Lambda Xis Degradation In Vivo by Lon and FtsH

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Lambda Xis, which is required for site-specific excision of phage lambda from the bacterial chromosome, has a much shorter functional half-life than Int, which is required for both integration and excision (R. A. Weisberg and M. E. Gottesman, p. 489–500, in A. D. Hershey, ed., The Bacteriophage Lambda, 1971). We found that Xis is degraded in vivo by two ATP-dependent proteases, Lon and FtsH (HflB). Xis was stabilized two- to threefold more than in the wild type in a lon mutant and as much as sixfold more in a lon ftsH double mutant at the nonpermissive temperature for the ftsH mutation. Integration of lambda into the bacterial chromosome was delayed in the lon ftsH background, suggesting that accumulation of Xis in vivo interferes with integration. Overexpression of Xis in wild-type cells from a multicopy plasmid inhibited integration of lambda and promoted curing of established lysogens, confirming that accumulation of Xis interferes with the ability of Int to establish and maintain an integrated prophage.

Bacteriophage lambda employs integrated regulatory mechanisms to ensure the appropriate equilibrium between lysogeny and lytic growth. In addition to well-characterized controls for transcription initiation and termination, lambda also utilizes rapid and specific degradation of key regulatory proteins to influence the direction of its development. RecA-dependent degradation of the repressor cI as part of the SOS response returns the dormant prophage to the lytic cycle (22–24). Once the lytic decision is made and the level of cII expression has decreased, rapid degradation of cII by FtsH (HflB) ensures the lytic decision is made and the level of cII expression has returned to establish and maintain an integrated prophage.

Our objectives in this study were to determine if the Xis protein is degraded in vivo and, if so, if any of the known ATP-dependent proteases are responsible for its degradation. We also wanted to test whether the instability of Xis has significant effects on the ability of lambda to establish and maintain lysogeny.

Xis is degraded in vivo by Lon and FtsH. Since the work by Weisberg and Gottesman, it has been found that most cytoplasmic protein degradation is energy dependent and that Escherichia coli has at least five different energy-dependent proteases with different substrate specificities (for a review, see reference 9). In order to determine if Xis is specifically degraded by any of the known ATP-dependent proteases, we examined an isogenic set of protease mutant strains, each possessing a chromosomal copy of lacP. We expressed Xis in the different genetic backgrounds from pRK5 (1), a multicopy plasmid with Xis under plac control, and inhibited protein synthesis by addition of the translational inhibitor spectinomycin, and the remaining Xis was measured as a function of time by immunoblotting (Fig. 1). Xis is in fact physically unstable in vivo, with a t1/2 of approximately 4 min at 32°C in wild-type cells (Fig. 1A). The only protease mutant that exhibited significant stabilization of Xis relative to that seen for the wild-type strain was the lon mutant. The t1/2 of Xis was extended to 10 to 12 min at 32°C in the lon background (Fig. 1A). None of the clp protease mutants stabilized Xis, either alone or in combination with a lon mutant (data not shown).

Because the FtsH (HflB) protease is essential to E. coli (2, 14), it was necessary to utilize a conditional lethal mutant (ftsH1 [25]) to assess the effects of FtsH activity on Xis stability. The temperature-sensitive ftsH mutation did not by itself appreciably stabilize Xis at 32 or 42°C but, in combination with the lon mutation, extended the t1/2 for Xis from approximately 4 min to about 25 min at the nonpermissive temperature (Fig. 1B). These results indicate that Xis is recognized and actively degraded in vivo by both the Lon and the FtsH proteases. The observations that Xis stabilization in the lon mutant background was not dramatic (two- to threefold) and that the ftsH genetic background by itself did not significantly stabilize Xis indicate that each protease degrades Xis rapidly, with Lon

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perhaps capable of degrading it more rapidly and thus playing the primary role in Xis degradation.

Integration of lambda is delayed in the lon ftsH double mutant. Based on the observed inhibition of lambda integration by Xis in vitro (6, 11, 18), the prediction is that accumulation of Xis in the cell due to its stabilization would lead to abortive lysogeny by preventing Int from mediating integration of repressed phage and/or by working with Int to excise phage that did integrate. Analysis of the effects of Xis stabilization on the establishment and maintenance of lysogeny is complicated by the fact that both the lon and the ftsH mutations have other significant effects on the biology of phage lambda.

To have an assay that was independent of the pleiotropic effects of the ftsH and lon mutations on the life cycle of lambda, we utilized a PCR-based approach to directly monitor the kinetics of integration following infection with λcI857. Primers from within the int region of the phage and from the region between gal and attB of the bacterial chromosome were used to amplify the unique attL sequence of the integrated prophage (schematically shown in Fig. 2A) at different times following infection (20). The amount of attL detectable in each infection mixture at each time point following infection was approximately the same for the wild-type, ftsH, and lon ftsH strains (Fig. 2B). The accumulation of attL sequence in the ftsH lon infection mixture was delayed in comparison to that in the other strains by more than 10 min. Under these infection conditions, the final frequency of lysogeny among survivors (plated on Luria-Bertani [LB] agar at 32°C) approached 100% for the wild-type, ftsH, and lon ftsH strains, with the frequency in the lon strain being about 20% lower (data not shown), in agreement with the expected effects of these mutations on cII and therefore on lysogeny. These results are consistent with moderate stabili-
tion of Xis in the double mutant, such that integration was delayed until the transiently expressed Xis (from pL) was degraded, and the more stable Int (expressed primarily from pI) could integrate the phage without interference. Although we did not see a consistent and large effect of the ftsH mutation in combination with lon on Xis degradation at permissive temperatures (data not shown), the delay in integration in the lon ftsH mutant suggests that temperature-sensitive FtsH is not fully functional at low temperatures.

Excess Xis inhibits integration of phage lambda into the bacterial chromosome. If moderate stabilization of Xis delays integration, complete stabilization and, therefore, higher levels of Xis would be expected to inhibit integration more fully. We assumed that high-level expression from pRK5 (plac-xis\textsuperscript{1}) would mimic to some degree the effects of stabilization by increasing the accumulation of Xis in the cell. We utilized the PCR approach described above to assess the degree of integration of \(\lambda\)I857 following infection of wild-type cells containing pBR322 or pRK5. Cultures of SG22163 (lac\textsuperscript{P}), SG22166 (lac\textsuperscript{P} ftsH\textsuperscript{1}), SG22185 (lac\textsuperscript{P} \Delta{lon}510), and GL008 (lac\textsuperscript{P} \Delta{lon}510 ftsH\textsuperscript{1}) were grown to early log phase, their cell concentrations were normalized by measuring the optical density at 600 nm, and the cultures were then chilled on ice. Cells were mixed with \(\lambda\)I857 (multiplicity of infection, ~17) and incubated on ice for 20 min in order to synchronize infection. Infection mixtures were warmed to 32°C, aliquots (~3 \times 10\textsuperscript{7} cells) were taken at the time points indicated, and the cells were pelleted at 4,000 \times 10\textsuperscript{3} g for 10 min at 4°C. Pelleted cells were washed twice with cold sterile water and resuspended in a final volume of 30 \(\mu\)l. A portion of each suspension was plated on LB-citrate agar at 32°C to determine titers for survivors, and 20 \(\mu\)l was used for PCR amplification as described elsewhere (20). Reconstruction experiments with a mixture of ~2 \times 10\textsuperscript{7} lysogens and ~2 \times 10\textsuperscript{7} wild-type cells demonstrated a strong attL amplification, suggesting that the absence of a signal in these samples represents an integration frequency of <1 \times 10\textsuperscript{−5} (data not shown). In multiple experiments, cell killing under these infection conditions ranged from 50 to 90%. Lysogeny frequencies among survivors were determined by testing for \(\lambda\) immunity in cross-streaks and by plating at 39°C for SG22163 and SG22185. Lysogeny frequencies under these conditions were determined to be nearly 100% for wild-type, ftsH, and lon ftsH infection mixtures, with the lon survivors having ~20% fewer lysogens. (C) Overexpression of Xis inhibits integration. SG22163/pBR322 and SG22163/pRK5 were grown to early log phase in LB medium with 50 \(\mu\)g of ampicillin per ml at 32°C and then treated either with no IPTG or with 1 mM IPTG for an additional 30 min at 32°C. The cells were then chilled on ice, infected as described above with lambda \(\lambda\)I857 (multiplicity of infection, ~14) and harvested as described above for use in the attL amplification reaction. The DNA standard for the gels in panels B and C was the 100-bp ladder (Gibco-BRL).
a role for Xis instability in ensuring rapid integration of repressed prophage.

Excess Xis promotes spontaneous curing of lambda lysogens. Would accumulation of Xis in vivo promote excision of the repressed prophage? The effects of excess Xis on the maintenance of established lysogens was assessed by measuring the curing frequency of lambda lysogen with and without overexpression of Xis from pRK5. Cultures of wild-type and lon lysogens (lambda Cmr) containing either pBR322 or pRK5 were grown to early log phase at 30°C in LB with 50 μg of ampicillin per ml (to simplify the screen for lysogens, a derivative of lambda containing a mini-temperature sensitive [Cmr] was isolated). The cultures were then divided into three subcultures and treated with IPTG as indicated for 1 additional h at 30°C. Titers for each subculture were then determined on LB plates at 32°C to determine the total cell concentration and at 39°C on LB-citrate plates to determine the numbers of cells cured of the temperature-inducible prophage (all survivors tested were Cmr).

Curing frequencies are expressed as CFU per ml at 39°C/CFU per ml at 32°C. Data presented are for a single experiment but are indicative of curing patterns seen in multiple experiments.

<table>
<thead>
<tr>
<th>Conc of IPTG (mM)</th>
<th>Curing frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Wild type</th>
<th>lon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pBR322</td>
<td>pRK5</td>
</tr>
<tr>
<td>None</td>
<td>1.1 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.8 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>2.1 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
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<tr>
<td>0.1</td>
<td>1.7 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>3.8 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>3.5 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>7.9 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>2.5 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>1.4 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
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</table>

<sup>a</sup> Cultures of GL076 (lac<sup>+</sup> lambda Cmr<sup>)</sup> and GL078 (lac<sup>+</sup> lon<sup>-</sup> lambda Cmr<sup>)</sup> containing either pBR322 or pRK5 were grown to early log phase at 30°C in LB with 50 μg of ampicillin per ml (to simplify the screen for lysogens, a derivative of lambda carrying a mini-temperature sensitive [Cmr] was isolated). The cultures were then divided into three subcultures and treated with IPTG as indicated for 1 additional h at 30°C. Titers for each subculture were then determined on LB plates at 32°C to determine the total cell concentration and at 39°C on LB-citrate plates to determine the numbers of cells cured of the temperature-inducible prophage (all survivors tested were Cmr).

Curing frequencies are expressed as CFU per ml at 39°C/CFU per ml at 32°C. Data presented are for a single experiment but are indicative of curing patterns seen in multiple experiments.

### Conclusions

We have determined that lambda Xis is rapidly degraded in vivo by two different ATP-dependent proteases, Lon and FtsH. The observations that large quantities of Xis in vivo, such as might be seen if Xis were stable, inhibit integration and promote excision are consistent with the proposal first made by Weisberg and Gottesman (30) that rapid degradation of Xis ensures rapid integration of lambda into the bacterial chromosome. Given the multiple strategies used by the phage to maintain the appropriate ratio of Int to Xis at different points in its life cycle, it is likely that the difference in stability between the two proteins does in fact represent another important layer of regulation.

This is the first case in which we have seen overlap in substrate specificity for Lon and FtsH in E. coli, although an overlap of chaperone activity has been reported for the mitochondrial analogs of these proteins in Saccharomyces cerevisiae (21). These two proteases, while both ATP dependent, differ in many respects (for reviews, see references 9 and 10). They have different proteolytic mechanisms (a serine-active site for Lon and a Zn metalloprotease site for FtsH) and do not share any noticeable sequence similarity beyond the appearance of a Walker ATPase consensus sequence in each. FtsH has a transmembrane domain, while Lon is a fully cytoplasmic protein. The existence of a shared substrate suggests that the membrane association of FtsH does not preclude full access to cytoplasmic substrate proteins. As noted above, FtsH is responsible for degradation of λ cII; lon mutations actually lead to increased cII degradation (3, 13). The involvement of FtsH in degradation of other known Lon substrates (N protein and the bacterial proteins SulA and RcsA, for instance) has not been tested, but it is known that a lon mutant alone significantly stabilizes these proteins (13, 17, 27, 28). Thus, if FtsH contributes to their degradation, it plays a much more minor role than it does for Xis. It will be interesting to determine if FtsH and Lon recognize similar sites or structures on this small (72-amino-acid) protein.

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