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# Effects of single amino acid substitutions at the predicted coiled-coil or hydrophobic region on the self-assembly of $\phi$29 replication protein, gp1 ☆

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## Abstract

Gp1, the product of one of the essential genes of $\phi$29 replication, is an RNA binding protein and self-associates to form large complexes. Furthermore, gp1 suppresses the synthesis of $\phi$29 DNA polymerase and primer protein in the post-transcriptional process. In this report, we have employed seven variants with single amino acid substitutions to analyze the self-assembly of gp1. Using chemical cross-linking and sedimentation assays, amino acid substitutions within the predicted coiled-coil or hydrophobic region were shown to strongly affect the formation of large complexes, suggesting that these two regions were required for the self-assembly of gp1. The self-association of gp1 was suggested to be necessary for the efficient binding to RNA and the translational repression.
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*Keywords:* Self-assembly; Single amino acid substitution; Glycerol gradient sedimentation; Chemical cross-linking; In vitro translation; Coiled-coil structure; Hydrophobic region; RNA binding protein

*Bacillus* phage $\phi$29 has a linear double-stranded DNA genome (19,285 bp) and contains 19 genes classified into early and late genes [1–3]. Among the early genes, genes 2 and 3 code $\phi$29 DNA polymerase and primer protein, respectively, and these two genes are essential for the $\phi$29 replication [2,3]. These genes are transcribed together with other replication genes, including gene 1, as a long polycistronic mRNA of 5.1 kb [4]. Although the function is not clearly understood, gene 1 was also shown to be essential for in vivo replication by genetic studies [5–7].

Previously, Takeuchi et al. [8] have reported that gene 1 product (gp1, 85 amino acids) suppresses the synthesis of $\phi$29 DNA polymerase (gp2) and primer protein (gp3) in *Escherichia coli*. However, the amount and integrity of the polycistronic mRNA were not influenced by gp1, and gp1 was shown to bind RNA [9], suggesting that it inhibits the translation through binding to the polycistronic mRNA. Although the size of gp1 is relatively small (10 kDa), multiple characteristics are reported or suggested including RNA binding, membrane localization, and interaction with primer protein [6,9,10]. In addition, gp1 self-associates into filamentous structures in vitro [7,11]. Serrano-Heras et al. [12] demonstrated that gp1 assembled into multimeric structures in infected cells. These observations suggested that gp1 could assemble in vivo to form large complexes, although the biological role of the self-assembly is unknown.

In the studies of the self-assembly of gp1, a secondary structure prediction program to look for coiled-coil

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☆ *Abbreviations:* IPTG, isopropylthio-β-D-galactoside; Chaps, 3-[(3-cholamidopropyl)dimethyl-ammonio]propanesulfonic acid; DSS, disuccinimidyl suberate; BSA, bovine serum albumin; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue.

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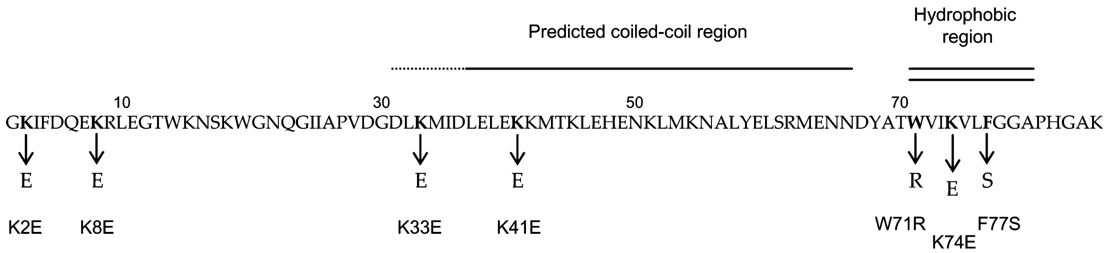
Predicted coiled-coil region
Hydrophobic region
10
30
50
70
GKIFDQEKRLEGTWKNSKWGNQGIIAPVDGDLKMIDLELEKKMTKLEHENKLMKNALYELSRMENNDYATWVIKVLFGGAPHGAK
E
E
E
E
R
E
S
K2E
K8E
K33E
K41E
W71R
K74E
F77S

Fig. 1. Schematic diagram of structure of gp1 and the location of substitutions. With names and sites of the seven variants, the 85 amino acid residues of gp1 are shown at the center of the figure. A secondary structure prediction program to look for coiled-coil domains (COILS) [13] revealed that the region of gp1 spanning amino acid residues Leu$^{37}$ to Asn$^{66}$ (thin line) had a high probability (0.70–0.98) [7], and that the region spanning amino acid residues Asp$^{31}$ to Asp$^{36}$ (dotted line) had a lower probability (0.30–0.40) to form a coiled-coil structure. The hydrophobic region spanning Trp$^{71}$ to Ala$^{80}$ is indicated by double line [12,14,15].

domains (COILS) [13] revealed that the region of gp1 spanning amino acid residues Leu$^{37}$ to Asn$^{66}$ had a high probability (0.70–0.98) to form a coiled-coil structure (Fig. 1) [7], and that the region spanning Asp$^{31}$ to Asp$^{36}$ also had a probability (0.30–0.40). Furthermore, the amino acid residues near the carboxyl terminus spanning Trp$^{71}$ to Ala$^{80}$ were highly hydrophobic (Fig. 1) [12,14,15]. Bravo et al. [10,11] have demonstrated that the predicted coiled-coil region and the hydrophobic region are essential for the self-assembly of gp1. However, the variants of gp1 used in these studies contained extensive truncation at either amino- or carboxyl terminal region.

To elucidate the function of the self-assembly in detail, we have already obtained 31 variants of full-length gp1 with single amino acid substitutions at various positions (to be published elsewhere). In this study, we selected seven of them from the predicted secondary structures. Using chemical cross-linking and sedimentation assays, we analyzed their self-association. Moreover, by using the variants, we examined the effects of self-association on the formation of large gp1–RNA complexes and on translational repression.

## Materials and methods

*Bacterial strain and plasmids. Escherichia coli* strain DH5α was our laboratory stock. The *E. coli* expression plasmid, pUV1-WT, encodes wild type gp1 with a polyhistidine tag (His-tag) at the carboxyl terminus under lacUV5 promoter. Other plasmids, pUV1-K2E, pUV1-K8E, pUV1-K33E, pUV1-K41E, pUV1-W71R, pUV1-K74E, and pUV1-F77S, encode single amino acid substituted variants of gp1 with His-tag (to be published elsewhere).

*Purification of wild type gp1 or variants.* The products of gene 1 of wild type or variants were purified as described [9] with some modifications. The recombinant gp1 in *E. coli* DH5α containing each plasmid was induced with IPTG. After induction for 2 h at 37 °C, cells were collected by centrifugation. After cell disruption, the bacterial lysate was separated into soluble and insoluble fractions by centrifugation (10,000*g*, 15 min, 4 °C). To determine the distribution of gp1 in the soluble or insoluble fractions, aliquots from each fraction were subjected to 13% SDS–PAGE and the gel was stained with CBB [16]. Fractions, either soluble (W71R and K74E) or insoluble (K2E, K8E, K33E, K41E, and F77S), which contained the majority of gp1 were solubilized by denaturing buffer containing 6 M guanidium–HCl, 100 mM sodium phosphate, and 10 mM imidazole at pH 7.4. Each gp1 was further purified by using a Ni column (HiTrap chelating) according to the method suggested by the manufacturer (Amersham Biosciences). The eluate in elution buffer (6 M guanidium–HCl, 100 mM sodium phosphate, and 300 mM imidazole at pH 7.4) was collected and analyzed by 13% SDS–PAGE. Fractions containing gp1 were separated into aliquots and stored at −80 °C until refolding.

*Refolding of gp1.* To refold the denaturated gp1 and to remove high concentrations of imidazole and guanidium–HCl, the stored eluate from Ni column was dialyzed against 125 volumes of refolding buffer containing 1 M urea, 50 mM Tris–HCl (pH 8.0), 1 M NaCl, 0.5% Chaps (Dojindo Laboratories), and 0.1 mM EDTA at 4 °C for 16 h followed by dialysis against 125 volumes of refolding buffer without urea (20 mM Tris–HCl, pH 8.0, 1 M NaCl, 0.5% Chaps, and 0.1 mM EDTA) at 4 °C for 1.5 h twice.

*Chemical cross-linking assay.* Gp1 was incubated with 0.25 mM of the covalent cross-linker, DSS (Pierce), in Hepes buffer containing 20 mM Hepes (pH 8.0), 1 M NaCl, 0.5% Chaps, and 0.1 mM EDTA at 4 °C. After quenching the free reactive groups with 50 mM glycine for 15 min at room temperature, cross-linked proteins were analyzed by 13% SDS–Tricine–PAGE [17] and visualized by staining with sypro orange (Molecular Probes).

*Sedimentation assay.* Eighty microliters of gp1 solution (25 μM) was loaded on 4.8 ml of linear glycerol gradient (15–35% in 20 mM Tris–HCl, pH 8.0, 1 M NaCl) and centrifuged for 24 h at 216,000*g* at 4 °C. BSA (66.5 kDa, Amersham Biosciences), chymotrypsinogen A (21.1 kDa, Amersham Biosciences), and aprotinin (6.5 kDa, Sigma), all of which behave as monomers in solution, were mixed with gp1 as internal size markers in every assay. After centrifugation, the gradient was fractionated into 20 aliquots from the tube bottom. The precipitate at the tube bottom was dissolved in 250 μl of loading buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 2.5% 2-mercaptoethanol, and 10% glycerol), and an aliquot was analyzed by 13% SDS–Tricine–PAGE [17].

*Gel mobility shift assay.* In this assay, we used an approximately 300 base RNA which was labeled with fluorescein at its 5′ end (named 10-4.5 FW). Wild type gp1 has been shown to bind this RNA preferentially, whose sequence was identical to a region within the polycistronic mRNA of major early genes of ϕ29 (to be published elsewhere). Prior to binding assays, the labeled RNA was once denatured in 10 mM Tris–HCl, pH 8.0, at 55 °C for 5 min and immediately chilled on ice for 2 min. Then for renaturation, the RNA was adjusted to contain 20 mM Tris–HCl, pH 8.0, 200 mM NaCl, and 0.1 mM EDTA, followed by incubation at 55 °C for 5 min and slow cooling (1 °C/min) to 25 °C. After renaturation, the RNA was immediately used in the binding assay. For binding reaction, the RNA (0.1 ng/μl) was incubated with gp1 in the presence of non-labeled poly(A) RNA (200 ng/μl) as a competitor, in a buffer con-

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