The Helix-Loop-Helix Motif at the N Terminus of HalI Is Essential for Its Immunity Function against Halocin C8^{▽†}

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Halocin C8 (HalC8) is a stable microhalocin exhibiting strong antimicrobial activity against a wide range of haloarchaea. HalI, a 207-amino-acid peptide derived from the N terminus of the HalC8 preproprotein, is the immunity protein of HalC8. In this study, the molecular mechanism of the immunity function of HalI was investigated. Both pull-down and surface plasmon resonance assays revealed that HalI directly interacted with HalC8, and a mixture of purified HalI and HalC8 readily formed a heterocomplex, which was verified by gel filtration. Interestingly, HalC8 tended to form a self-associated complex, and one immunity protein likely sequestered multiple halocins. Significantly, the helix-loop-helix (HLH) motif containing a 4-amino-acid repeat (RELA) at the N terminus of HalI played a key role in its immunity activity. Disruption of the HLH motif or mutagenesis of the key residues of the RELA repeat resulted in loss of both the immunity function and the ability of HalI to bind to HalC8. These results demonstrated that HalI sequestered the activity of HalC8 through specific and direct binding.

Halocins are proteinaceous antibiotics that are ribosomally synthesized by extremely halophilic archaea and excreted into the environment to kill or inhibit other haloarchaeal cells (15). Halocins were first discovered in 1982 (23) and later were found to be produced by most rod-shaped haloarchaea (12, 32). So far, several halocins have been purified and characterized (7, 11, 15, 17, 31), and three halocin genes coding for H4 (*halH4*), S8 (*halS8*), and C8 (*proC8*) have been cloned (2, 19, 29). Although the amino acid sequences of these halocins are quite diverse, the halocins share many features. For example, halocins are usually produced upon transition from the exponential to stationary phase and are processed from larger precursor proteins (2, 19, 29). In addition, halocins appear to be exported by a twin-arginine translocation (Tat) pathway (24, 29), as their precursors usually contain a Tat signal at their N termini (2, 19, 29). Accordingly, halocin has been considered a good model for examining gene expression regulation and protein processing and transportation in haloarchaea.

HalC8 is an extremely stable microhalocin with a wide inhibition spectrum (7). The HalC8 gene encodes a 283-amino-acid preproprotein (ProC8), which is processed into two functional peptides, the C-terminal peptide antibiotic HalC8 (76 amino acids) and the N-terminal immunity protein HalI (29). As far as we know, HalI is the only halocin immunity protein that has been characterized. It is likely oriented toward the

outside of the cellular membrane and hence inhibits HalC8 by sequestration (29). However, the molecular details of the interaction between HalI and HalC8 have not been elucidated yet. In this study, we demonstrated that HalI blocked HalC8 activity by direct interaction with HalC8. The motif and residues of HalI required for this functional interaction were also determined.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* DH5 α (Clontech) and *E. coli* BL21(DE3) (Novagen) were grown in LB medium (25) at 37°C. The halophilic archaeon *Natrinema* sp. strain AS7092 (formerly *Halobacterium* sp. strain AS7092, which was renamed because its 16S rRNA gene [accession number AY899297] exhibits 99.1% identity with that of the newly described organism *Natrinema altunense* [accession number AY208972]) (7, 34), *Haloarubrum saccharovorum* ATCC 29252, and *Haloferax volcanii* DS70 (33) were cultivated at 37°C in AS-169 or AS-168 medium as described previously (7). When needed, kanamycin, ampicillin, or mevinolin was added to a final concentration of 50, 100, or 3 μ g/ml for selection.

Mutagenesis, plasmid construction, and protein expression. The plasmids used in this study are listed in Table 1, and the primers used are listed in Tables S1 and S2 in the supplemental material.

For expression of the HalI protein and mutant proteins with an N-terminal polyhistidine tag (His₆), the pET28a system (Table 1) was used. Briefly, the coding sequence of HalI was amplified with primers HalIF and HisHalI28R (see Table S1 in the supplemental material) and cloned into the expression vector pET28a, resulting in pET-HalI (Table 1), which was used to express His₆-HalI in *E. coli*. Similarly, primers HalI41F, HalI61F, and HalI66F combined with primer HisHalI28R (see Table S1 in the supplemental material) were used for construction of the expression plasmids pET-HalI Δ N41, pET-HalI Δ N61, and pET-HalI Δ N66, respectively, which were used to express N-terminally truncated HalI mutants (Table 1). In the same way, primers HalI180R, HalI170R, HalI150R, and HalI120R combined with primer HalIF (see Table S1 in the supplemental material) were used to construct the expression plasmids pET-HalI Δ C180, pET-HalI Δ C170, pET-HalI Δ C150, and pET-HalI Δ C120, respectively, which were used to express C-terminally truncated HalI mutants (Table 1). To search for the functional motifs and the key amino acids in HalI, site-directed mutagenesis of *halI* was performed as previously described (6). Briefly, pET-HalI containing the *halI* sequence was used as the template. Primers shown in Table S2 in the supplemental material were designed to introduce desired mutations into pET-HalI with KOD-plus DNA polymerase (Toyobo, Japan). The parental template DNA and the newly synthesized mutagenesis-primer-containing DNA were

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TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
pET28a	5.4 kb, isopropyl- β -D-thiogalactopyranoside-inducible expression vector with His ₆ tag	Novagen
pET-Hall	pET28a-derived expression plasmid for Hall (207 amino acids) with an N-terminal His ₆ tag (His ₆ -Hall)	This study
pET-Hall Δ N41	pET28a-derived expression plasmid for Hall Δ N41 (amino acids 41 to 207 of Hall)	This study
pET-Hall Δ N61	pET28a-derived expression plasmid for Hall Δ N61 (amino acids 61 to 207 of Hall)	This study
pET-Hall Δ N66	pET28a-derived expression plasmid for Hall Δ N66 (amino acids 66 to 207 of Hall)	This study
pET-Hall Δ C180	pET28a-derived expression plasmid for Hall Δ C180 (amino acids 1 to 180 of Hall)	This study
pET-Hall Δ C170	pET28a-derived expression plasmid for Hall Δ C170 (amino acids 1 to 170 of Hall)	This study
pET-Hall Δ C150	pET28a-derived expression plasmid for Hall Δ C150 (amino acids 1 to 150 of Hall)	This study
pET-Hall Δ C120	pET28a-derived expression plasmid for Hall Δ C120 (amino acids 1 to 120 of Hall)	This study
pET-H1m	pET-Hall-derived expression plasmid for H1m (RELA at amino acids 62 to 65 of Hall changed to PGPG)	This study
pET-H2m	pET-Hall-derived expression plasmid for H2m (RELA at amino acids 71 to 74 of Hall changed to PGPG)	This study
pET-HLHm	pET-Hall-derived expression plasmid for HLHm (RELA at amino acids 65 to 62 and RELA at amino acids 71 to 74 of Hall changed to PGPG)	This study
pET-H3m	pET-Hall-derived expression plasmid for H3m (DFAR at amino acids 100 to 103 of Hall changed to GPGP)	This study
pET-H4m	pET-Hall-derived expression plasmid for H4m (AIRE at amino acids 183 to 186 of Hall changed to GPGG)	This study
pGEX-4T-1	4.9 kb, isopropyl- β -D-thiogalactopyranoside-inducible expression vector for GST fusion	Pharmacia
pGEX-Hall	pGEX-4T-1-derived expression plasmid for the Hall protein (207 amino acids) with an N-terminal GST tag (GST-Hall)	This study
pET26b	5.4 kb, isopropyl- β -D-thiogalactopyranoside-inducible excreted-expression vector with His ₆ tag and PelB leader	Novagen
pET26-HalC8	pET26b-derived expression plasmid for HalC8 (76 amino acids) with a C-terminal His ₆ tag (HalC8-His ₆)	This study
pWL102	10.5 kb, shuttle vector, Amp ^r Mev ^r	5
pWL-Hall	pWL102-derived expression plasmid with Hall coding sequence under a haloarchaeal promoter	This study
pWL-HallH1m	Coding sequence of Hall in pWL-Hall replaced by that of H1m	This study
pWL-HallH2m	Coding sequence of Hall in pWL-Hall replaced by that of H2m	This study
pWL-HallHLHm	Coding sequence of Hall in pWL-Hall replaced by that of HLHm	This study

treated with DpnI and then introduced into *E. coli*. In this way, the pET-Hall-derived plasmids pET-H1m, pET-H2m, pET-HLHm, pET-H3m, and pET-H4m with specific mutations in the corresponding helix motifs of Hall (Table 1) were obtained. Fifteen other plasmids expressing Hall mutants, designated A61D, R62A, E63A, L64A, A65P, K66A, T67A, P68A, A69V, F70A, R71A, E72A, L73A, A74V, and Q75A (see Table S2 in the supplemental material), with a point mutation for each amino acid (from residue 61 to residue 75) in the helix-loop-helix (HLH) motif, were also constructed. All these plasmids were constructed in *E. coli* DH5 α , verified by DNA sequencing, and then transformed into *E. coli* BL21(DE3) (Novagen) for protein expression. Overexpressed His₆-tagged Hall and Hall mutants were purified on Ni-nitrilotriacetic acid (NTA) columns used according to the manufacturer's instructions (Novagen). The concentrations of the purified proteins (purity, >95%) were determined with a bicinchoninic acid protein assay kit (Pierce).

To express Hall and its variants in haloarchaea, the *hall* gene and corresponding mutant genes were amplified from plasmids pET-Hall, pET-H1m, pET-H2m, and pET-HLHm (Table 1) with primers PrF and HallIR (see Table S1 in the supplemental material) and cloned into the shuttle vector pWL102 (5), resulting in plasmids pWL-Hall, pWL-HallH1m, pWL-HallH2m, and pWL-HallHLHm (Table 1), respectively. These plasmids were transformed into *H. volcanii* DS70 as described by Cline et al. (3). All the PCR-amplified DNA sequences were confirmed by DNA sequencing.

Pull-down assay. To detect the direct interaction between HalC8 and Hall, plasmid pET26-HalC8 (Table 1) containing the *halC8* sequence (amplified with primers HisC8F and HisC8R [see Table S1 in the supplemental material]) was used to express the HalC8 protein with a C-terminal polyhistidine tag (HalC8-His₆) in *E. coli* BL21(DE3), while the Hall protein with an N-terminal glutathione *S*-transferase (GST) tag (GST-Hall) was expressed by pGEX-Hall (Table 1), which was constructed by cloning the *hall* sequence (amplified with primers HallIF and GST-HallIR [see Table S1 in the supplemental material]) into pGEX-4T-1 (Table 1). For the pull-down assay, purified HalC8-His₆ (as the bait) was mixed with Ni-NTA beads (Novagen) and then washed three times with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole; pH 8.0). After this,

cell lysates of BL21(DE3)/pGEX4T-1 and BL21(DE3)/pGEX-Hall cultures containing overexpressed GST and GST-Hall, respectively, were incubated with HalC8-His₆-immobilized Ni-NTA beads at room temperature for 1 to 2 h. After three washes with the washing buffer, the bound proteins on the Ni-NTA beads were eluted by using elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole; pH 8.0), separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and visualized by immunoblotting with antiserum against GST-Hall as described previously (29).

SPR assay. The surface plasmon resonance (SPR) assay was carried out by using a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden) (16). For immobilization of Hall Δ N41 on a CM5 chip (BIAcore AB, Uppsala, Sweden), the chip surface was first activated with 35 μ l of a 1:1 mixture of *N*-hydroxysuccinimide (0.1 M) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (0.4 M), and then Hall Δ N41 (5 to 20 μ l; 50 μ g/ml in 10 mM sodium acetate buffer [pH 4.0]) was injected and immobilized to obtain 900 resonance units. After this, the remaining active groups were blocked with 35 μ l of ethanolamine (1 M, pH 8.5). Throughout this immobilization procedure, the flow rate was kept at 5 μ l/min. For detection of the interaction between Hall Δ N41 and HalC8, natural HalC8 protein purified from the culture medium of the halophilic archaeon *Natrinema* sp. strain AS7092 (7) was diluted in HBS buffer (10 mM HEPES [pH 7.5], 150 mM NaCl, 3 mM EDTA, 0.005% [vol/vol] surfactant P20) to obtain different concentrations (5 to 100 μ M) and injected with the K-inject command. At the end of each cycle, 50 mM NaOH was used as regeneration buffer to remove the bound HalC8. A blank flow cell was used as a control for each concentration of HallC8. HBS buffer was used as the running buffer throughout the binding analysis at a flow rate of 30 μ l/min.

Gel filtration chromatography and electrophoresis. For examination of the Hall-HalC8 interaction complex, gel filtration chromatography was performed. Samples (100 μ l) of purified Hall Δ N41 protein from *E. coli*, purified natural HalC8 from *Natrinema* sp. strain AS7092, and a mixture of both these proteins (after incubation at 37°C for 2 to 4 h) were subjected to analytical gel filtration chromatography on a Superdex 200 10/300 GL column (GE Healthcare), which was equilibrated and eluted with elution buffer (50 mM Tris-HCl, 150 mM NaCl;

FIG. 1. Pull-down assay of HalC8-His₆ and GST-HalI. Lysates from *E. coli* cells expressing GST-HalI (pGEX-HalI) and GST (pGEX-4T-1) were incubated with HalC8-His₆-immobilized Ni-NTA beads. The bound HalC8-His₆ (lane 1), HalC8-His₆ incubated with GST-HalI (lane 2), and GST (lane 3) were separated by SDS-PAGE and immunoblotted with anti-GST-HalI antibody. Purified GST-HalI (lane 4) and GST (lane 5) were used as positive controls.

pH 8.0). The columns used in this study were calibrated by using a set of gel filtration markers (Sigma), including β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa), and horse cytochrome *c* (12.4 kDa). In all gel filtration experiments, the flow rate was 0.5 ml/min, and samples were collected by using 1 ml/fraction. The eluted samples of Hal Δ N41 or the Hal Δ N41-HalC8 mixture were analyzed by Western blot analysis with anti-GST-HalI polyclonal antibodies.

For investigation of the possible oligomerization of HalI, purified Hal Δ N41 was divided into two aliquots. One aliquot was mixed with an equal volume of 2 $\times$ denaturing buffer (100 mM Tris-HCl, 400 mM dithiothreitol, 4% SDS, 20% glycerol, 0.2% bromophenol blue; pH 6.8) and boiled for 10 min, while the other aliquot was mixed with native sample buffer (100 mM Tris-HCl, 20% glycerol, 0.2% bromophenol blue) and kept on ice before loading. Both aliquots were subjected to 5 to 15% gradient native PAGE (14) with Tris-glycine buffer (pH 8.8).

To determine the oligomerization of HalC8, the active fractions of natural HalC8 eluted from a Superdex 75 10/300 GL column (GE Healthcare) were concentrated and split into two aliquots. One aliquot was denatured as described above for Hal Δ N41, while the other aliquot was not treated. Both aliquots were subjected to Tricine-SDS-PAGE as previously described (26).

Evaluation of the immunity function of HalI and HalI mutants. The microtiter plate assay (7) with *H. saccharovorum* cells as the indicator was used to evaluate the immunity function of HalI and HalI mutants against HalC8. Briefly, natural HalC8 (10 nM) was mixed with the same volume of purified His₆-HalI or His₆-HalI mutants, which were diluted in 50 mM Tris-HCl (pH 8.0) using the double-dilution method and the same starting concentration (1 μ M). The mixtures were loaded into the wells in an indicator plate and incubated at 37°C for 2 to 3 days. The highest dilution of HalI or a HalI mutant that exhibited inhibition activity against HalC8 was considered the relative activity of the immunity function.

Evaluation of the binding affinity of HalI mutants. An enzyme-linked immunosorbent assay (ELISA) was used to evaluate the binding affinity of HalI mutants, as described by Quadri et al. (20). Briefly, a 96-well ELISA plate was first coated with purified natural HalC8 (10 μ g/well, diluted in 0.05 M NaHCO₃ [pH 9.6]) and blocked with 0.5% bovine serum albumin (diluted in phosphate buffer [pH 7.4]). Subsequently, 200 μ l of a 1.5 μ M solution of His₆-HalI or a His₆-HalI mutant was added to each well and incubated for 2 h at room temperature. Then anti-GST-HalI antibody and horseradish peroxidase-conjugated anti-immunoglobulin G antibody were added sequentially, with incubation for 2 h for each step. The plate was always washed three times with phosphate buffer (pH 7.4) before reagents were changed. After this, a substrate solution containing tetramethyl benzidine (Tiangen, People's Republic of China) was added and incubated at 37°C for 10 to 15 min; then the reaction was terminated by using 2 M H₂SO₄, and the absorbance at 450 nm was determined with a plate reader. The binding activities of HalI and the mutants were evaluated by determining the absorption at 450 nm after the absorption of the bovine serum albumin negative control was subtracted; the binding activity of HalI was arbitrarily defined as 100%.

Heterologous expression of HalI and HalI mutants in haloarchaea. The expression plasmids pWL-HalI, pWL-HalIH1m, pWL-HalIH2m, and pWL-

FIG. 2. SPR assay of HalC8 binding to HalI. The binding affinity of natural HalC8 with Hal Δ N41 (amino acids 41 to 207 of HalI with complete immunity activity) is expressed in reference-corrected resonance units (RU), and the equilibrium dissociation constant (K_D) is also shown. The sensorgrams were obtained with different concentrations of protein HalC8 (0, 5, 10, 25, 50, and 100 μ M).

HalIHLHm (Table 1) were transformed into *H. volcanii* DS70. For detection of the expression of HalI and HalI mutants, the *H. volcanii* DS70 transformants were collected after they entered stationary phase and subjected to Western blot analysis as described previously (29). For the HalC8 sensitivity assay, the *H. volcanii* DS70 transformants were streaked onto an assay plate containing 3 μ g/ml mevinolin and 50 nM natural HalC8. A mevinolin-containing plate without HalC8 was used as a negative control. The plates were incubated at 37°C for up to 1 week.

RESULTS

HalI interacts with HalC8 through direct binding. Our previous investigation revealed that HalC8 is likely intercepted, but not destroyed, when it is inhibited by the immunity protein HalI (29). However, the molecular details of the interaction between HalI and HalC8 remain unclear. To further investigate this interaction, both HalC8-His₆ and GST-HalI were expressed in *E. coli* and used in a pull-down assay. Purified HalC8-His₆ protein was immobilized on Ni-NTA beads and then incubated with cell lysates containing similar amounts of GST-HalI or GST (as a negative control). The bound proteins were identified by a Western blot assay with the anti-GST-HalI antibody. As shown in Fig. 1, GST-HalI, but not GST, was specifically pulled down by the HalC8-His₆ protein. This result indicated that HalI likely interacted with HalC8 through direct and specific binding.

To confirm this, the interaction between purified natural HalC8 and the HalI peptide without its signal sequence (designated Hal Δ N41), which had complete HalI activity, was analyzed by the SPR assay. The Hal Δ N41 peptide was immobilized on the CM5 sensor chip, and different concentrations of purified natural HalC8 (isolated from *Natrinema* sp. strain AS7092) were injected. After subtraction of the blank cell signals, the signals specific for binding of HalC8 on Hal Δ N41 were clearly observed (Fig. 2). The SPR data were further analyzed by using the BIAEVAL software, and the equilibrium dissociation constant was calculated to be about 1.2 μ M, indicating that the interaction between HalI and HalC8 in vitro was a moderate-affinity interaction.

HalI and HalC8 form a large heterocomplex in vitro. Although HalC8 might directly bind to HalI, it is likely that an

FIG. 3. Characterization of the HalI and HalC8 heterocomplex. (A) Gel filtration chromatography of HalIAN41, HalC8, and the HalIAN41-HalC8 complex. The molecular masses of the calibration proteins (12.4 kDa to 200 kDa) are indicated at the top. (B and C) Western blots (WB) for HalIAN41 for eluted collections of HalIAN41 (B) and HalIAN41-HalC8 (C) samples. The fraction numbers are indicated at the bottom. (D) Native PAGE and Coomassie brilliant blue (CBB) staining of HalIAN41. HalIAN41 was denatured with dithiothreitol (200 mM) and heated in boiling water for 10 min (lanes 1, 2, and 3, 12, 8, and 4 μ g, respectively) or not denatured and boiled (lanes 4, 5, and 6, 12, 8, and 4 μ g, respectively). Lane M contained the Brilliant Blue Plus prestained protein standard (TransGen, People's Republic of China), and the molecular masses are indicated on the left.

unexpected large heterocomplex between these molecules was formed, as the resulting interaction complex was not able to enter a native gel when an attempt to collect the complex was made (29). To obtain more information about this interaction, gel filtration chromatography was performed to identify the interaction complex. To do this, purified HalIAN41 was incubated with excess natural HalC8 at 37°C for 2 to 4 h and then subjected to gel filtration chromatography. Significantly, the HalIAN41-HalC8 heterocomplex eluted as a single peak, which formed only after HalC8 and HalIAN41 were mixed. However, its molecular mass was more than 200 kDa as estimated by gel filtration chromatography (Fig. 3A), which is much greater than the predicted molecular mass (31 kDa) of a heterocomplex consisting of a 1:1 mixture of single molecules

FIG. 4. Characterization of the oligomerization of HalC8. (A) Natural HalC8 was purified using a Superdex 75 size exclusion column. The molecular masses of the calibration proteins (12.4 to 66 kDa) are indicated at the top. mAU, milli-absorbance units. (Inset) Tricine-SDS-PAGE (28) of denatured (lane 1) and nondenatured (lane 2) HalC8 with activity. Lane M contained markers. (B) Activity analysis of eluted HalC8. Samples (50 μ l) taken from each fraction were loaded into the wells of an indicator plate. The fraction numbers are indicated at the bottom.

of HalIAN41 (23.5 kDa) and natural HalC8 (7.4 kDa). The identities of the HalIAN41 peptide and the constituents of the HalIAN41-HalC8 complex in the eluted collections were confirmed by Western blot analysis with the anti-GST-HalI antibody (Fig. 3B and C).

To understand why the molecular mass of the complex was much greater than predicted, we analyzed purified HalIAN41 and natural HalC8. HalIAN41 eluted as a single peak with an estimated molecular mass of about 50 kDa, which is greater than the predicted molecular mass, 23.5 kDa (Fig. 3A). It is likely that this functional region of HalI has a tendency to form a dimer. However, analysis by native gradient PAGE indicated that HalI did not form oligomers, as the molecular weight of native HalIAN41 was the same as that of denatured HalIAN41 (Fig. 3D). Hence, the higher apparent molecular weight of HalI deduced from gel filtration chromatography was likely due to its specific molecular shape (e.g., chainlike structure) and/or amino acid features (e.g., strong hydration caused by the acidic residues in HalI). Interestingly, while the molecular mass of HalC8 is 7.4 kDa (29), the samples of HalC8 eluted in gel filtration chromatography were a main peak corresponding to a molecular mass of about 23 kDa and a minor peak corresponding to a molecular mass of about 45 kDa (Fig. 3A and 4A), which were likely a trimer and a hexamer of HalC8, respectively. To confirm this, the active fractions (Fig. 4B) were concentrated and split into two aliquots. One aliquot was denatured, and the other aliquot was not denatured; both aliquots were subjected to Tricine-SDS-PAGE. The molecular

FIG. 5. Activities of HalI and HalI mutants. (A) Immunity activities of HalI and HalI truncated mutants. The protein regions of HalI and HalI truncated mutants are indicated by solid lines, and the helix motifs (cylinders) are indicated at the top (not to scale). The immunity activities of HalI and the truncated mutants are indicated on the right. (B) Amino acid sequences of the four helices (boxes). The amino acids subjected to mutation are indicated by shading. (C) Immunity and binding activities of HalI and HalI mutants. The light gray bars indicate the immunity activity against HalC8, and the dark gray bars indicate the binding activity against HalC8. The mutants with single-amino-acid mutations in the HLH motif of HalI are underlined.

mass of the denatured HalC8 was indeed found to be about 7.4 kDa, while the molecular mass of the nondenatured form of HalC8 appeared to be 23 kDa (Fig. 4A, inset), as revealed by gel filtration chromatography. However, the 45-kDa band was not detectable, likely due to a low protein concentration for the minor peak. These results demonstrated that HalC8 had a strong tendency to form self-associated oligomers. Therefore, the large HalIAN41-HalC8 heterocomplex may be due to oligomerization of HalC8, which might associate with more HalIAN41 molecules.

The HLH motif of HalI is essential for the immunity function. To search the putative active domain of HalI, the amino acid sequence of HalI was analyzed by using the PredictProtein program (<http://www.predictprotein.org/>). It was found that there were four helices (Fig. 5A) in the immunity function region of HalI (amino acids 41 to 207) downstream of the putative signal sequence (amino acids 1 to 40). The first two helices constituted a typical HLH motif at the N terminus of HalI, and there was a 4-amino-acid repeat (RELA) in these two helices (Fig. 5B). The other two helices were located in the middle (amino acids 98 to 112) and at the C terminus (amino acids 180 to 188) (Fig. 5A and B).

To investigate the function of these helices in the immunity activity of HalI, we constructed a series of truncated HalI mutants by deletion from the N-terminal and C-terminal regions. As shown in Fig. 5A, the HalIAN41 truncated mutant without the signal sequence (amino acids 1 to 40) and HalIAN61 without amino acids 1 to 60 showed complete immunity activity *in vitro*. However, when amino acids 61 to 65 were also deleted, the resultant mutant, HalIAN66, exhibited no immunity activity. Since the sequence from amino

acid 62 to amino acid 65 is one unit of the RELA repeat in the HLH motif (Fig. 5B), we presumed that the repeat or the HLH motif was probably important for HalI activity. In contrast to the N terminus, the C terminus likely has no key section for HalI activity (Fig. 5A). HalIAC180 without amino acids 181 to 207, including helix 4 (residues 180 to 188), showed complete immunity activity. From HalIAC170 to HalIAC120, the immunity activity was gradually reduced along with the C-terminal deletion. The C terminus of HalI is rich in acidic residues, which are thought to be important for proteins to adapt to high-salt conditions (4, 8, 9, 13). Thus, the C terminus of HalI probably makes the structure more stable at high salt concentrations.

To confirm this hypothesis, we constructed several mutants with mutations in these helices (designated H1m, H2m, H3m, H4m, and HLHm) by using site-directed mutagenesis (see Table S2 in the supplemental material). Significantly, once the HLH motif at the N terminus of HalI was disrupted (H1m, H2m, or HLHm), there was little or no immunity activity of the mutant. In contrast, the activities of helix 3 and helix 4 mutants (H3m and H4m) were the same as the activity of wild-type HalI (Fig. 5C). These results supported our hypothesis that the HLH motif is essential for the HalI immunity function.

To determine which amino acid in the HLH motif plays a key role in the immunity function of HalI, we constructed HalI mutants with single-amino-acid mutations at amino acids 61 to 75 (see Table S2 in the supplemental material). The activities of the L64A and L73A mutants were much lower than that of wild-type HalI (Fig. 5C), indicating that the L64 and L73 res-

idues in the RELA repeat (Fig. 5B) play a very important role in the immunity activity of HalI.

Direct binding of HalI to HalC8 is required for the immunity function. To establish the relationship between the immunity function and the binding ability of HalI, a modified ELISA was performed to quantify the binding affinity of HalI mutants with HalC8. After HalC8 was coated in the ELISA plate, HalI and mutants H1m, H2m, H3m, H4m, HLHm, A61D, R62A, E63A, L64A, A65P, K66A, T67A, P68A, A69V, F70A, R71A, E72A, L73A, A74V, and Q75A (see Table S2 in the supplemental material) were incubated with HalC8. The absorption for each ELISA reaction representing binding activity of HalI or a HalI mutant was determined. The binding activity of HalI was arbitrarily defined as 100%, and the activities of the mutants were expressed as percentages of the HalI activity, as shown in Fig. 5C. The binding activities of mutants H1m, H2m, L64A, and L73A were much lower than that of wild-type HalI, consistent with their immunity activities. Most significantly, the binding activity of mutant HLHm was hardly detectable, which was verified by SPR and gel filtration chromatography (data not shown), and the immunity function was completely lost (Fig. 5C). These results demonstrated that the binding ability was directly correlated with the immunity function of HalI and further confirmed that HalI sequestered HalC8 activity by direct binding.

The HLH motif is necessary for the immunity function of HalI in vivo. To determine whether the HLH motif is important for the biological function of HalI in vivo, the expression plasmids pWL-HalI, pWL-HalIH1m, pWL-HalIH2m, and pWL-HalIHLHm (Table 1) were transformed into a HalC8-sensitive haloarchaeon, *H. volcanii* DS70. Western blot analysis revealed that both HalI and HalI mutants were successfully expressed in the transformants, and the expression levels were almost the same (Fig. 6A). After culturing for up to 1 week in plates, all these strains grew very well on the control plate with 3 μ g/ml mevinolin but without HalC8 (Fig. 6B, left panel), and DS70/pWL-HalI also grew well on the assay plate containing 3 μ g/ml mevinolin and 50 nM HalC8 (Fig. 6B, right panel). In contrast, mutants DS70/pWL-HalIH1m and DS70/pWL-HalIHLHm were not able to grow, and DS70/pWL-HalIH2m exhibited only poor growth on the assay plate (Fig. 6B, right panel). These results showed that the HLH motif was indeed essential for the immunity function of HalI, which could provide the HalC8-sensitive haloarchaeal strain with HalC8 resistance.

DISCUSSION

Halocin production is a practically universal feature of most haloarchaeal rods (32), like bacteriocins produced in the domain *Bacteria* (22). However, the halocin immunity protein has been identified only for HalC8 (29). This immunity protein, designated HalI, is localized in cytoplasmic membrane of haloarchaeal strain AS7092, the producer of HalC8 (29). The immunity function of HalI against HalC8 is specific and efficient, but the underlying mechanism of this immunity function is not clear. In the present study, we found that HalI directly binds to HalC8 and forms a stable heterocomplex, inhibiting HalC8 from approaching and killing the producer cells. We also demonstrated that the HLH motif at the N terminus of HalI plays

FIG. 6. Immunity function analyses of HalI and HalI mutants expressed in *H. volcanii* DS70. (A) Western blot analysis. The cellular proteins (200 μ g) from DS70/pWL-HalIH1m, DS70/pWL-HalIH2m, DS70/pWL-HalIHLHm, DS70/pWL-HalI, and DS70/pWL102 (negative control) cells were analyzed by SDS-PAGE and visualized by immunoblotting with anti-GST-HalI antibodies. HalIAN41 protein expressed in *E. coli* was directly loaded as a positive control. (B) Comparison of the immunity activities of HalI and HalI mutants expressed in *H. volcanii* DS70. Cells of DS70/pWL-HalIH2m (region 1), DS70/pWL-HalIH1m (region 2), DS70/pWL-HalIHLHm (region 3), and DS70/pWL-HalI (region 4) were cultured on an assay plate containing 3 μ g/ml mevinolin and 50 nM HalC8 (right panel), and a mevinolin-containing plate without HalC8 was used as a negative control (left panel).

a key role in both the binding ability and the immunity function.

This immunity mechanism for HalI against HalC8 involving specific binding resembles that of the bacteriocin immunity protein NisI, which intercepts nisin at the surface of the cytoplasmic membrane (28). The equilibrium dissociation constant (1.2 μ M) for the binding interaction between HalI and HalC8 evaluated by the SPR assay was similar to that for the NisI-nisin interaction (range, 0.6 to 2 μ M) (30). The moderately strong binding may be helpful for releasing the captured peptide antibiotic into the environment again by some unknown mechanism. Interestingly, similar to genes observed in the nisin gene cluster, genes encoding a system homologous to an ABC transporter were found in the *halC8* gene cluster (10). The transporter system may also play an additional role in the halocin tolerance of the producer, like NisFEG, which seems to work by expelling cell-attached nisin molecules into the environment (28). It is reasonable to suggest that the HalC8 captured by HalI could be released into the environment with the help of the ABC transporter. However, the molecular details of the HalC8 released from HalI or cellular membrane remain to be elucidated.

Interestingly, the direct interaction between HalI and HalC8 likely involved the HLH motif of HalI. The HLH motifs common to several known transcription factors usually control the affinity of proteins for homo- or heterodimerization (1) and play an important role in some protein-protein interactions (18). Although HalIAN41 had a higher apparent molecular mass ($\sim$ 50 kDa) as determined by gel filtration chromatography (Fig. 3A), this molecular mass was likely not due to HLH-

directed dimerization, as the HLH mutants had the same apparent molecular weight as HalI Δ N41 (data not shown) and no HalI dimer was observed in the native gradient PAGE gel (Fig. 3D). In addition, Leu64 (in helix 1) and Leu73 (in helix 2) of the HLH motif at the N terminus of HalI played an important role in both the immunity and binding activities of HalI against HalC8 (Fig. 5C). As Leu64 and Leu73 are hydrophobic amino acids located in a hydrophobic region of the HLH motif, we believe that HalI probably interacts with HalC8 via this HLH motif due to the molecular force of the hydrophobic interaction, which most resembles the interaction between colicin E1 and its immunity protein (35).

It is noteworthy that the HalI-HalC8 complex was much larger than predicted. It seems likely that this interaction complex consists of multiple molecules of HalI and HalC8. As HalC8 tends to form oligomers, multiple molecules of HalC8 should be present in the HalI-HalC8 complex, and the oligomerized HalC8 might bind excessive molecules of HalI, forming a macromolecule heterocomplex. However, the interaction between HalI and HalC8 in vivo might be different. Because HalI is known to be anchored on the cell membrane of the producer, possibly through its Tat signal region, the interaction of HalI and HalC8 might occur at the two-dimensional surface of the outer membrane. The immobilized HalI could be bound by the oligomeric HalC8, but the redundant HalC8 might not bind to other HalI molecules. This is a highly efficient mechanism since one molecular immunity protein binds multiple molecules of halocin. As HalI and HalC8 are usually derived from ProC8 at a ratio of 1:1 (29), one molecule of HalI binding multiple molecules of HalC8 might protect the producer cell more efficiently.

This is the first report indicating that a halocin forms oligomers, which might be an important feature of some microhalocins. As observed for purification of HalC8 (7), halocins R1 (3.8 kDa) and S8 (3.6 kDa) also exhibit activities with both high-molecular-weight forms (>30 kDa) and low-molecular-weight forms (19, 21). Although the presence of a "carrier protein" for the larger forms of these halocins has been presumed (15), oligomerization might be an alternative explanation for these halocins. Oligomerization generally occurs for the pore-forming antimicrobial peptides (27, 36), which implies that oligomerization of HalC8 might be important for its function, and the mode of action of HalC8 is probably similar to the modes of action of the pore-forming antimicrobial peptides.

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