C1pX Inhibits FtsZ Assembly in a Manner That Does Not Require Its ATP Hydrolysis-Dependent Chaperone Activity

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C1pX is a well-characterized bacterial chaperone that plays a role in many processes, including protein turnover and the remodeling of macromolecular complexes. All of these activities require ATP hydrolysis-dependent, C1pX-mediated protein unfolding. Here we used site-directed mutagenesis in combination with genetics and biochemistry to establish that C1pX inhibits assembly of the conserved division protein FtsZ through a noncanonical mechanism independent of its role as an ATP-dependent chaperone.

The highly conserved chaperone C1pX has been implicated in numerous cellular processes, including the turnover of regulatory proteins (6, 13, 19, 20, 32), the destruction of unfinished polypeptides (8, 30, 31), and the remodeling of macromolecular complexes (3–5). While C1pX-mediated proteolysis is dependent on the ClpP protease, C1pX alone is sufficient for remodeling of macromolecular complexes, such as the phage Mu transpososome (3–5). All of these functions share a requirement for ATP hydrolysis-dependent, C1pX-mediated protein unfolding.

In Bacillus subtilis, C1pX modulates assembly of the conserved cytoskeletal protein FtsZ to help control the process of cell division (29). FtsZ, a homolog of the eukaryotic cytoskeletal protein tubulin, assembles into a ring structure at the nascent division site in response to an unidentified cell cycle signal. This ring serves as the foundation for assembly of the cell division apparatus (25). In previous work, we determined that C1pX inhibits FtsZ assembly and maintains the pool of subunits available for ring formation (29). Surprisingly, our biochemical data suggested that C1pX inhibits FtsZ assembly through a ClpP-independent mechanism that does not appear to require ATP hydrolysis (29).

Here we employed site-directed mutagenesis in combination with genetic and biochemical analyses to establish that C1pX has two functions in vivo: an ATP hydrolysis-dependent chaperone activity, required for the unfolding of proteolytic substrates, and an ATP hydrolysis-independent activity, required for interaction with FtsZ.

Generating mutations in C1pX residues required for chaperone activity. To generate mutations that render B. subtilis C1pX defective for ATP-dependent protein unfolding, we took advantage of an alignment with the well-characterized Escherichia coli C1pX protein (Fig. 1A) to engineer single alanine substitutions in B. subtilis C1pX residues that are predicted to be required for C1pX-mediated proteolysis. These substitutions included mutations in the putative Walker A [C1pX(K122A)] and Walker B [C1pX(E182A)] boxes, which in E. coli are required for ATP binding and hydrolysis, respectively, as well as a mutation in the substrate-processing pore loop [C1pX(Y150A)] (12). ATP binding is necessary for formation of the active C1pX hexamer (2, 9, 27), whereas ATP hydrolysis provides the energy required for protein unfolding and translocation (12). Mutations in the E. coli C1pX Walker A and Walker B motifs disrupt C1pX-mediated proteolysis and macromolecular complex remodeling (reviewed in reference 12). Mutations in the pore loop of E. coli C1pX impair substrate binding and/or processing without affecting ATP binding and hydrolysis (26).

To generate wild-type and mutant c1pX strains, we first cloned a promoterless version of c1pX into a suicide vector (pIL74 [15]), upstream of a spectinomycin (Spc) resistance cassette (spc). This fragment contained the c1pX Shine-Dalgarno sequence and stop codon but only part of the c1pX terminator. B. subtilis DNA encoding lonB, the gene immediately downstream of c1pX, was cloned downstream of spc such that both fragments were in the same orientation as spc. Transformation of either the wild-type construct (pAL16) or one of the three mutant versions (pAL17, pAL18, and pAL19) into a B. subtilis strain encoding a Tn10-cat insertion in c1pX (either AHL125 or AHL127) resulted in marker replacement of the c1pX region by double-crossover recombination. Subsequent screening for Spc-resistant, chloramphenicol-sensitive cells allowed us to identify those strains encoding a copy of c1pX at its native locus under the control of the native c1pX promoter.

C1pX levels in the strains described above were approximately 50% of those in wild-type cells, most likely due to truncation of the c1pX terminator. To increase C1pX expression to wild-type levels, we also generated a second, xylose-inducible construct by cloning the appropriate c1pX allele into the integration vector pRDC19 (the gift of Fabrizio Arigoni). The resulting wild-type (pAL11) or mutant (pAL12, pAL13, and pAL14) plasmids were then transformed into wild-type B. subtilis cells, selecting for macrolincosomide resistance and

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screening for Spc. DNA from these cells was then used to transform congenic strains expressing wild-type or mutant clpX (AHL143, AHL147, and AHL151) from the native promoter, generating strains with two copies of clpX (wild type or mutant). Experiments described in this article were performed with strains encoding both constructs. Table 1 lists the strains used in this study.

When it was expressed only from the native promoter, levels of ClpX in the mutant and congenic wild-type strains were 2-fold lower than those in wild-type cells (Fig. 1B). However, when it was expressed simultaneously from both the native promoter and the xylose-inducible Pxy promoter, levels of ClpX in both the mutant and the congenic wild-type backgrounds were identical to those in the parental wild-type strain (JH642) (Fig. 1B). Quantitative immunoblotting indicated that FtsZ levels were constant in all strains regardless of ClpX expression levels, consistent with previous results (29).

Mutations in clpX prevent proteolysis of substrates by ClpXP in vivo. Assays for ClpXP-mediated proteolysis confirmed that all three ClpX point mutants were defective in ATP-dependent protein unfolding, as expected. As a preliminary test of ClpX function, we first examined motility in cells encoding either one of the three clpX mutants or the congenic wild-type clpX allele. Motility requires ClpXP-mediated proteolysis of the global transcriptional regulator Spx (21) and is thus an excellent indicator of ClpX chaperone activity.

Consistent with a defect in ClpXP-mediated proteolysis, all three mutant strains were severely defective for motility on swarm plates regardless of the addition of xylose. In contrast, the congenic wild-type strain exhibited robust motility in both the presence and absence of xylose (Fig. 1C). We next used quantitative immunoblotting experiments as described previously (33) to confirm that Spx was accumulating in the clpX mutant backgrounds (Fig. 1D) in a manner consistent with defects in ClpXP-mediated proteolysis. As expected based on previous reports (23), Spx accumulated to considerably higher levels in the clpX null cells than was the case with wild-type B. subtilis. Similarly, we observed an accumulation of Spx in all three clpX point mutant strains but not in the congenic wild-type strain (Fig. 1D), indicating that the clpX point mutations prevent proteolysis of Spx by ClpXP. These results show that the three B. subtilis mutants abrogate ClpX activity similarly to their E. coli mutant counterparts, consistent with a loss of ClpX ATP binding or hydrolysis or with defects in substrate processing, respectively.

The accumulation of Spx in the clpX mutants results in

![FIG. 1. Mutations in B. subtilis ClpX residues predicted to be required for interaction with canonical substrates disrupt ClpXP degradation of Spx. (A) Alignment of B. subtilis and E. coli ClpX polypeptides. Amino acid identity is indicated by black boxes and amino acid similarity by gray boxes. Bars indicate Walker A, pore loop, and Walker B boxes, respectively. Numbers refer to the B. subtilis ClpX sequence. Arrows indicate residues that were changed to alanine for this study. (B) Quantitative immunoblot of lysates from mid-exponential-phase cultures grown in the absence (H11002) or presence (H11001) of xylose. In strains encoding clpX under the control of both its native promoter and the exogenous Pxy promoter, ClpX levels were ~2-fold lower than those in the parent strain in both the mutant and congenic wild-type backgrounds in the absence of xylose. Xylose-induced expression from the Pxy promoter raised the intracellular concentration of ClpX to wild-type levels in these strains. Lysates were blotted with anti-ClpX sera (top) or anti-FtsZ sera (bottom). ClpX appears as a doublet on immunoblots. Mutations in clpX do not affect FtsZ levels. (C) Colonies stabbed onto tryptone broth swarm plates in the presence and absence of xylose. Note that only the congenic wild-type strain is motile, indicating appropriate turnover of Spx. Minor differences in swarm size in the congenic wild-type strain are most likely the result of experimental variation and are not a reflection of changes in ClpX levels. (D) Immunoblot of lysates from mid-exponential cultures probed with antisera against the ClpX target Spx or with anti-FtsZ sera. Note the increased levels of Spx in the four clpX mutant strains, confirming a defect in the ClpX-Spx interaction. FtsZ serves as a loading control.

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genetic instability and the rapid accumulation of suppressor mutations in spx itself or in the alpha subunit of RNA polymerase (22, 33). To circumvent this issue, the above assays were performed on spx alleles encoding wild-type and mutant versions of the thrC:Pxyl-clpX construct that were freshly transformed with DNA encoding the corresponding clpX allele [clpX (wild type), clpX(K122A), clpX(E182A), or clpX(Y150A)] at the clpX locus.

Residues implicated in ATP-dependent protein unfolding are dispensable for ClpX-mediated inhibition of FtsZ assembly. We next examined whether ClpX mutants with defects in residues implicated in ATP binding, ATP hydrolysis, or canonical substrate binding/processing inhibited FtsZ assembly in vivo. For these experiments, we assayed the relative stability of FtsZ assembly in vivo by determining the sensitivity of individual clpX alleles to overexpression of the MinCD division inhibitor. During exponential growth, the MinCD complex functions to prevent inappropriate FtsZ assembly and division at cell poles (17, 18). However, increasing MinCD levels more than 12-fold in B. subtilis leads to a global inhibition of FtsZ assembly, blocking medial FtsZ ring formation and causing lethal filamentation (16). Loss-of-function mutations in division inhibitors, including clpX, stabilize FtsZ assembly at mid-cell and permit FtsZ ring formation and division even in the presence of excess MinCD (16, 28, 29). The ability to suppress MinCD-induced lethality is thus a good measure of the effect of the clpX point mutations on FtsZ assembly dynamics in vivo.

We evaluated relative MinCD suppression by testing the plating efficiencies of strains encoding the three clpX mutations versus that of the congenic wild-type clpX strain when combined with an isoprropyl-β-D-thiogalactopyranoside (IPTG)-inducible allele of minCD (amyE:hypermPspc-clpX). The plating efficiency was determined as described previously (16, 29). Cells were plated on LB media with 1 mM IPTG in the presence or absence of 0.5% xylose and scored for CFU after 16 to 18 h at 37°C. If any of the clpX mutations disrupt the interaction between ClpX and FtsZ, then replacing the wild-type clpX allele with that clpX mutant allele should restore viability to cells overproducing MinCD. Conversely, if residues predicted to affect ATP binding, ATP hydrolysis, and/or interaction with the substrate-processing pore loop region are dispensable for the ability of ClpX to inhibit FtsZ assembly, then the respective clpX mutants should remain sensitive to MinCD overproduction.

As mentioned in the previous section, B. subtilis clpX mutants are genetically unstable due to aberrant accumulation of the Spx transcription factor (22, 33). We therefore performed these assays in the presence of an spx null allele (spx::neo). The loss of spx has no impact on FtsZ assembly or cell division (29).

MinCD suppression data indicate that residues that are required for ClpXP-mediated proteolysis are largely dispensable for ClpX’s ability to inhibit FtsZ assembly (Fig. 2A). Both the clpX(E182A) and clpX(Y150A) mutants behaved similarly to wild-type B. subtilis cells with regard to MinCD sensitivity. Cells encoding either clpX(E182A) or clpX(Y150A) and the inducible minCD overexpression construct exhibited a reduction in CFU similar to that in congenic wild-type clpX cells when plated on medium containing 1 mM IPTG. This is in contrast to results with a loss-of-function mutation in clpX, which almost completely suppresses the growth defect associated with overexpression of MinCD. Furthermore, the presence or absence of xylose had little effect on the sensitivity of the clpX(E182A) and clpX(Y150A) mutants to MinCD overproduction, indicating that the ClpX(E182A) and ClpX (Y150A) proteins effectively inhibit FtsZ assembly even when expressed at levels twofold below that of the wild type. These results are consistent with a model in which ClpX inhibits FtsZ assembly in a manner that is independent of its ability to unfold proteins.

In contrast to results for the two other mutants, clpX

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**TABLE 1. B. subtilis strains**

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<tr>
<th>Strain name</th>
<th>Genotype</th>
<th>References</th>
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<td>trpC2 pheA1</td>
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<tr>
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<tr>
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<sup>a</sup> All other strains are derivatives of JH642.  
<sup>b</sup> wt, wild type.
(K122A) cells exhibited an intermediate phenotype, suggesting that ATP binding is important for the interaction between ClpX and FtsZ, consistent with our previous biochemical data (29). When ClpX was expressed at wild-type levels, clpX(K122A) cells behaved similarly to the clpX(E182A) and clpX(Y150A) mutants with regard to MinCD sensitivity (Fig. 2A). However, and in contrast to results for the two other point mutants, the twofold reduction of clpX(K122A) expression by growth in the absence of xylose rendered these cells close to clpX null mutants with regard to suppression of MinCD-induced lethality (Fig. 2A). Quantitative immunoblotting indicated that MinD levels were unaffected by defects in clpX activity (see Fig. S1 in the supplemental material).

To further confirm that the loss of ATP hydrolysis was dispensable for ClpX-mediated inhibition of FtsZ assembly, we examined the ability of ClpX(E182A), which is predicted to be defective in ATP hydrolysis, to inhibit FtsZ assembly in vitro using FtsZ and ClpX, purified as described previously (11). For these experiments, we used 90°-angle light scattering to measure relative FtsZ assembly in real time (Fig. 2B and C), essentially as described previously (10), using a DM-45 spectrophotometer (Olis).

As expected based on previous results (29), wild-type ClpX was a robust inhibitor of FtsZ assembly. At a one-to-one wild-type ClpX:FtsZ ratio, we observed an \( \frac{70}{100} \) reduction in FtsZ assembly by 90°-angle light scattering (Fig. 2B and C). Importantly, ClpX(E182A) also perturbed FtsZ assembly, albeit to a somewhat lesser degree. We observed an \( \frac{45}{100} \) reduction in FtsZ assembly in the presence of the equimolar ClpX(E182A) protein (Fig. 2B and C).

Note that all these experiments were done in the absence of nucleotide. Our previous light scattering data indicated that ClpX inhibits FtsZ assembly equally well in the presence of ATP or ADP (29) but that the addition of adenine nucleotide enhances the ability of purified ClpX to inhibit FtsZ assembly to a small but significant degree. However, in this work we found that the addition of adenine nucleotide had a negligible effect on the ability of wild-type ClpX to inhibit FtsZ assembly (data not shown). It has been reported that Helicobacter pylori ClpX purifies with sufficient nucleotide to promote hexamerization (14); hence this discrepancy may be the result of variations in the nucleotide content of our ClpX preparations.

Although ClpX(E182A) exhibited wild-type levels of FtsZ inhibition in vivo (Fig. 2A), it was somewhat reduced in its ability to inhibit FtsZ assembly in vitro (Fig. 2B). This reduced potency may be due to partial misfolding of the purified mutant protein. Alternatively, ATP hydrolysis may be required for maximum inhibition in vitro although dispensable in vivo.

FIG. 2. Mutations in residues required for ClpXP-mediated proteolysis have a negligible effect on the ability of ClpX to inhibit FtsZ assembly. (A) Suppression of the lethality associated with >12-fold overexpression of the MinCD division inhibitor. Both the Walker B (E182A) and pore loop (Y150A) mutants are sensitive to MinCD overexpression in the presence or absence of xylose, indicating that they are wild type with regard to inhibiting FtsZ assembly. In contrast, the predicted ClpX Walker A mutant (K122A) is sensitive to high levels of MinCD only when expressed at wild-type levels in the presence of xylose. A twofold reduction in the ClpX(K122A) concentration by growth in the absence of xylose results in an increase in resistance to MinCD overexpression. Error bars indicate standard deviations from at least three separate experiments. (B) Representative traces of 90°-angle light-scattering reactions containing 3 \( \mu \)M FtsZ assembled alone or in the presence of wild-type ClpX (top) or a mutant that is defective in ATP hydrolysis, ClpX(E182A). (C) Histogram of FtsZ assembly reactions conducted in the presence of the wild-type or mutant ClpX protein. Each bar represents at least three replicate experiments. Error bars indicate standard deviations from three separate experiments.
An ATP hydrolysis-independent function for ClpX. Our results indicate that ATP hydrolysis-dependent protein unfolding is not required for ClpX-mediated inhibition of FtsZ assembly and argue that ClpX interacts with FtsZ in a manner distinct from its interaction with canonical substrates. Notably, cells encoding a clpX allele that is predicted to be defective in ATP hydrolysis [ClpX(E182A)] exhibited wild-type FtsZ assembly (Fig. 2A and B). Moreover, purified ClpX(E182A) inhibited FtsZ assembly in vitro despite a loss of canonical ClpX activity (Fig. 2B and C). These results are consistent with our previous data indicating that both a nonhydrolyzable nucleotide analogue, γ-S-ATP, and ADP support ClpX-mediated inhibition of FtsZ assembly in vitro (29). Moreover, a mutation in the predicted ClpX pore loop domain [ClpX(Y150A)] did not significantly alter the ability of ClpX to inhibit FtsZ assembly in vivo (Fig. 2A), indicating that ClpX likely interacts with FtsZ in a manner distinct from its interaction with proteolytic targets. This conclusion is consistent with our previous work demonstrating that ClpXP is unable to degrade FtsZ in an in vitro assay (29).

Intriguingly, nucleotide binding appears to promote ClpX interaction with FtsZ in vivo. A clpX mutant that is predicted to be defective in ATP binding, clpX(K122A), exhibits an intermediate phenotype with regard to FtsZ assembly. When ClpX was expressed at levels ~2-fold less than those of the wild type, clpX(K122A) mutants behaved like clpX null mutants, exhibiting increased FtsZ polymer stability and resistance to the lethality associated with overexpression of the MinCD division inhibitor (Fig. 2). In contrast, when ClpX was expressed at normal levels, clpX(K122A) cells displayed near-wild-type sensitivity to MinCD overexpression (Fig. 2A), consistent with normal FtsZ assembly dynamics. Because nucleotide binding but not hydrolysis is required for ClpX hexamerization (2, 9, 27), these results suggest that FtsZ may interact efficiently only with hexameric ClpX.

Taken together, our data suggest that ClpX does not need to unfold FtsZ to inhibit assembly. This is in contrast to the role of ClpX in the remodeling of other macromolecular complexes, in which protein unfolding is a critical step. This difference may be due to the highly dynamic nature of the FtsZ polymer, in which monomers have a high on/off rate (1). ClpX masking a surface required for interaction between FtsZ monomers would thus be sufficient to prevent polymerization of new polymers and enhance disassembly of preformed polymers. In contrast to the case with FtsZ polymers, disassembly of intrinsically stable complexes, such as the Mu transpososome, necessitates the input of energy in the form of ATP hydrolysis-dependent protein unfolding (4).

In summary, our data argue that ClpX modulates FtsZ assembly in Bacillus subtilis through an ATP hydrolysis-independent mechanism that is distinct from all other previously described ClpX activities. Given the high degree of conservation of FtsZ and ClpX across bacterial species, it will be interesting to determine if ClpX modulates FtsZ assembly in a similar manner in other organisms. Of particular interest is E. coli, where an interaction between ClpX and FtsZ has already been established in a proteomic screen (7). Our results with Bacillus subtilis begin to address the significance of this interaction.

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