The Small Heat-shock Protein HspL Is a VirB8 Chaperone Promoting Type IV Secretion-mediated DNA Transfer*

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Abstract

Agrobacterium tumefaciens is a plant pathogen that utilizes a type IV secretion system (T4SS) to transfer DNA and effector proteins into host cells. In this study we discovered that an α-crystallin type small heat-shock protein (α-Hsp), HspL, is a molecular chaperone for VirB8, a T4SS assembly factor. HspL is a typical α-Hsp capable of protecting the heat-labile model substrate citrate synthase from thermal aggregation. It forms oligomers in a concentration-dependent manner in vitro. Biochemical fractionation revealed that HspL is mainly localized in the inner membrane and formed large complexes with certain VirB protein subassemblies. Protein-protein interaction studies indicated that HspL interacts with VirB8, a bitopic integral inner membrane protein that is essential for T4SS assembly. Most importantly, HspL is able to prevent the aggregation of VirB8 fused with glutathione S-transferase in vitro, suggesting that it plays a role as VirB8 chaperone. The chaperone activity of two HspL variants with amino acid substitutions (F98A and G118A) for both citrate synthase and glutathione S-transferase was reduced and correlated with HspL functions in T4SS-mediated DNA transfer and virulence. This study directly links in vitro and in vivo functions of an α-Hsp and reveals a novel α-Hsp function in T4SS stability and bacterial virulence.

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§§ The abbreviations used are: α-Hsp, α-crystallin-type small heat-shock proteins; CS, citrate synthase; AS, ascorbyl synthase; GST, glutathione S-transferase; SEC, size exclusion chromatography; DDM, dodecyl-β-D-maltopyranoside; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; NiNTA, nickel-nitrilotriacetic acid; DDM, dodecyl-β-D-maltopyranoside; AS, acetosyringone; T4SS, type IV secretion system; SEC, size exclusion chromatography; DDM, dodecyl-β-D-maltopyranoside; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; NiNTA, nickel-nitrilotriacetic acid.
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...ing as an inner membrane base complex to connect the VirB7/VirB9/VirB10 core channel across the double membrane (26–28). Crystallography and protein-protein interaction studies also suggest that VirB8 dimerization and interactions with VirB4 and VirB10 are important for its role in T4SS assembly (29, 30). As monitored by transfer DNA immunoprecipitation assays, the T-complex (a covalent complex of single-stranded DNA and VirD2) is translocated across the cell envelope via four discrete steps of sequential interactions with VirD4, VirB11, VirB6/VirB8, and VirB2/VirB9 (31). In addition to transporting the T-complex and effector proteins from bacteria into plant cells, the T4SS can translocate the Q group incompletely (32) or between agrobacteria (33).

...We previously discovered that HspL is a VirB-induced α-Hsp involved in VirB protein accumulation, T4SS-mediated DNA transfer, that is required for full virulence of A. tumefaciens (18). Here, we demonstrate that HspL is a typical molecular chaperone interacting not only with the model substrate CS but also with VirB8. HspL is able to protect both target proteins from aggregation in vitro. The altered chaperone activity of two HspL variants correlated well with reduced HspL functions in promoting T4SS-mediated DNA transfer and virulence. The provided evidence strongly suggests that HspL functions as a VirB8 chaperone and that this chaperone activity is responsible for its role in DNA transfer and virulence of A. tumefaciens.

EXPERIMENTAL PROCEDURES

Plasmids, Bacterial Strains, and Growth Conditions—The bacterial strains and plasmids constructed or used in this study were summarized in supplementary Table 1. The sequences of primers used for plasmid construction were listed in supplementary Table 2. A. tumefaciens strains were routinely grown at 28°C in 523 medium (34), and Escherichia coli strains were grown at 37°C in Luria-Bertani medium (35). Antibiotics were grown at 37 °C in Luria-Bertani medium (35). Antibiotics were provided evidence strongly suggests that HspL functions as a promoting T4SS-mediated DNA transfer and virulence. The...
centration of 100, 50, or 25 μg/ml for 10 min on ice. The reaction was stopped by adding 2× SDS sample buffer (35), and the samples were then boiled immediately for 15 min.

Dodecyl-β-D-maltopyranoside (DDM)-extracted HspL Complex and Its Interacting Protein(s)—The membrane isolation, extraction of membrane-associated proteins with DDM and SEC for analysis of HspL and VirB protein complexes, were conducted as described (45) with minor modifications. Membrane fractions isolated from *A. tumefaciens* strains NT1RE(pJK270) or Δ*hspL* (pHspL-His) grown in AS-induced 1-medium at 25 °C for 16 h were resuspended at 0.2 mg/ml protein concentration in lysis buffer (50 mM sodium phosphate, 250 mM NaCl, pH 8.0) containing 2% DDM and incubated with rocking at 4 °C overnight to extract membrane protein complexes. The DDM-extracted soluble fractions were collected by centrifugation at 150,000 × g at 4 °C for 1 h, which sedimented the insoluble fraction. 1 ml of DDM-extracted proteins was analyzed by SEC on a Superdex 200 HiLoad 16/60 column as described above. When applicable, the samples were 4-fold-diluted in lysis buffer and used to pull-down potential HspL-interacting protein(s) purified via a Ni-NTA His Bind Resin column (Novagen). Purified HspL-His and its interacting protein(s) were resolved by glycine-SDS-PAGE and detected by Western blotting with specific antibodies.

**Yeast Two-hybrid Analysis**—Matchmaker™ yeast two-hybrid analysis was performed according to the instructions of the user manual (Clontech, Mountain View, CA). The PCR-amplified full-length *virB8* (VirB8-Y2H, digested by NdeI/EcoRI) and *hspL* (HspL-Y2H, digested by NdeI/BamHI) were cloned into pGADT7 and pGBKT7 to create an N-terminal fusion to the activating domain and into pGBK7 to create an N-terminal fusion to the DNA binding domain (Clontech) at the same restriction enzyme sites, respectively. Each pair of plasmids was transformed into *Saccharomyces cerevisiae* strain AH109 and selected on synthetic dextrose minimal medium in the absence of leucine and tryptophan grown at 28 °C for 3 days. The positive clones were selected twice by their growth on synthetic dextrose minimal medium in the absence of Ade, His, Leu, and Trp grown at 28 °C for 3 days.

**Co-purification of HspL-His and VirB8 from E. coli**—The proteins encoded by pETHspL and pTrcB8 plasmids were co-expressed in *E. coli* BL21(DE3) by isopropyl 1-thio-β-D-galactopyranoside induction at 37 °C (0.4 mM) for 2 h. Soluble HspL-His and its interacting protein(s) were bound to a Ni-NTA His Bind Resin column (Novagen) and washed with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 50 mM imidazole, pH 8.0) following the manufacturer’s instructions. The bound proteins were eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0), resolved by glycine-SDS-PAGE, and detected by Western blotting with VirB8- and HspL-specific antibodies.

**Conjugal Transfer Analysis of Q Group Incompatibility Plasmid RSF1010**—The conjugation assays were carried out using NT1RE(pJK270), and its derivatives as donor strains and NT1RE-Sp as the recipient strain as is described (18).

**Tumor Assay on Potato Tuber Discs**—Quantitative tumorigenesis assays using potato tuber discs were performed following the method described (18) with minor modifications.

The potato tuber discs were placed on water agar, infected with 10 μl of bacterial culture, and incubated at 24 °C for 24 h. To prevent bacterial growth, infected potato discs were dipped into phosphate-buffered saline containing Timentin (25 μg/ml) for a few seconds before further incubation at 24 °C on water agar supplemented with Timentin (100 μg/ml). Tumors were scored after 3 weeks.

**RESULTS**

**HspL Possesses Concentration-dependent Chaperone Activity and Oligomerizes in Vitro**—To understand the molecular mechanism underlying the contribution of HspL to T4SS-mediated DNA transfer and virulence of *A. tumefaciens*, we first investigated whether HspL possesses typical chaperone activity in preventing a model substrate from thermal aggregation. His-tagged HspL was purified and co-incubated with the standard heat-labile substrate CS at 43 °C. Although CS alone or in the presence of bovine serum albumin as a control aggregated rapidly as measured by light scattering (absorbance at 360 nm), thermal aggregation was inhibited in the presence of HspL (Fig. 1A). CS aggregation was reduced with increasing HspL concentrations, reaching full protection of CS from aggregation when HspL/CS co-incubation was set at a molar ratio of 2:1 (Fig. 1A). This result indicated that HspL is a typical α-Hsp possessing chaperone activity in protecting CS from thermal aggregation in a concentration-dependent manner.

Another property of α-Hsp is the formation of large oligomers, which is a prerequisite for chaperone activity (2). SEC analysis of purified soluble HspL resulted in two major peaks, the first corresponding to the void volume. Both peaks increased with increasing protein concentrations, and the second peaks corresponded to molecular masses of 208, 264, and 330 kDa at 0.5, 1.0, and 2.0 mg/ml protein concentration, respectively (Fig. 1B). Based on SDS-PAGE analysis, the majority of HspL eluted in the second peaks, but we also detected low amounts eluted in the void volume (≈44 ml of elution volume) (supplemental Fig. S1). The data suggest that native HspL predominantly forms large complexes containing appropriately 12–18 subunits.

**Two HspL Variants Causing Reduced HspL Chaperone Activity and Altered Oligomerization Patterns**—The α-crystallin domain of α-Hsps plays a critical role in its oligomerization and chaperone activity (9, 41). Multiple amino acid sequence alignments of HspL and selected homologs encoded by Rhi zobiaecae and *Homo sapiens* α-crystallin A and B revealed the presence of a typical α-crystallin domain with conserved amino acid residues in HspL. (supplemental Fig. S2). Several amino acid residues are strikingly conserved in all α-Hsps, strongly suggesting that these conserved amino acids are critical for α-Hsp function. Here, we generated two HspL variants in which the conserved residues Phe-98 or Gly-118 were substituted by Ala (F98A and G118A, supplemental Fig. S2). The importance of these two conserved amino acids has been documented previously in assays for both *in vitro* chaperone activity and *in vivo* function (9, 41). Although CS was fully protected from thermal aggregation by wild type HspL, HspLF98A completely lost the ability to prevent CS aggrega-
tion, and HspLG118A possessed reduced chaperone activity (Fig. 2A). The defect in chaperone activity was not due to precipitation of the HspL variants that remained mostly soluble after heat treatment (supplemental Fig. S3).

To determine whether the altered chaperone activity correlated with changes in oligomerization, we examined HspLF98A and HspLG118A by SEC and found that oligomerization was different from the wild type in both cases (Fig. 2B and supplemental Fig. S1). In comparison to the 264-kDa oligomer of wild type HspL, HspLF98A formed two different complexes, one eluting with the void volume and the other at 130 kDa. HspLG118A formed a large 600-kDa oligomer. These results suggested that formation of a stable and defined oligomer is important for HspL chaperone activity.

HspL Chaperone Activity Correlates with Its Function in Promoting T4SS-mediated DNA Transfer and Virulence—We next examined whether the altered in vitro chaperone activity of HspLF98A and HspLG118A correlated with a change in HspL function in vivo. The expression of wild type hspL driven by its native promoter on an IncP plasmid rescued the defect of the hspL deletion mutant (ΔhspL) in tumorigenesis efficiency on potato tuber discs (Ref. 18, Fig. 3A, and supplemental Table 3). In contrast, the expression of pHspLF98A in ΔhspL did not restore wild type tumor formation, and expression of pHspLG118A gave an intermediate phenotype. Similar to our previous findings (18), the deletion of hspL had only modest effects on T4SS-mediated plasmid transfer, but the expression of HspL stimulated transfer to levels higher than wild type (Fig. 3B and supplemental Table 4). This effect may be explained by the modest overexpression of HspL from the IncP plasmid (18), and the stimulation underlines the positive contribution of HspL to T4SS function. The expression of pHspLF98A in ΔhspL did not rescue the reduced transfer efficiency caused by the absence of HspL (Fig. 3B, supplemental Table 4). Consistent with its residual in vitro activity, pHspLG118A caused the increased T4SS-mediated transfer efficiency in ΔhspL to a level between ΔhspL and ΔhspL expressing wild type HspL. The positive correlation between chaperone activity, T4SS-mediated DNA transfer, and virulence strongly suggested that the chaperone activity of HspL is critical for its in vivo functions participation in T4SS.

HspL Co-fractionates with Inner and Outer Membranes—We showed the importance of HspL chaperone activity in promoting DNA transfer, and this raises the question of how HspL impacts T4SS functions. Our previous study revealed that HspL protein accumulation was triggered in response to certain VirB proteins, and optimal VirB protein accumulation required HspL (18). Thus, we hypothesize that HspL may function as a VirB chaperone to prevent VirB proteins from aggregation and/or degradation. To assess this possibility, we first examined the subcellular localization of HspL to determine whether it co-fractionates with VirB proteins in the membrane. Biochemical fractionations revealed that HspL was present in the insol-
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HspL protein was tightly associated with membranes, because it was not solubilized by high salt concentrations (1 M NaCl or 0.1% Na₂CO₃) or mild nonionic detergent (1% Triton X-100). Only strong denaturant (6 M urea) or ionic detergent (1% SDS) succeeded in solubilizing the chaperone (Fig. 4B). Further analysis of the membrane fractions by sucrose gradient separation revealed that HspL localized primarily in the inner membrane but also associated with the outer membrane (Fig. 4C). The presence of HspL in both inner and outer membranes provoked us to determine whether HspL is exposed across the inner membrane. We converted agrobacterial cells to spheroplasts and performed protease sensitivity assays. We observed the complete digestion of HspL without traces of truncated products, and this suggested that membrane-associated HspL is exposed toward the periplasm (Fig. 4D). As controls, cytoplasmic GroEL and the integral inner membrane VirB2 proteins were analyzed and shown to be resistant to protease digestion, whereas the bitopic inner membrane VirB8 was amenable to proteolytic cleavage, resulting in truncated products.

HspL Co-fractionates with VirB Complexes and Interacts with VirB8, a Key T4SS Assembly Factor—HspL is predicted to be a soluble protein without signal peptide or transmembrane domain (23), and therefore, its co-fractionation with membranes may be due to its association with membrane-associated VirB protein complexes. To assess this possibility, we extracted membrane proteins with the mild detergent DDM and then size-fractionated the DDM-extracted proteins by SEC (Fig. 5A). The eluted fractions were analyzed by Western blot with VirB10 as a marker of high molecular mass T4SS core components, VirB2 as a marker of low molecular mass pilus assembly complex components, and VirB8 as a marker present in both subassemblies (45, 46). HspL was detected in fractions across both T4SS subassemblies (Fig. 5A), raising the possibility that HspL may associate with certain VirB proteins such as VirB8 and VirB2 based on their largely overlapped eluted fractions. To test this possibility, we expressed HspL-His driven by its native promoter in the hspL deletion mutant under AS-inducing conditions, collected the DDM-extracted membrane
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![Graph](image)

**DISCUSSION**

The chaperone activity of α-Hsps is usually analyzed by testing their ability to prevent aggregation of thermally denatured artificial model substrates *in vitro*. In most cases it remains an open question of whether the *in vitro* chaperone activity reflects the respective biological function. In this study we provided strong evidence that the virulence-induced *A. tumefaciens* small heat-shock protein HspL is a VirB8 chaperone. The correlation between reduced chaperone activity of two HspL variants and the effects on T4SS-mediated DNA transfer and virulence of *A. tumefaciens* provided a direct link between *in vitro* and *in vivo* functions. Our study also revealed the first example of a small heat-shock protein as a molecular chaperone involved in T4SS functions and bacterial virulence.
Similar to many α-Hsps including HspH of Bradyrhizobium japonicum (41), HspL oligomerizes in a concentration-dependent manner ranging from appropriately 12 to 18 subunits in vitro (Fig. 1B). Two available α-Hsp crystal structures, MjHsp16.5 from the archaeon Methanococcus jannaschii (47) and TaHsp16.9 from wheat (48), both revealed a hollow, foot-ball-like 24-mer structure of native α-Hsp. Thus, it is possible that HspL may form a spherical structure yet to be determined.

Two HspL protein variants with amino acid substitutions at specific amino acids in the conserved α-crystallin domain (HspLF98A and HspLG118A) had reduced chaperone activity (Figs. 2A and 6), and the oligomeric states were altered (Fig. 2B). We noticed that all SEC analysis resulted in two major peaks, the first corresponding to the void volume, and both peaks contained HspL proteins (Fig. 2B and supplemental Fig. S1), but those of HspL and HspLG118A in the void volume represent only small amounts and do not likely represent a significant fraction. Although the majority of wild type HspL forms the 264-kDa oligomer, HspLF98A eluted as a smaller 130-kDa oligomer and in the void volume (Fig. 2B). HspL and its variants were purified as soluble proteins, and the majority remained soluble after heat treatment (supplemental Fig. S3). Therefore, the significant amounts of HspLF98A eluted in the void volume, and earlier fractions likely represent a soluble complex larger than 669 kDa; the largest protein marker could be resolved by Superdex 200 (GE Healthcare).

The absence of chaperone activity of the HspLF98A is consistent with respective HspH F94A (or F94D) protein of B. ja-
Although HspH G114A in *B. japonicum* caused a complete loss of chaperone activity, the HspL G118A still retained partial chaperone activity, similar with that observed in the respective Hsp16.6 G124A protein in *Synechocystis* (9). The importance of G118 for proper oligomerization is consistent with critical roles of equivalent *Synechocystis* Hsp16.6 G124 and *B. japonicum* HspH G114 in maintaining oligomers (9, 41). The HspL F98A protein formed both larger and smaller oligomers (Fig. 2B) but did not render the protein monomeric (19-kDa) like its counterparts, HspH F94A or F84D in *B. japonicum* (41), or form dimers (38 Da), like the equivalent Hsp16.6 F102A in *Synechocystis* (9). The somewhat distinct functions of conserved amino acids in different α-Hsps in dimerization or oligomerization imply that different α-Hsps may not fold into oligomers in the same manner. Nevertheless, our results clearly indicate that the amino acid residues Phe-98 and Gly-118 of HspL are critical for maintaining proper oligomerization (Fig. 2B).

The lost or reduced chaperone activity observed in case of the two HspL variants (F98A and G118A) (Figs. 2A and 6) correlated with the loss or reduction of their ability in promoting T4SS-mediated RSF1010 transfer between agrobacteria and tumorigenesis (Fig. 3). The link between *in vitro* and *in vivo* functions has also been observed for the equivalent Hsp16.6 F102A and G124A that resulted in loss and severe reduction in thermotolerance of *Synechocystis*, respectively (9). Basha *et al.* (49) showed that Hsp16.6 interacts with several heat-labile proteins during heat stress *in vivo*, and these substrates could be released by ATP-dependent chaperones *in vitro*. Importantly, this work also demonstrated that Hsp16.6 interacts with serine esterase, one of the identified substrates, and protects it from aggregation in a heat-dependent manner *in vitro*. Consistent
with its induction by the virulence gene inducer AS (and not heat), we here demonstrate that HspL interacts with its substrate VirB8 in a large complex in A. tumefaciens (Fig. 5, A and B). Moreover, we provided direct evidence that HspL is a VirB8 chaperone in protecting it from aggregation in vitro (Fig. 6). This argument is further supported by the observation that more soluble VirB8 was detected when VirB8 was co-expressed with HspL-His than when it was expressed alone in E. coli (Fig. 5, C and D), suggesting that HspL protects VirB8 from aggregation in vivo.

The identification of HspL tightly associated with the membrane and exposed to the periplasm (Fig. 4) suggested that HspL may interact with membrane lipids and/or with membrane proteins such as VirB8. Despite the lack of any obvious transmembrane domain or Sec-dependent signal peptide, HspL was found to interact with many VirB proteins, including VirB2 and VirB9 and Ban-Yang Chang for GroEL antibody. We also thank Chan Gao and Sonja Klüsener for assistance at the initial stage of this work and the Lai laboratory members for discussion and reading the manuscript.

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