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The T4 RI Antiholin Has an N-Terminal Signal Anchor Release Domain That Targets It for Degradation by DegP▽

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Lysis inhibition (LIN) of T4-infected cells was one of the foundational experimental systems for modern molecular genetics. In LIN, secondary infection of T4-infected cells results in a dramatically protracted infection cycle in which intracellular phage and endolysin accumulation can continue for hours. At the molecular level, this is due to the inhibition of the holin, T, by the antiholin, RI. RI is only 97 residues and contains an N-terminal hydrophobic domain and a C-terminal hydrophilic domain; expression of the latter domain fused to a secretory signal sequence is sufficient to impose LIN, due to its specific interaction with the periplasmic domain of the T holin. Here we show that the N-terminal sequence comprises a signal anchor release (SAR) domain, which causes the secretion of RI in a membrane-tethered form and then its subsequent release into the periplasm, without proteolytic processing. Moreover, the SAR domain confers both functional lability and DegP-mediated proteolytic instability on the released form of RI, although LIN is not affected in a *degP* host. These results are discussed in terms of a model for the activation of RI in the establishment of the LIN state.

For double-stranded DNA bacteriophages, the length of the infection cycle is controlled by a holin, which triggers to disrupt the membrane at an allele-specific time (28, 31). This allows a phage-encoded endolysin to attack the cell wall, leading to the rupture of the cell and the release of the progeny virions. Many phages also encode an antiholin which contributes to the timing of host lysis by inhibiting the holin. In phage T4, the holin, endolysin, and antiholin are the products of genes *t*, *e*, and *rI*, respectively (22, 23, 26). Normally, *Escherichia coli* cells lyse ~25 min after infection by T4. If the infected cell undergoes a secondary infection beginning at 3 to 5 min after the primary infection, lysis is inhibited and the infection cycle is extended. At a low multiplicity of secondary infection, the lysis-inhibited (LIN) state is unstable and repeated superinfections are required for its maintenance (1). In practice, LIN can lead to a greatly prolonged phase of intracellular virus accumulation, with each cell containing thousands of progeny virions, compared to the normal burst size of $\sim 10^2$. The LIN state can be terminated by depolarization of the membrane, after which lysis is essentially instantaneous. The LIN phenomenon, the T4 genes associated with it, and the interaction of these genes with host genes were the basis of the classic studies by which the fundamentals of genetic structure were defined (4). Nevertheless, nearly sixty years after Hershey's (10) first reports of T4 "*r*" mutants ("*r*" for *r*apid lysis, or LIN defective), the molecular basis of LIN is still obscure.

The picture has begun to clarify in the past few years, when it has been established that although a number of other T4 genes can affect LIN, including *rIIAB*, *rIII*, *rIV*, *stI*, and *stIII* (1), *rI* and *t* are the only phage genes required for LIN in all *E. coli* hosts (19, 22). T is unique among holins in that it has a large periplasmic domain and only a single transmembrane domain (TMD), whereas other holins have two or more TMDs and no significant periplasmic component (28). RI is predicted to be a secreted protein with a periplasmic domain of ~75 residues (Fig. 1A) (19). Recently, we have shown that LIN requires interaction of the periplasmic domains of these two proteins (27). A chimera, ^{ss}PhoAΦRI$_{CTD}$, (Fig. 1B) in which the N-terminal domain (NTD) of RI was replaced by the cleavable signal sequence of periplasmic alkaline phosphatase, PhoA, was found to be more effective at LIN than the wild-type RI. By contrast, the complementary chimera RI$_{NTD}$ΦPhoA, constructed of the NTD of RI and the periplasmic domain of PhoA, had no effect on T-mediated lysis.

Strikingly, the unprocessed RI$_{NTD}$ΦPhoA protein was found to be present in both the membrane and periplasmic compartments, leading us to suspect that the RI$_{NTD}$ might be a signal anchor release (SAR) domain (30, 31). The first SAR domain to be characterized was the predicted N-terminal TMD of the bacteriophage P1 endolysin, Lyz. We have provided evidence that the N-terminal TMD of Lyz is initially retained in the bilayer and that this membrane-inserted form is enzymatically inactive (29, 30). At a low rate, the Lyz TMD exits the bilayer, spontaneously resulting in the activation of the enzyme. Although the mechanism by which the SAR domain of Lyz moves from the membrane to the periplasm is unknown, this process is accelerated by depolarization of the membrane, either artificially by the addition of exogenous energy poisons or physiologically by the action of the P1 holin. Similar SAR domains are found in many bacteriophage endolysins and are characterized by a high content of neutral residues with low hydrophobicity. More-recent studies have shown that SAR domains are not restricted to phage-encoded endolysins. The N-terminal TMD of the holin S^{21} is also a SAR domain and must exit the bilayer in order for the holin to trigger and form its lethal membrane lesion (20). Here we report the results of experiments to determine whether the NTD of RI is also a SAR

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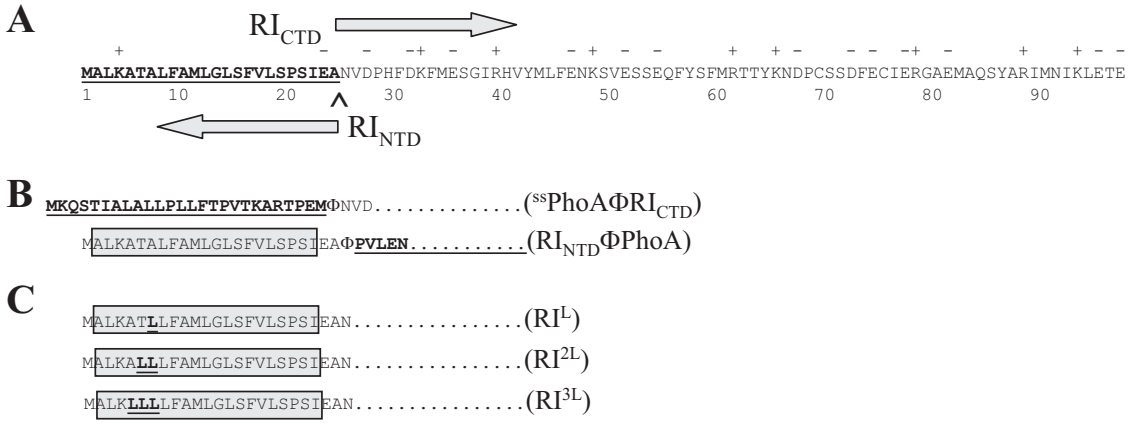

FIG. 1. Amino acid sequence of RI and its derivatives. (A) A secretory signal sequence (bold, underlined) and a leader peptidase I cleavage site (carat) between residues 24 and 25 predicted at 99.8% probability by the SignalP program (http://www.cbs.dtu.dk/services/SignalP/) (3). The proposed SAR domain of RI is boxed in panels B and C. (B) The RI/PhoA chimeras. PhoA sequences are in underlined bold text. In the ^{ss}PhoAϕRI$_{CTD}$ construct, the signal sequence of PhoA was substituted for residues 1 to 24 of RI. In the RI$_{NTD}$ϕPhoA construct, the predicted signal sequence of RI replaced residues 1 to 26 of PhoA. (C) The positions of the leucine substitutions in the NTD of the RI^{L}, RI^{2L}, and RI^{3L} proteins are in underlined bold text.

domain and present a new model for the molecular basis of LIN.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, and culture growth. The bacterial strains, bacteriophages, and plasmids used in this work are described in Tables 1 and 2. Growth and lysis of cultures were monitored at A_{550}. T4 phage stocks were prepared as described previously (23). Bacterial cultures were grown in standard LB medium supplemented with ampicillin (Amp) (100 μg/ml), kanamycin (Kan) (40 μg/ml), or chloramphenicol (Cam) (10 μg/ml) when appropriate. When indicated, isopropyl β-D-thiogalactoside (IPTG), NaN_3, or $CHCl_3$ was added to give final concentrations of 1 mM, 1 mM, or 1%, respectively.

Standard DNA manipulations, PCR, and DNA sequencing. Isolation of plasmid DNA, DNA amplification by PCR, DNA transformation, and DNA sequencing were performed as previously described (27). Oligonucleotides for PCR were obtained from Integrated DNA Technologies, Coralville, IA, and were used without further purification; sequences of the oligonucleotides used are available on request. Enzymes were purchased from New England Biolabs, except for *Pfu* polymerase, which was from Stratagene. Automated fluorescent sequencing was performed at the Laboratory for Plant Genome Technology at the Texas Agricultural Experiment Station. Site-directed mutagenesis of plasmids was performed as described previously (27).

Subcellular fractionation, SDS-PAGE, and Western blotting. Subcellular fractionation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were performed as described previously (27).

TABLE 1. Phages and strains used in this study

Phage or strain	Genotype(s)/feature(s)	Source or reference
Phages		
Wild-type T4	T4D	I. Molineux
T4*rI*	*rI48*, single-base deletion at position 195; N66-E97 replaced with MTRALLILNV	D. Hall
T4Δ*rI*	Deletion of *rI* gene from nt 59204 to nt 59496 in T4 genome	This study
T4*23$_{am}$*	T4D with an amber codon in gene *23*	5
λ-*t*	λ*stf*::*cat*::*tfa cI$_{857}$* Δ*S*::*t bor*::Kan^r	23
Strains		
CQ21	*E. coli* K-12 *ara leu lacI^q purE gal his argG rpsL xyl mtl ilv*	21
CQ21(λ-*t*)	Lysogen carrying (λ-*t*) prophage	This study
MG1655	F$^-$ *ilvG rfb-50 rph-1*	*E. coli* Genetic Stock Center
MG1655 *tonA*::Tn*10 lacI^q*		20
KS474	F$^-$ Δ*lacX74 galE galK thi rpsL*(*strA*) Δ*phoA*(PvuII) *degP*	15
NJH110	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR degP*::Cm^r	11
MDS12	MG1655 with 12 deletions, totaling 376,180 nt, including cryptic prophages	12
MDS12 *tonA*::Tn*10 lacI^q*		27
MDS12 *tonA*::Tn*10 lacI^q degP*::Kn^r		This study
MDS12 *tonA*::Tn*10 lacI^q degP*::Cm^r		This study
XL1-Blue	*E. coli* K-12 *recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacZ*Δ*M15*::Tn*10*]	Stratagene
LE392	*E. coli* K-12 *hsdR574*(r_K^- m_K^+) *supE44 supF58* Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55*	17
LE392 *tonA*::Tn*10*		This study

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TABLE 2. Plasmids used in this study

Plasmid	Genotype(s)/feature(s)	Source or reference
pZA-RI	pZA32Δ*luc*::*rI*	27
pZA-^{ss}PhoAΦRI$_{CTD}$	Codons 1–24 of *rI* in pZA-RI replaced by codons 1–26 of *phoA*, encoding the PhoA signal sequence	27
pZA-RI$_{TMD}$ΦPhoA	The first 24 codons of *rI* inserted in front of codon 27 of *phoA* in pZA-PhoA	27
pZA-RI^{L}	pZA32 Δ*luc*::*rI*$_{A7L}$	This study
pZA-RI^{2L}	pZA32 Δ*luc*::*rI*$_{A7L,T6L}$	This study
pZA-RI^{3L}	pZA32 Δ*luc*::*rI*$_{A7L,T6L,A5L}$	This study
pZE12-luc	ColE1 origin, P$_{LlacO-1}$ promoter; Amp^{r}	14
pZE12-Δluc	pZE12 Δ*luc*	This study
pZE12-RI	pZE12 Δ*luc*::*rI*	22
pZE12-RI^{L}	pZE12 Δ*luc*::*rI*$_{A7L}$	This study
pZE12-RI^{2L}	pZE12 Δ*luc*::*rI*$_{A7L,T6L}$	This study
pZE12-RI^{3L}	pZE12 Δ*luc*::*rI*$_{A7L,T6L,A5L}$	This study
pZE12-500RI	pZE12 Δ*luc*::*500rI*	This study
pZE12-500ΔRI	pZE12-500RI with in-frame deletion of RI reading frame	This study
pBAD-18	pBR322 origin; P$_{BAD}$ promoter; Amp^{r}	9
pBAD-RI	pBAD-18 with *rI* gene insertion using EcorI and PstI	This study
pJF118EH	ColE1 origin of replication; P$_{tac}$; *lacI*^{q1}; Amp^{r}	7
pJF-FtsI^{cmyc}	pJF118EH harboring *ftsI*^{cmyc} gene	30
pJF-PhoA	pJF118EH harboring *phoA* gene	30
pT4T	pBR322 derivative carrying late promoter and lysis cassette of λ with the *S* gene replaced by T4 *t*	I. N. Wang
pZS-24*MCS	pSC101 origin of replication; P $_{lac/ara-1}$; Kan^{r}	14
pQ	pZS24* with *Q* gene insertion using Kpn1 and Cla1	8

Briefly, to establish whether a protein was a membrane-associated or soluble species, cultures were chilled and concentrated ~150:1 by centrifugation and disrupted by use of a French press. The lysates were cleared of debris by minicentrifuge centrifugation and separated into membrane and soluble fractions by ultracentrifugation at 100,000 × *g*. To establish periplasmic localization, cultures were concentrated 120:1 by centrifugation and incubated in 12.5% sucrose, 30 mM Tris-HCl, pH 8.0 supplemented with 200 μg of lysozyme and 1 mM EDTA. When ~95% of cells were converted to spheroplasts, the samples were centrifuged at 9,000 × *g* for 10 min to separate the periplasm from the spheroplasts (membrane and cytosol).

A rabbit polyclonal antiserum against polypeptide AQSYARIMNIKLETE of RI was obtained from Sigma-Genosys. The blocking solution for Western blotting was a 1:16 mixture of 1% gelatin (Difco) with 1% bovine serum albumin (ICN) in Tris-buffered saline (150 mM NaCl and 10 mM Tris [pH 7.7]).

Construction of plasmids and T4 mutants. All DNA manipulations were done by standard techniques described previously (27). Empty vector pZE12-Δluc was constructed by deleting the *luc* insert between the KpnI and XbaI sites and ligating the backbone. Plasmid pZE12-500RI was constructed by inserting T4 DNA from nucleotide (nt) 58681 to nt 60000 between the KpnI and XbaI sites of pZE12, and plasmid pZE12-500ΔRI was constructed by deleting the *rI* sequence from pZE12-500RI.

The codons for Ala7, Thr6, and Ala5 of RI were changed sequentially to leucine codons (Fig. 1C) by use of a QuikChange kit from Stratagene with pZA-RI (27) as the template, generating plasmids pZA-RI^{L}, pZA-RI^{2L}, and pZA-RI^{3L}, respectively. The KpnI-AvrII fragments encoding these *rI* derivatives were transferred to the pZE-12 vector by ligation into similarly digested pZE12-luc, yielding pZE12-RI^{L}, pZE12-RI^{2L}, and pZE12-RI^{3L}. The same approach was used to generate pZE12-RI$_{TMD}$ΦPhoA from the starting plasmids, pZA-RI$_{TMD}$ΦPhoA and pZE12-luc.

T4Δ*rI* phage was made by homologous recombination between pZE12-500ΔRI and T4D. Plasmid pZE12-500ΔRI was transformed into MDS12 *tonA*::*Tn*10 *lacI*^{q}, and the transformants were grown to an A_{550} of ~0.4. The culture was infected with T4D at a multiplicity of infection (MOI) of ~10 and was allowed to grow for an additional 3 h. The infected cells were lysed using $CHCl_3$, and the T4Δ*rI* in the lysate was enriched three times as follows. A culture of MDS12 *tonA*::*Tn*10 *lacI*^{q} at an A_{550} of ~0.3 was infected with the lysate containing T4Δ*rI* at an MOI of ~0.1. Five minutes later, the infected culture was superinfected with T4*23$_{am}$* at an MOI of ~10 and incubated for an additional 30 min. The infected culture was rapidly filtered through a 0.22-μm syringe filter, and the filtrate was used for the next round of enrichment. At the end of the third enrichment, the filtrate was screened for phage that produced large plaques indicative of the *rI* deletion. The presence of the deletion in the phages recovered from the large plaques was confirmed by PCR.

Complementation of T4Δ*rI* with *rI* alleles expressed from plasmids. Fresh transformants of strains harboring either pZE12-RI, pZE12-^{ss}PhoAΦRI$_{CTD}$, or pZE12-Δluc were grown in 25 ml of LB-Amp to an A_{550} of ~0.2 and split into three 7-ml aliquots. Each aliquot was diluted with LB-Amp to make a 25-ml culture. The cultures were grown until reaching an A_{550} of ~0.3 and induced with IPTG for 10 min. One of these cultures was left uninfected, while the other two were infected with either T4D or T4Δ*rI* at an MOI of ~10, and the culture growth was monitored at A_{550}.

Stability of the RI protein. The host strain harboring the plasmid with the indicated allele of *rI* was grown in 30 ml of LB-Amp to an A_{550} of ~0.4. The culture was then split into two 15-ml aliquots, and each aliquot was diluted to 60 ml with LB-Amp. These cultures were grown until reaching an A_{550} of ~0.4 and induced with IPTG for 1 h. Protein synthesis in one of the cultures was inhibited using Cam at a 300-μg/ml final concentration. At ~1.5-min intervals for 10 min after the addition of Cam, 5 ml from each culture was taken and added to 5 ml of 10% ice-cold trichloroacetic acid (TCA). The TCA precipitates were collected by centrifugation and subjected to SDS-PAGE and Western blotting using anti-RI antibodies. The intensities of the bands corresponding to RI were analyzed using the Image J program from the NIH website (http://rsb.info.nih.gov/ij/).

Effect of protein synthesis and *sec* inhibitors on LIN. MDS12 *tonA*::*Tn*10 *lacI*^{q} was grown to an A_{550} of ~0.4 and infected with either T4D or T4Δ*rI* at an MOI of ~10. Fifteen minutes after infection with T4Δ*rI*, Cam (in 95% ethanol) was added to a final concentration of 300 μg/ml. As a control, an equivalent volume of 95% ethanol was added to a parallel culture. To cultures infected with T4D, the same additions were made 30 min after infection. For the T4D-infected cultures, total and free phage were determined at 1 h after infection. Total phage was assessed by determining the titer of a $CHCl_3$-treated aliquot of the infected culture. A second aliquot was rapidly filtered through a 0.22-μm filter to obtain free phage. The ratio of PFU in the filtrate to that in the $CHCl_3$-treated sample was used as the fraction of total phage that had been released.

Fresh transformants of MG1655 *tonA*::*Tn*10 *lacI*^{q} harboring pQ and pT4T, with or without pZA-^{ss}PhoAΦRI$_{CTD}$, were grown at 37°C until reaching an A_{550} of ~0.2. The cultures were split into three 7-ml aliquots and diluted with LB containing appropriate antibiotics to a final volume of 25 ml. The cultures were grown to an A_{550} of ~0.2, and NaN_3 was added to one aliquot. Ten minutes after the addition of NaN_3, IPTG was added, and the A_{550} was followed until lysis occurred.

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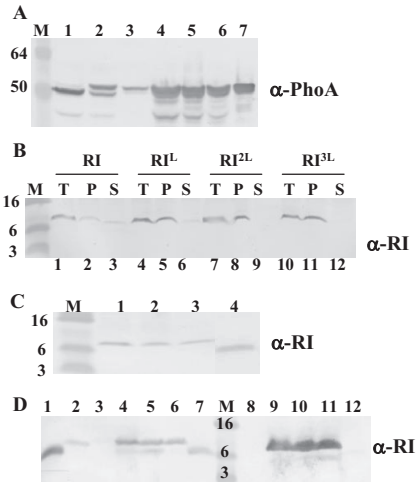

FIG. 2. Subcellular localization of RI and its derivatives. Subcellular fractions were prepared and analyzed by SDS-PAGE and Western blotting as described in Materials and Methods. The primary antibodies (α) used are indicated on the right of each panel. For each blot, lane M contains molecular mass markers (in kDa). (A) The RI_{NTD} directs the secretion of RI_{NTD}ΦPhoA to the periplasm without processing. Cells carrying pZE12-RI_{NTD}ΦPhoA were induced and fractionated into spheroplast and periplasmic fractions. Lanes: 3, uninduced cells; 4, induced cells; 5, cells after spheroplasting; 6, spheroplasts (membranes and cytoplasm); 7, periplasm. To show the positions of the precursor and mature forms of PhoA, unfractionated samples from cells expressing the wild-type *phoA* gene from pJF-PhoA and grown in the absence (lane 1) or presence (lane 2) of 1 mM NaN_3 were also analyzed. (B) Increasing the hydrophobicity of the RI_{NTD} inhibits the membrane release of RI. Cells harboring either pZE12-RI, pZE12-RI^L, pZE12-RI^{2L}, or pZE12-RI^{3L} were induced, disrupted by passage through a French pressure cell, and separated into membrane and soluble fractions by centrifugation. T, total lysate; P, membrane pellet; S, soluble fraction. (C) RI is found in the periplasm in an unprocessed form. Cells harboring pZE12-RI were induced and separated into spheroplast and periplasmic fractions. Lanes: 1, total cells after spheroplasting; 2, spheroplast fraction; 3, periplasmic fraction; 4, partially purified RI_{CTD} (27). (D) Cleavage and export of ^{ss}PhoAΦRI_{CTD} is blocked by NaN_3. Cells harboring pZE12-^{ss}PhoAΦRI_{CTD} were grown in the absence (lanes 3 to 7) or presence (lanes 8 to 12) of 1 mM NaN_3 10 min before induction. Sixty minutes after induction, the cells were harvested and fractionated into spheroplasts and periplasm. Lanes: 3 and 8, uninduced cells; 4 and 9, induced cells; 5 and 10, cells after spheroplasting; 6 and 11, spheroplasts; 7 and 12, periplasm; 1 and 2, control samples containing RI_{CTD} and full-length RI, respectively.

RESULTS

The N-terminal TMD of RI is a SAR domain. The fact that *sec*-mediated secretion of the C-terminal periplasmic domain of RI (RI_{CTD}) was both necessary and sufficient to block lysis mediated by the T4 holin (27) suggested that the NTD, RI_{NTD}, might be a cleavable signal sequence, as suggested by Paddison et al. (19) and as predicted, at a 99.8% probability, by SignalP analysis (Fig. 1A). However, in the course of these studies, we noted that the chimeric protein RI_{NTD}ΦPhoA (Fig. 1B) existed in both soluble and membrane-associated forms indistinguishable by SDS-PAGE (27). Upon further examination, we found that a considerable fraction of the soluble portion is located in the periplasm (Fig. 2A, lane 7). This species had a mass similar to that of the unprocessed form of PhoA, which accumulates during the inhibition of the *sec*-mediated translocation pathway by sodium azide (Fig. 2A, lane 2). Thus, by several criteria, RI_{NTD}ΦPhoA behaves like the endolysin of bacteriophage P1 Lyz, suggesting that the RI_{NTD} can function as a SAR domain. The N-terminal SAR domain of Lyz directs its *sec*-mediated integration into the cytoplasmic membrane and allows its subsequent release into the periplasm independent of proteolytic processing. Using antisera prepared against a C-terminal peptide of RI revealed that the RI protein itself was present in both the soluble and membrane fractions of cells induced for a plasmid-borne *rI* gene (Fig. 2B, lanes 2 and 3). Similarly, the soluble RI protein appeared to be periplasmic (Fig. 2C, lane 3). Since the masses of the soluble and membrane bound forms of RI were indistinguishable (Fig. 2B and C), the former was not generated from the latter by proteolysis. As a control, a Western blot of the samples from cells expressing $FtsI^{cmyc}$ was probed with the antibodies for the c-*myc* tag; this inner membrane protein was detected only in the membrane and spheroplast fractions (not shown). The ability of both RI_{NTD}ΦPhoA and RI to distribute between the membrane and periplasmic fractions strongly argues that the NTD of RI functions as a SAR domain, instead of the cleavable signal sequence predicted by SignalP.

The RI_{CTD} is most active after its release to the periplasm. The SAR domains of P1 Lyz and related endolysins have a high content of weakly hydrophobic and uncharged polar residues compared to conventional TMDs. This suggested that this compositional feature is one factor allowing SAR domains to exit the membrane. For this reason, we tested the effect of increasing the hydrophobicity of the SAR domain of RI on its ability to distribute in the soluble fraction and on its function as an antiholin. The progressive substitution of leucines for the Ala/Thr residues at positions 5 to 7 of RI (RI^L, RI^{2L}, and RI^{3L}; Fig. 1C) had the effect of increasing the fraction of RI that was membrane associated upon the separation of cellular contents into soluble and membrane fractions (Fig. 2B). Moreover, increasing the hydrophobicity of RI_{NTD} resulted in decreasing the ability of RI to inhibit T-mediated lysis (Fig. 3A).

Previously, we had observed that when the periplasmic C-terminal domain of RI (RI_{CTD}) was fused to the cleavable signal sequence of alkaline phosphatase (PhoA), the resulting chimera, ^{ss}PhoAΦRI_{CTD} (Fig. 1B), was more effective at eliciting LIN than RI itself (see the work of Tran et al. [27]). Induction of the *^{ss}phoAΦrI$_{CTD}$* gene resulted in the accumulation of two anti-RI-reactive species (Fig. 2D, lane 5). The larger product had a mass approximately equal to that of RI (Fig. 2D, lane 2), while the mass of the smaller product was approximately the same as that of a polypeptide fragment comprising residues 25 to 97 of RI (Fig. 2D, lane 1). During subcellular fractionation, the larger product was spheroplast associated (Fig. 2D, lane 6), while the smaller product was periplasmic (Fig. 2D, lane 7) and, moreover, was absent from cultures treated with azide (Fig. 2D, lane 12). Since azide at 1 mM is known to inhibit the *sec* translocation pathway (6, 18), it is clear that the smaller product detected after induction of the *^{ss}phoAΦrI$_{CTD}$* gene represents the soluble, periplasmic RI_{CTD} released by cleavage of the ^{ss}PhoAΦRI_{CTD} precursor by signal peptidase. The full-length ^{ss}PhoAΦRI_{CTD} product seen in the presence and absence of azide probably represents protein trapped in the cytoplasm. Since azide treatment also

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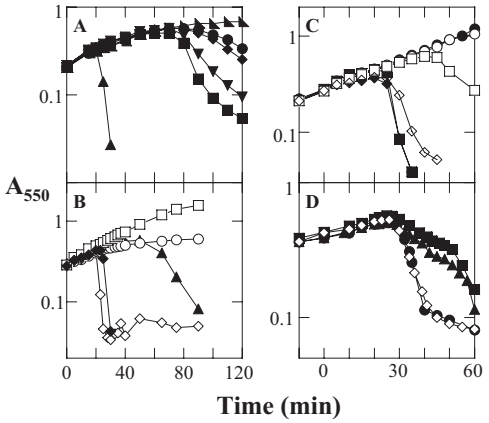

FIG. 3. LIN by RI and its derivatives. Abscissa is time after induction (A and C) or infection (B and D). (A) Increasing the hydrophobicity of the RI_{NTD} decreases LIN. CQ21(λ-t) cells carrying the indicated plasmids were grown to an A_{550} of ~0.2 and induced by a shift to 42°C and the addition of IPTG. Symbols: ▲, no plasmid; ◣, pZE12^{ss}phoAΦRI_{CTD}; ●, pZE12-RI; ◆, pZE12-RI^{L}; ▼, pZE12-RI^{2L}; ■, pZE12-RI^{3L}. (B) Complementation of the LIN defect in T4Δ*rI* infections with RI and ^{ss}PhoAΦRI_{CTD}. MG1655 *tonA*::*Tn*10 *lacI*^{q} cells carrying the indicated plasmids were induced with IPTG 10 min prior to infection with either T4D or T4Δ*rI* at an MOI of ~10. Symbols: ■, uninfected cells with pZE12-Δluc; ○, cells with pZE12-Δluc infected with T4D; ◇, cells with pZE12-Δluc infected with T4Δ*rI*; ◆, cells with pZE12-RI infected with T4Δ*rI*; ▲, cells with pZE12^{ss}PhoAΦRI_{CTD} infected with T4Δ*rI*. (C) Azide prevents ^{ss}PhoAΦRI_{CTD}-mediated LIN. The host used, MG1655 *tonA*::*Tn*10 *lacI*^{q} cells carrying either pQ and pT4T (●, ■, and ◆) or pQ, pT4T, and pZA-^{ss}PhoAΦRI_{CTD} (○, □, and ◇), were grown to an A_{550} of ~0.2 and induced with IPTG. Circles, uninduced cultures; squares, induced cultures; diamonds, cultures pretreated with 1 mM azide 10 min before induction. (D) LIN is not significantly prolonged in hosts with mutations in *degP* or *cpxP*. MDS12 *tonAI*::*Tn*10 *lacI*^{q} (◇), MDS12 *tonA*::*Tn*10 *lacI*^{q} *degP*::Cm^{r} (■), MDS12 *tonA*::*Tn*10 *lacI*^{q} *cpxP*::Kn^{r} (●), and MDS12 *tonA*::*Tn*10 *lacI*^{q} *degP*::Cm^{r} *cpxP*::Kn^{r} (▲) harboring pZE12-RI were induced for 10 min and then infected with T4Δ*rI* at an MOI of ~5.

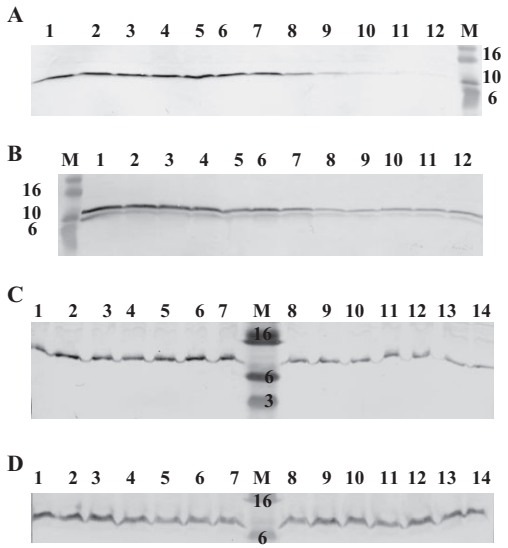

FIG. 4. Stability of the RI protein and its derivatives. (A to D) Western blots of samples that were collected by TCA precipitation and analyzed by SDS-PAGE. In each blot, lane M contains molecular mass markers (in kDa). (A) Wild-type RI. Lanes: 7 to 12, 0, 2, 3.5, 5.5, 7.5, and 9 min after addition of Cam; 1 to 6, same time intervals but with no Cam added. (B) ^{ss}PhoAΦRI_{CTD}. Lanes: 7 to 12, 0, 2.3, 3.9, 5.5, 7, and 10 min after Cam addition; 1 to 6, same time intervals but with no Cam added. (C) RI^{3L}. Lanes: 8 to 14, 0, 2.3, 3.7, 5.3, 6.7, 8.2, and 10 min after Cam addition; 1 to 7, same time intervals but without Cam addition. (D) RI in a $DegP^{-}$ strain. Lanes: 8 to 14, 0, 2.4, 3.8, 5.3, 6.8, 8.3, and 9.2 min after addition of Cam; 1 to 7, same time intervals but with no Cam added.

eliminated LIN (Fig. 3C), it was the periplasmic RI_{CTD} and not the precursor that inhibited the T holin.

RI is an unstable protein. At a low multiplicity of superinfection, LIN is transient and requires continued reinfection for its maintenance. This could be explained if RI were an unstable protein that is rapidly degraded in the absence of a signal generated by repeated superinfections. As one test of this hypothesis, we determined the half-life of RI protein. In this experiment, the *rI* gene under the control of the *lac* promoter was induced by the addition of IPTG, and 1 hour later Cam was added to block further protein synthesis. The level of RI present as a function of time after the addition of Cam was assessed by SDS-PAGE and Western blotting. In this case, the RI protein rapidly disappeared (Fig. 4A) after the addition of the protein synthesis inhibitor, showing a half-life of ~2 min (Fig. 5). Similar results were obtained when RI protein synthesis was induced from the arabinose-inducible plasmid pBAD-RI and then repressed with fucose (not shown).

It was not possible to measure RI stability in T4-infected cells, since the level of RI produced was below the limits of detection with our antibody, even when 5 ml of T4-infected culture at an A_{550} of ~0.5 was used for SDS-PAGE and Western blotting. Instead, to provide evidence that the continued synthesis of RI is necessary to maintain LIN during a T4 infection, we added Cam to cultures infected with wild-type T4. This treatment resulted in a gradual decrease in the culture A_{550} beginning immediately after the addition of Cam (Fig. 6A). After 1 h of infection, nearly 80% of the total phage present in the treated culture had been released to the medium, compared to 6% released in a parallel culture that was not treated with Cam (Fig. 6B). The ability of Cam to partially abrogate LIN is consistent with the rapid turnover of RI during phage infection.

The SAR domain is a major determinant of RI instability. When cultures expressing the ^{ss}*phoA*Φ*rI*$_{CTD}$ gene were treated

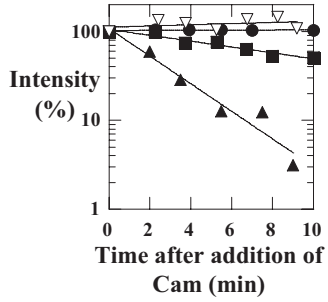

FIG. 5. The N-terminal SAR domain of RI dictates its rapid degradation. The intensities of the bands in the Western blots shown in Fig. 4 were digitized and plotted as a function of time after Cam addition, relative to intensity at time 0. Symbols: ▲, RI; ▽, RI in an isogenic *degP* strain; ●, ^{ss}PhoAΦRI_{CTD}; ■, RI^{3L}.

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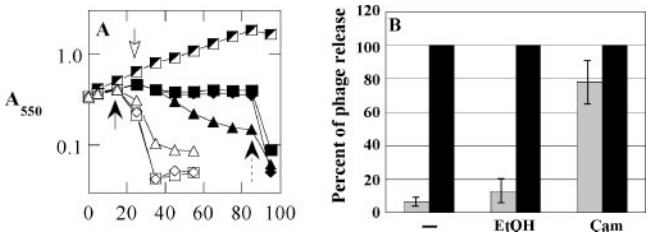

FIG. 6. Cam collapses LIN during T4 infections. (A) Cam triggers lysis. MDS12 *tonA*::*Tn*10 *lacI*^{q} was infected at an MOI of ~10 with either T4D (solid symbols) or T4Δ*rI* (open symbols). The solid arrows with solid or open arrowheads indicate the times of Cam or ethanol addition to T4Δ*rI* or T4D-infected cultures, respectively. The dashed arrow indicates the time of addition of chloroform. ◩, no infection; ■ and □, infected cultures without treatment; ◆ and ◇; infected cultures treated with 95% ethanol; ▲ and △, infected cultures treated with 300 μg/ml of Cam. (B) Cam facilitates phage release from T4D-infected cells. Total and extracellular phage were measured as described in Materials and Methods. For each condition, the black bar indicates total phage and the gray bar indicates extracellular phage. −, no additions; EtOH, treatment with 95% ethanol; Cam, treatment with 300 mg/ml of Cam.

with Cam, the periplasmic RI_{CTD} polypeptide was found to be relatively stable (Fig. 4B and 5). Moreover, the membrane-restricted RI^{3L} protein also had a half-life considerably longer than that of RI (Fig. 4C and 5). These data indicate that the degradation of RI occurred after its release from the membrane and was promoted by the exposure of its SAR domain to the periplasm. Additional evidence for the destabilization of RI by its SAR domain was obtained by complementing the LIN-defective phage, T4Δ*rI*, with plasmids carrying either the *rI* or the *^{ss}phoAΦrI$_{CTD}$* gene. In these experiments, the plasmids were induced 10 minutes before infection with T4Δ*rI*. Since T4 infection results in the rapid destruction of host DNA (16), synthesis of RI or ^{ss}PhoAΦRI_{CTD} should cease shortly thereafter. Attempts to complement the LIN defect of T4Δ*rI* with *rI* resulted in a 5-min delay in lysis, compared to the 30- to 40-min delay seen with *^{ss}phoAΦrI$_{CTD}$* (Fig. 3B). This result is consistent with the increased stability of the RI_{CTD} compared to that of RI with its N-terminal SAR domain.

The DegP protease is required for the rapid turnover of RI. We reasoned that the rapid degradation of periplasmic RI might due to the recognition of its exposed SAR domain by proteolytic machinery responsible for removing misfolded proteins from this compartment. Since DegP is known to be involved in the degradation of misfolded periplasmic proteins (13, 24, 25), we determined the effect of mutations that eliminate DegP activity on the half-life and function of RI. The RI protein was found to have a prolonged half-life in a *degP* mutant (Fig. 4D and 5). Unexpectedly, no significant lysis delay was observed when T4Δ*rI* was complemented with *rI* expressed from a plasmid in this mutant (Fig. 3D). Moreover, T4 plaque morphology is unaffected on *degP* lawns (not shown).

Recently, Isaac et al. (11) showed that degradation of a subset of misfolded periplasmic proteins by DegP required the adaptor protein CpxP. We hypothesized that both DegP and CpxP were necessary for RI degradation. According to this idea, even when DegP is absent and RI is stable, LIN might be compromised because CpxP binds to RI and prevents the interaction of RI with T. To test this hypothesis, we performed the complementation experiments in the absence of CpxP, DegP, or both. The lysis delay in the double mutant was not different from that seen for the *degP* mutant alone (Fig. 3D), indicating that CpxP did not interfere with RI antiholin function.

DISCUSSION

LIN in T-even phage infection is a historically significant phenomenon that was used to elucidate many basic concepts of molecular biology. However, its molecular basis has been obscure. With T4-infected cells, LIN occurs upon superinfection with intact T4 particles but not with T4 ghosts, indicating that the intact phage delivers a signal necessary to activate LIN. Recently, it was shown that T and RI, the holin and antiholin of phage T4, play a major role in LIN and that LIN can be reconstituted by expressing T and RI from plasmids (22). In this system, RI is expressed at higher levels than in T4 infection, suggesting that the overexpression of RI compensates for the lack of the LIN signal associated with superinfection. Here, we have established that RI possesses an N-terminal SAR domain, which allows it to be released into the periplasm. Increasing the hydrophobicity of the SAR domain by leucine substitutions has two effects. First, as the leucine content increases, the fraction of RI that is released to the periplasm is progressively decreased (Fig. 2B). Second, as RI is increasingly restricted to the membrane, its ability to function as an antiholin is significantly reduced (Fig. 3A). Together, these observations indicate that it is the soluble periplasmic form of RI that normally participates in LIN.

Unexpectedly, the SAR domain was found to affect the function of the RI protein even after its release to the periplasm. The wild-type protein is extremely unstable, with a half-life of 2 to 3 min. By contrast, the RI_{CTD} delivered to the periplasm after signal peptidase cleavage of ^{ss}PhoAΦRI_{CTD} was much more stable, with a half-life considerably in excess of 10 min. There are five residues from the mature domain of PhoA retained in the processed form of this chimera, so it cannot be ruled out that these residues contribute to the enhanced stability. However, the fact that the membrane-restricted RI^{3L} protein appeared to be as stable as the RI_{CTD} strongly suggests that the SAR domain not only controls the release of RI to the periplasm but also limits its persistence in this compartment by facilitating its rapid degradation. The instability of RI appears to be largely due to the periplasmic protease DegP. In a *degP* host, RI is just as stable as the RI_{CTD} and RI^{3L} proteins. Surprisingly, the absence of DegP does not affect the plating phenotype of T4, nor does it have a significant effect on the kinetics of T4-mediated lysis in liquid culture. These findings lead us to conclude that RI_{CTD} must have two distinct conformations in the periplasm. One conformation, adopted by the membrane-tethered RI and maintained for a short period after its release from the membrane, is both active in LIN and insensitive to DegP. Once released to the periplasm, however, this active form of RI rapidly decays into a form that is inactive in LIN and is sensitive to DegP. Thus, exposure of the RI SAR domain to the periplasm has two functions: it promotes the inactivation of the RI_{CTD} and facilitates the degradation of the inactive protein by DegP. This would explain the enhanced stability and antiholin activity of

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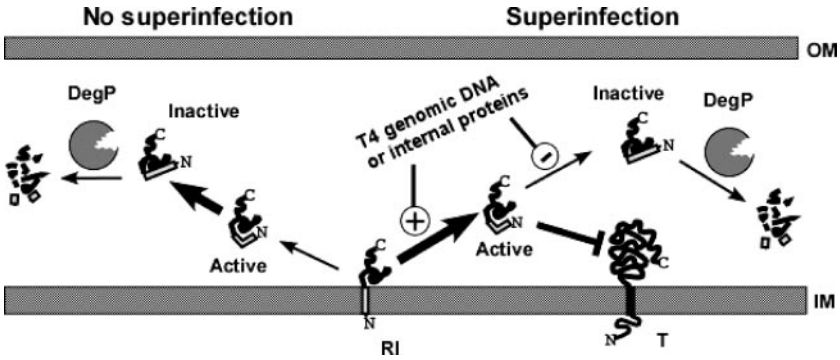

FIG. 7. Model for LIN. The RI protein is initially anchored to the cytoplasmic membrane by its SAR domain but undergoes slow, spontaneous release to the periplasm. The periplasmic form of RI that serves as the T4 antiholin is rapidly inactivated and is eventually degraded by DegP. The LIN signal delivered by superinfecting phage either increases the rate of RI release from the membrane or stabilizes the active form of RI in the periplasm. See the text for further discussion.

the RI_{CTD} produced from $^{ss}PhoA\Phi RI_{CTD}$ compared to those of RI itself.

Based on these findings, we propose the model for LIN shown in Fig. 7. During a primary infection, RI is made and released into the periplasm, where it undergoes spontaneous inactivation and ultimate degradation by DegP. Change from the active to the inactive form in the absence of superinfection is rapid, since the persistence of RI in DegP mutants is not accompanied by a measurable change in lysis kinetics. Thus, the inability of RI to inhibit lysis in the absence of superinfection is due to the conformational instability of RI and not to its degradation by periplasmic proteases. Upon superinfection, a signal is transmitted to the periplasm which either accelerates the rate at which RI is released from the membrane or stabilizes the active conformation of RI after its release to the periplasm. We favor the latter mechanism, since LIN is maintained for 20 min after the addition of Cam to the infected cultures, a time period greatly in excess of the half-life of RI. This suggests to us that superinfection served to stabilize a pool of active RI that was present at the time of Cam addition. In any case, since RI can protect cells from T-mediated lysis in the absence of superinfection, it seems likely that the LIN signal operates by increasing the concentration of active RI in the periplasm, resulting in the phenomenon we know as LIN. In primary infection, both T4 genomic DNA and internal protein are injected into the cytoplasm of the infected host (16). In T4 superinfection, it was shown that T4 DNA is ectopically localized in the periplasm (2), and it seems reasonable to extrapolate that this mislocalization also applies to the internal proteins. Thus, either T4 DNA or the internal proteins, or both, might serve as the LIN signal.

Why should RI possess a SAR domain instead of a signal sequence or simply a TMD? While there is not yet a definitive answer to this question, it is clear that the SAR domain of RI endows the protein not only with DegP-dependent proteolytic instability but, more importantly, with an extreme functional lability that may be critical for its function as the receptor of the LIN signal. The RI protein that accumulates in a *degP* host appears to be incapable of blocking T-mediated lysis but also apparently does not act as a "sink" for the LIN signal, since the plating phenotype of wild-type T4 is not affected by the *degP* genotype of the host. Thus, RI is capable of interacting with T or the LIN signal only for a brief period after its release to the periplasm. The net effect of this is to ensure that the total concentration of RI molecules capable of both inhibiting T-mediated lysis and receiving the LIN signal is maintained at a low level, which may be important in imparting the requisite sensitivity necessary to respond to individual superinfections. In any case, the availability of the functionally and chemically stable form of RI, i.e., the proteolytically processed form of $^{ss}PhoA\Phi RI_{CTD}$, will facilitate investigation into the nature of the LIN signal and the interaction of RI and the T holin.

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