Characterization of DLP12 Prophage Membrane Associated Protein: HolinGFP

K. V. Srividhya · S. Krishnaswamy

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Abstract Lysis cassette genes from phages determine the final lytic event of the host cells. The lysis cassette genes are conserved in phages and prophages. The membrane associated holin from DLP12 prophage, available as a GFP fusion construct, was shown to be overexpressed, using confocal microscopy analysis, in bacterial cells. The protein expression caused cell death in *E. coli* AG1 strain suggesting the protein was functional. The His-tag HolinGFP protein was purified using cobalt affinity column and was eluted in the presence of different non-ionic detergents DDM (*n*-dodecyl- β -D-maltoside), LDAO (Lauryldimethylamine-oxide), OG (*n*-octyl β -D-glucopyranoside) and C₁₂E₉ (dodecyl nonaoxyethylene ether). HolinGFP existed predominantly as a dimer in LDAO in Superdex S200 gel filtration chromatography. Circular dichroism and fluorescence spectroscopy of the fluorescent HolinGFP in all four detergents (C₁₂E₉, DDM, LDAO, and OG) confirmed the folded state. Both dithiobis succinimidyl propionate and glutaraldehyde crosslinking revealed the existence of higher order oligomers and dimers. HolinGFP has been functionally and biophysically characterised and is being explored for crystallographic structure determination.

Keywords HolinGFP · DLP12 · Prophage · Oligomerisation · Crosslinking

Introduction

Holins are defined as simple “molecular clocks”, controlling host lysis at the end of vegetative phase [1]. These small membrane proteins allow translocation of endolysins across the membrane [2]. Holins belong to the α -type channels class of channels and pores family of transporters [3]. Holins have two membrane spanning α helical domains separated by β turn, a short hydrophilic turn and highly charged residues at C-terminus. They are grouped into three classes on the basis of primary sequence and based on the number of potential transmembrane domains. Lambda holin S (S^{λ}) has three transmembrane helices with N-out:C-in topology. By contrast S^{21} group, the class II holins have N-in:C-in membrane topology. The third class, T4 has a topology of N-in:C-out with one transmembrane domain [4–6].

S^{λ} is extensively studied in terms of genetic analysis and biochemical approaches [7–10]. Dual start motif and availability of two protein products namely S105 (holin) and S107 (anti-holin) are critical for S^{λ} function [2].

S107 which is supposed to be lysis inhibitor in physiological conditions caused rapid lysis in T7 expression system [11]. S105 (holin) with C-terminal His tag, characterized by circular dichroism spectroscopy, takes up 40 % alpha helical conformation corresponding to three transmembrane helices in the primary sequence. Further in vitro assay using calcein-loaded liposome revealed the active nature of holin as evident with permeabilization of fluorescent dye [12]. Solubilization of megadalton complexes of S^{λ} has been attempted using GroEL [13].

Lambda holin was found to be cytotoxic to tumor cell xenografts and some eukaryotic cell lines [14]. Hence holin has been suggested for potential therapeutic use in cancer gene therapy [15].

K. V. Srividhya · S. Krishnaswamy (✉)
Department of Genetic Engineering, School of Biotechnology,
Madurai Kamaraj University, Madurai 625021, Tamilnadu, India
e-mail: krishna@mrna.tn.nic.in

Electron microscopic analysis revealed that S105 in DDM and OG exist as rings [16, 17]. By single particle analysis on S105, rings are proposed to have 18–20 protomers with outer diameter of 23 nm and inner diameter of 9 nm for upper ring and 8 nm for inner or lower ring. The upper and lower rings are suggested to have a head to tail arrangement. Very recently the use of thiol specific reagents (2,2'-dithiodipyridine) in S105 and S²¹68 purification has enabled the generation of purified protein with its cysteine residue in native thiol state [18]. Recently, the possible structure of pinholins (S²¹68) has been examined by combining the results of negative-stain transmission electron-microscopy and structure modeling [19]. Extensive mutational and mapping studies of P21 pinholin transmembrane domains has been reported [20, 21].

The lysis systems of lambda, P21 and P22 are considered to be functional homologs due to their conserved dual start motif [22]. Recent reports suggest the possibility of different regulation mode operative in P21 holin involving dynamic topology changes of the TMD1 (transmembrane domain 1) [23]. The phage 21 holin, S²¹, forms small membrane holes to depolarize the membrane and is designated as pinholins. Pinholins require secreted SAR endolysins, a pairing that may represent an intermediate in the evolution of canonical holin-endolysin systems [24]. Understanding of holin function further requires an insight into protein structure, protein–protein interaction, membrane structure and electrophysiology. Very few prophage holins have been experimentally studied (Table 1). In this study holin encoded by DLP12 prophage was characterized.

Materials and Methods

Bacterial Strains and Plasmids

The HolinGFP clone in pCA24N vector were received as stabs from GenobaseTM in AG1 strain (Dr. Hiroto Mori, Nara Institute of technology, Japan) [25]. The clone has N terminal His tag and C terminal GFP tag.

Purification Methods for HolinGFP

Over expression and purification was done as earlier [26]. In brief, crude lysate was incubated with Talon resin for 2–3 h at 4 °C to permit binding of HolinGFP to the resin. pH of lysis buffer was optimized to 7.5 and Triton X 100 (TX 100) in elution buffer was replaced with detergents like DDM (*n*-dodecyl-β-D-maltoside), LDAO (Lauryldimethylamine-oxide), OG (*n*-octyl β-D-glucopyranoside) and C₁₂E₉ (dodecyl nonaoxyethylene ether) in talon affinity column. The elutions from talon were pooled and upconcentrated using Amicon Ultra 10 kDa (MilliPore) in

Table 1 Experimentally characterized holins from prophages

Host	Prophage	Lysis cassette genes	Computational analysis/functional characterization	Reference
<i>Lactococcus gasseri</i>	ϕ gaY	holgaY	Prophage ϕ gaY excised by Mitomycin C treatment and is realed to phage ϕ gle. holgaY has three TMDs	[30]
<i>S. warneri</i>	ϕ WMY	holWMY	holWMY is toxic to <i>E. coli</i> . holWMY has three TMDs	[31]
<i>Borrelia burgdorferi</i>	Cp32 plasmid/ ϕ BB1 prophage	BlyA holin	Complements lambda Sam7 mutant. BlyA is membrane localised	[32]
<i>Staphylococcus aureus</i> MRSA NU3-1	Genome	HolNU3-1	BlyA has two transmembrane domains and C-terminal charged residues Functional as its elicits growth inhibition against <i>S. aureus</i> . TEM showed translucent cells	[33]
<i>Bacillus subtilis</i>	PBSX	xhIA export protein xhIB holin	xhIA and xhIB needed to effect host cell lysis xhIB and xlyA cannot mediate lysis without xhIA	[34]

the presence of 1 mM PMSF and 1 mM EDTA. Gel filtration chromatography was performed in AKTA prime on a Superdex s200 10/300 analytical column. 500 μ l of HolinGFP was loaded onto column at a flow rate of 0.5 ml/min. 1 ml fractions were collected. The resultant elution volume was compared with the retention volume of standard proteins (as given for Superdex s200 10/300 from GE Healthcare) and probable molecular weight of eluted protein was calculated. Chromatograms were generated using ÄKTA PrimeViewTM and Microsoft Excel sheet.

Confocal Microscopy

AG1 cell pellets harbouring HolinGFP before and after induction with 1 mM IPTG were used for observation under confocal microscope (Leica Microsystems Heidelberg GmbH). Cells pellets after IPTG induction were suspended in 100 μ l of 0.9 % saline and 4 μ l of the cell suspension was placed in slide and air dried before viewing under 63 $\times$ magnification in Leica Confocal microscope.

Circular Dichroism (CD) and Dynamic Light Scattering Studies (DLS)

Circular dichroism was measured at school of chemistry, Madurai Kamaraj University in a JASCO spectropolarimeter using a 0.1 cm path-length cell in the far-UV range (190–250). The CD spectrums were recorded at resolution of 0.5 nm, bandwidth 1.0 nm, sensitivity 50 m deg, response 4 s and accumulation 3. The CD values were expressed in $[\theta]$ MRW deg cm²/dmol. DLS (VISCOTEK 802 DLS) facility available at Molecular Biophysics Unit, Indian Institute of Science was used for measuring hydrodynamic radius of HolinGFP. The parameters set in

OMNISIZE 3.0 software includes run duration of 0.67, with total experiments of 50, Spike tolerance of 20 %, Base Line Draft 15 % and resolution 300. HolinGFP samples in detergents 0.2 % LDAO, 0.2 % OG and 0.2 % C₁₂E₉ were used for measurement.

Crosslinking Studies

DSP Crosslinking was performed using protocol mentioned for S⁺ [16]. 10 μ l of purified protein (130 μ M) was diluted into 90 μ l of HEPES buffer [50 mM HEPES pH 7.2, 150 mM NaCl, 0.2 % (v/v) LDAO or 0.2 % (w/v) DDM or 0.2 % (w/v) OG, 0.2 % (w/v) C₁₂E₉]. 20 μ l of 6 mM DSP dissolved in anhydrous DMSO was added to the diluted protein and 20 μ l of DMSO for un-cross-linked samples. The samples were incubated with gentle shaking at 25 °C for 30 min. Reactions were terminated by addition of 0.1 M glycine. Crosslinked products were analysed by 12 % SDS-PAGE under reducing and non reducing conditions. HolinGFP in varying detergents were also cross-linked with 0.01 % (v/v) glutaraldehyde [27]. All the samples were prepared checked on 12 % SDS PAGE under non reducing conditions.

Results and Discussion

Confocal Microscopy of Host Cells

Phage holins are reported to localise on the cell membrane and oligomerise resulting in lesion on the membrane. The target protein in our study is HolinGFP (holin from a prophage, DLP12 and has C-terminal GFP fusion tag). The expression of HolinGFP was then investigated by

Fig. 1 Confocal image of induced host cells harbouring HolinGFP: **a** HolinGFP images in confocal microscope. **b** Intensity distribution of the fluorescence for the selected cells

fluorescence microscopy. GFP fluorescence was detected from first hour after induction. By confocal microscopy, localization could not be exactly tracked. However, relatively higher distribution of fluorescence was observed in periphery of the cell (Fig. 1a, b). Till date apart from HolinGFP, another GFP fusion holin which is reported and functionally studied is YwcE protein from *Bacillus subtilis*.

Fig. 2 Effect of HolinGFP on the growth of *E. coli*. In plotted graph, empty and filled triangles represent uninduced and induced HolinGFP. Empty and filled squares represent uninduced and induced IntEGFP respectively

YwcE-GFP fusion construct was found to be functional and also found to localise in cell membrane by fluorescence microscopy [28].

Effect of HolinGFP on the Viability of Host Cells

The effect of HolinGFP on the growth of *E. coli* was followed by measuring OD₆₀₀ decrease upon induction with 1 mM IPTG. As seen from Fig. 2 HolinGFP expression arrests cell growth of *E. coli* with a OD₆₀₀ of 0.754 being maintained even after 5 h of induction. Induction of control of IntEGFP did not decrease cell density confirming that holin counterpart in the HolinGFP is purely responsible for the lethal effect and GFP has no role to play in this physiological change.

Purification in Different Detergents

DLP12 HolinGFP fusion construct (36 kDa) was overexpressed using glucose repression followed by IPTG induction. Further the protein could only be solubilized in buffer containing 0.2 % Triton X 100. HolinGFP was initially solubilized with 0.2 % Triton X 100 and was later purified in other detergents namely, DDM, LDAO, OG and C₁₂E₉ using cobalt based affinity chromatography (Fig. 3)

Fig. 3 Cobalt affinity purification profiles of His-tag HolinGFP in different detergents. Lane: 1 marker, 2 crude protein, 3 flow through, 4 10 mM imidazole wash, 5 50 mM imidazole elution, 6 100 mM

imidazole elution (not present in panels 'd' and 'e'), 7 150 mM imidazole elution (a—TX100, b—C₁₂E₉, c—OG, d—LDAO, e—DDM)

for further biophysical characterization and structural studies. From a two litre culture, purification yielded around 2–3 mg protein irrespective of the detergent and could be further concentrated to 5 mg in a volume of 500 μ l.

Gel Filtration Chromatography of HolinGFP

The Superdex S200 chromatography was done in varying detergents for HolinGFP (Fig. 4). For HolinGFP- $C_{12}E_9$ (Fig. 4a) a total of four peaks were obtained (Table 2). The first two peaks correspond to higher oligomeric assemblies and the third broad peak is equivalent to a monomer. Similar profile is obtained with HolinGFP in DDM (Fig 4b) except for the monomer peak. HolinGFP in LDAO (Fig. 4c) predominantly elutes as dimer along with a minor oligomeric peak. HolinGFP OG (Fig. 4d) elutes immediately after the void volume of the column indicating the oligomeric state. S105 purified with DDM has been shown to have a similar profile and exists in higher oligomeric assemblies [16]. HolinGFP, although a class II holin, behaves like S105. Interestingly HolinGFP, which is not crosslinked, exists predominantly as a dimer only in

Table 2 Relative molecular weight and proposed HolinGFP oligomer in S200 column

Sample	Retention volume	kDa	Probable oligomer
HolinGFP- $C_{12}E_9$	9	669	Oligomer
	11	440	Oligomer
	16	35	Monomer
HolinGFP-DDM	9	669	Oligomer
	11	440	Oligomer
HolinGFP-LDAO	10.5	440	Oligomer
	14	68	Dimer
HolinGFP-OG	9	669	Oligomer

detergent LDAO. By native gel analysis (6 %) HolinGFP in DDM alone enters the gel whereas the protein in LDAO does not (data not shown).

Biophysical Characterization of HolinGFP

Circular dichroism of HolinGFP in all the four detergents ($C_{12}E_9$, DDM, LDAO, and OG) showed the molecule to be folded (Fig 5). The holin as predicted from sequence

Fig. 4 Superdex 200 chromatogram for HolinGFP in varying detergents (a— $C_{12}E_9$, b—DDM, c—LDAO, and d—OG)

features should ideally have α helical structure and the GFP counterpart is of β class. As seen in Fig. 5, the intensities of CD spectra related to α helix usually predominate those of β sheet. Generally for such class of proteins the spectrum

comprises of two negative CD bands at 222 and 208–210 nm for β part. S105 exhibited similar CD spectrum in DDM and EBB (Empigen BB) with α helical structure [16]. The GFPuv4 when excited at 494 nm gives

Fig. 5 CD spectrum of HolinGFP in different detergents

Fig. 6 Crosslinking of HolinGFP. HolinGFP monomers are indicated as *asterisk*, dimers as *double asterisk* and oligomers as *hash*.

a DSP crosslinking: lane 1 protein marker, lane 2 HolinGFP-LDAO, lane 3 HolinGFP-LDAO + 6 mM DSP, lane 4 HolinGFP-OG, lane 5 HolinGFP-OG + 6 mM DSP, lane 6 HolinGFP-LDAO-5X protein loading dye not boiled.

b DSP crosslinking: lane 1 protein marker, lane 2 HolinGFP-DDM, lane 3 HolinGFP-DDM + 6 mM DSP, lane 4 HolinGFP-C₁₂E₉, lane 5 HolinGFP-C₁₂E₉ + 6 mM DSP, lane 6 HolinGFP-LDAO-5X protein loading dye not boiled.

c Glutaraldehyde crosslinking: lanes 1—protein marker, 2—HolinGFP-C₁₂E₉, 3—HolinGFP-C₁₂E₉ + GL, 4—HolinGFP-DDM, 5—HolinGFP-DDM + GL, 6—HolinGFP-LDAO, 7—HolinGFP-LDAO + GL, 8—HolinGFP-OG, 9—HolinGFP-OG + GL

an emission peak at 512 nm when properly folded [29]. Eluted HolinGFP protein fluorescing green was additionally confirmed for proper folding by measurement of fluorescence emission at 512 nm in all the detergents.

Crosslinking Studies of HolinGFP

DSP crosslinking studies showed that lambda holin (S105 and S107) could be cross-linked into dimers, trimers and higher order oligomers [16]. Since gel filtration and DLS studies on HolinGFP clearly revealed the existence of higher order oligomers and dimers, DSP crosslinking was carried out with HolinGFP with different detergents. As seen in Fig. 6a and b, HolinGFP in DDM, OG and C₁₂E₉ upon crosslinking with DSP showed higher oligomer formation. In LDAO samples, tetramer, trimer, dimer and monomer populations could be seen. In a screen with DSP, a concentration of 2 mM was sufficient to crosslink HolinGFP. Interestingly, HolinGFP crosslinking could be induced using glutaraldehyde. As seen in Fig. 6c, HolinGFP in all four detergents crosslinked as higher order oligomers. Upon boiling, only the dimer and monomer could be observed in all crosslinked samples.

Conclusion

HolinGFP protein was found to be functional. The protein solubilized using Triton X 100 was purified in the presence of different detergents like DDM, LDAO, OG, and C₁₂E₉. HolinGFP existed predominantly as dimer in LDAO in Superdex S200 gel filtration chromatography. Circular dichroism of HolinGFP in all the four detergents (C₁₂E₉, DDM, LDAO, and OG) was found to have a typical spectrum characteristic of GFP and holin. Eluted HolinGFP protein was confirmed as properly folded by measurement of fluorescence emission at 512 nm in different detergents. DSP cross linking revealed the existence of higher order oligomers and dimers. Glutaraldehyde was also found to crosslink HolinGFP. Resolving the reason for the different oligomers seen for holinGFP will enable crystallographic structure determination.

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