of plasmid construction, western analysis, intein processing analysis, lariat and LexA purification, LexA and Ni^{2+}-NTA chromatin precipitation assays, and LexA proteolysis assay.

Reagents

Oligonucleotides were synthesized by Integrated DNA Technologies and are listed in Table S1. The biotin-labeled L2 lactone peptide was synthesized by Protein Peptide Research. The lactone peptide was synthesized on 2-chlorotrityl resin, using serine(trityl) for the serine to be cyclized. The fully side chain and N-terminal protected peptide was cleaved from the chlorotrityl resin using 20% trifluorethanol/dichloromethane and then the trityl group was removed from the side chain of serine using 1% trifluoroacetic acid/dichloromethane. The peptide was cyclized using benzotriazol-1-yloxy tris(dimethylamino)phosphonium hexafluorophosphate/diisopropylethylamine in dichloromethane, having first solubilized the peptide in a small quantity of dimethylformamide. Once cyclized the remaining protecting groups were removed using 95% trifluoroacetic acid scavengers. The biotin-labeled linear L2 was synthesized by GenScript.

Strains and Plasmids

E. coli Strains

BL21(DE3) and BL21-CodonPlus(DE3)-RIL (BL21-CP) were obtained from Novagen and Stratagene, respectively.

S. cerevisiae Strains

EY93 (MATa ura2 his3 trp1 leu2 ade2::URA3) was derived from EGY42 (Cohen et al., 1998). EY111 (MATα his3 trp1 ura3::LexA8op-lacZ ade2::URA3-LexA8op-ADE2 leu2::LexA6op-LEU2) was derived from EGY48 (Golemis and Brent, 1992).

Plasmids

pEG202

pEG202 expresses the LexA target (GenBank accession U89960) (Gyuris et al., 1993).

pIN01

pIN01 expresses an inactive Synechocystis spp. strain PCC6803 (Ssp) DnaE lariat intein construct with a CPGC peptide in the noose region.

Lariat Library (pIL-XX)

We constructed pIL-XX by replacing the CPGC linker peptide in pIN01 with a combinatorial seven amino acid peptide using oligonucleotide K. The library was cloned into RsrII-digested pIN01 using in vivo homologous recombination (Ma et al., 1987) in EY93. We performed 100 lithium acetate transformations (Schiestl and Gietz, 1989) with each transformation containing 400 ng of PCR-amplified oligonucleotide K and 1 μg of RsrII-digested pIN01. In total, 20 million yeast colonies were obtained.

pIL-L2

pIL-L2 expresses L2 lariat isolated from the pILXX library. The noose amino acid sequence of pIL-L2 is RSWDLPGEY.

pIL-L2 Scrambled

pIL-L2-scrambled expresses the L2 scrambled lariat, where the noose amino acid sequence is DPGLRSWEY. pIL-L2-scrambled was constructed using the same strategy as pIL-XX, except oligonucleotide S was used in place of oligonucleotide K.

pIL-L2 Inverted

pIL-L2-inverted expresses the L2 inverted lariat where the noose amino acid sequence is GPLDWSREY. pIL-L2-inverted was constructed using the same strategy as pIL-XX, except oligonucleotide T was used in place of oligonucleotide K.

pIN-L2

pIN-L2 expresses the L2 inactive intein. pIN-L2 was constructed by mutating cysteine at I$_{N+1}$ to alanine using PCR site-directed mutagenesis.

pLIN-L2

pLIN-L2 expresses the L2 linear peptide. pLIN-L2 was constructed by introducing two stop codons at position I$_{N+1}$ and I$_{N+2}$ in pIL-L2.

pACT-L2

pACT-L2 expresses the L2 active intein. pACT-L2 was constructed by mutating alanine at position I$_{C+1}$ in pIL-L2 to asparagine using PCR site-directed mutagenesis.

E. coli intein expression plasmids were constructed by PCR amplifying the entire intein gene including the stop codon from the corresponding yeast expression vector using primers P and Q. The PCR product was digested with EcoRI and XhoI (Fermentas) and cloned into pET28b (Novagen) using standard restriction enzyme-based cloning procedures (Raleigh et al., 2002). The following E. coli expression plasmids were constructed: pETIL-L2, expresses L2 lariat; pETIL-L2scrambled, expresses the L2 scrambled lariat; pETIN-L2, expresses the L2 inactive intein; pETLIN-L2, expresses the L2 linear peptide; pETACT-L2, expresses the L2 active intein.

Screening Combinatorial Lariat Intein Library

The lariat library was screened for interactions with LexA using Y2H interaction mating (Kolonin et al., 2000). We transformed the LexA bait plasmid (pEG202) (Gyuris et al., 1993) into EY111 and mated EY111::pEG202 to EY93::pIL-XX. EY111::pEG202 was cultured in 500 ml of synthetic dextrose. His⁻ media to an OD$_{600}$ of 0.6–0.9. EY111:pEG202 cells were pelleted by centrifugation and resuspended in an equal volume of yeast peptone dextrose (YPD) media. EY93::pIL-XX cells were mixed with EY111::pEG202 cells at a ratio of 1:20. Yeast cells were mated on YPD plates at 30°C for 24 hr. Mated yeast cells were pooled and 200 million diploid yeast cells were screened to detect lariats that interacted with LexA using the LEU2, ADE2, and LacZ reporter genes. Diploid yeast cells were cultured on synthetic galactose/raffinose (SGR) His⁻, Trp⁻, Leu⁻, and Ade⁻ plates containing X-Gal for approximately 7 days. Positive colonies were selected and positive interactions were reconfirmed by isolating pILXX from the positive colonies and repeating the Y2H assay as described above.

Characterization of Intein Processing and Lariat Product

We monitored expression of L2 lariat and intein constructs in EY93 using western analysis with an anti-HA antibody. Samples were normalized using their OD$_{600}$ and 20 μl of supernatant was analyzed by western analysis with an anti-HA tag antibody (Santa Cruz Biotechnology, Inc.) (1:200 dilution). The LI-COR Odyssey infrared imaging system (LI-COR Biosciences) was used to visualize and quantify blots.

We used LC-ESI-TOF MS to confirm the molecular weight of the His-tagged L2 lariat purified from E. coli. To confirm the presence of the lariat lactone prior to MS analysis, lyophilized His-tagged L2 lariat, which had been purified using Ni^{2+}-NTA affinity chromatography and reverse-phase HPLC, were treated with 0.5 M Na^{18}OH. The products were purified using reverse-phase HPLC and digested with trypsin, and the molecular weights of the trypsin fragments were analyzed using LC-ESI-TOF MS.

Surface Plasmon Resonance Assay

SPR was performed using a BiaCore3000 (BiaCore Life Sciences). LexA was purified using HIS-Select Nickel affinity gel (Sigma) and diluted in 50 mM Tris-HCl and 150 mM NaCl prior to use in SPR analysis. Purified His-tagged LexA was immobilized on a Series S Sensor Chip NTA (BiaCore Life Sciences). L2 lariat or L2 linear peptides were dissolved in 50 mM Tris-HCl and 150 mM NaCl. Sequential injections of 2-fold serial dilutions of either synthetic L2 lariat or L2 linear peptides ranging from 125 μM to 7.8 μM were injected over the surface at a flow rate of 10 μl/min. The K$_D$ for the L2 lariat-LexA interaction

was calculated by plotting maximum response units versus peptide concentration and fitting the data to a one site binding model using Prism4.0 (GraphPad) statistical software.

LexA ChIP Assay

The LexA ChIP assay was based on a previously described procedure (Wade and Struhl, 2004). BL21-CP cells transformed with *E. coli* lariat expression plasmids were grown to an OD_{600} of ~0.6 in LB with 50 μg/ml of kanamycin (LB-KAN) and then induced for 2 hr with 0.2 mM IPTG at 30°C. The culture was split into two samples; one sample was treated with MMC (0.25 μg/ml) and the other untreated. Both samples were incubated for 1 hr at 30°C. ChIP assays were performed as previously described (Wade and Struhl, 2004). Eluted *lexA* promoter DNA was quantified by real-time PCR. The occupancy of LexA at *lexA* promoter was measured as a ratio of binding of LexA to the *lexA* promoter and to a control region in the *sgrR (yabN)* gene (occupancy units).

Ni²⁺-NTA Chromatin Precipitation Assay

Ni^{2+}-NTA chromatin precipitation assay was based on a previously described procedure (Tamimi et al., 2004). BL21-CP cells transformed with *E. coli* lariat expression plasmids were grown to an OD_{600} of ~0.6 in LB-KAN. Cultures were either induced for 2 hr with 0.2 mM IPTG at 30°C or left uninduced. Chromatin was precipitated using the protocol for the LexA ChIP assay except Ni^{2+}-NTA beads were used to precipitate His-tagged L2 and L2 scrambled lariats. Eluted *lexA* promoter DNA was quantified by real-time PCR and normalized to the corresponding input DNA.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using iQ SYBR Green (Bio-Rad) in a Miniopticon (Bio-Rad). Primers W and X were used to amplify a region of the *lexa* gene. Primers Y and Z were used to amplify a region in the *sgrR (yabN)* gene. A two step PCR cycle was used with an initial denaturation of 95°C for 5 min followed by 95°C for 30 s and 61°C (*lexa*) or 59°C (*sgrR*) for 1 min. The product was amplified for 40 cycles.

Analysis of LexA Autoproteolysis

BL21 cells transformed with *E. coli* lariat expression plasmids were induced with 1 mM IPTG and the cells were cultured at 30°C to an OD_{600} ~0.4–0.6. Cells were then treated with 100 μg/ml chloramphenicol and the culture was split in two; one culture was treated with 0.1 μg/ml MMC for 1 hr and the second culture was left untreated. The cells were lysed and cleared supernatants were analyzed by western analysis using an anti-LexA antibody (Invitrogen) (1:5000 dilution). The LI-COR Odyssey infrared imaging system (LI-COR Biosciences) was used to visualize and quantify blots.

Bacterial Viability Assays

We performed cell viability assays as described by Lin and Little (1988). BL21-CP transformed with *E. coli* lariat expression plasmids were cultured at 37°C in LB-KAN to an OD_{600} of 0.4. The culture was split in two and one sample was induced with 1 mM IPTG for 1 hr and the other sample was left uninduced. The samples were diluted 100-fold in 5 ml 0.85% NaCl with or without 0.1 μg/ml MMC. A 10 μl aliquot was diluted 1000-fold in ice cold LB (1 ml) for a zero time point control. We incubated the remaining sample at 37°C for 30 min and removed a 10 μl aliquot and diluted it 1000-fold into 1 ml ice-cold LB. We plated a 60 μl aliquot from the time points on LB plates and incubated them at 37°C overnight. Normalized percent cell survival was calculated by dividing the number of colony forming units (cfu) after 30 min by the number of cfu at the zero hour time point.

Statistical Analysis

Data was reported as mean ± standard deviation. P-values were calculated using a two-tailed t test with Prism 4.0c for Macintosh (GraphPad).

SUPPLEMENTAL DATA

Supplemental data include Supplemental Experimental Procedures, eight figures, and one table and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00363-9.

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