The Oligomeric T4 Primase is the Functional Form during Replication

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Running Head: Gp61 primase oligomerization

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ABSTRACT

Replisome DNA primases are responsible for the synthesis of short RNA primers required for the initiation of repetitive Okazaki fragment synthesis on the lagging strand during DNA replication. In bacteriophage T4, the primase (gp61) interacts with the helicase (gp41) to form the primosome complex, an interaction that greatly stimulates the priming activity of gp61. Since gp41 is hexameric, a question arises as to whether gp61 also forms a hexameric structure during replication. Several results from this study support such a structure. Titration of the primase/single-stranded DNA (ssDNA) binding followed by fluorescence anisotropy implicated a 6:1 stoichiometry. The observed rate constant, $k_{cat}$, for priming was found to increase with the primase concentration, implicating an oligomeric form of the primase as the major functional species. The generation of heterooligomeric populations of the hexameric primase by controlled mixing of wild type (wt) and an inactive mutant primase confirmed the oligomeric nature of the most active primase form. Mutant primases defective in either the N- or C-terminal domain and catalytically inactive could be mixed to create oligomeric primases with restored catalytic activity suggesting an active site shared between subunits. Collectively, these results provide strong evidence for the functional oligomerization of gp61. The potential roles of gp61 oligomerization during lagging strand synthesis are discussed.

INTRODUCTION

Primases play an indispensable role in DNA replication by synthesizing the RNA primers needed for initiation of de novo DNA synthesis (1). In eukaryotic cells, both leading and lagging strand syntheses are primed by the polymerase α−primase complex (2). In bacteriophage T4, the leading strand primer is synthesized by the E. coli RNA polymerase during the early phase of phage DNA replication, or provided through strand invasion via homologous recombination in the late phase of replication (3). T4 gp61 primase then makes the short pentaribonucleotide primers required on the lagging strand to initiate repetitive Okazaki fragment synthesis (4).

Gp61 primase recognizes two trinucleotide sequences in vitro, 5'-GTT-3' and 5'-GCT-3', but uses only the former sequence in vivo as the priming site (5, 6). Although gp61 alone is able to synthesize primers on a single-stranded DNA (ssDNA) substrate, its maximum activity is achieved only through interaction with the helicase (gp41) to form the primosome complex (6, 7). Several other T4 proteins, including the helicase accessory protein (gp59) and the single-stranded DNA binding protein (gp32), also modulate primase activity presumably by either facilitating the formation of the primosome or directly interacting with it (7).

Among all the T4 replisome proteins, the helicase exhibits the greatest stimulatory effect on the priming activity of the primase. T4 helicase, like T7 and E.coli DnaB helicases (8, 9), acts as a hexamer and forms a ring structure encircling the lagging strand (10, 11). Movement of this hexameric ring is driven by ATP hydrolysis along the lagging strand in the 5’ to 3’ direction and simultaneously unwinds the duplex DNA in front of the entire replisome (12-14). The ring structure of the hexameric helicase provides the basis for its high processivity during replication that is
commensurate with a dissociation half life of \( \sim 11 \) min (15). On the other hand, gp61 dissociates during lagging strand synthesis, which results in repeated assembly and disassembly of the primosome during replication (16).

Since the primase interacts closely with the helicase, it has been of interest to understand the structural features of such an interaction. One question is whether gp61 also forms a multimeric structure like gp41. Oligomerization of the replicative primase has been observed in several other systems. T7 primase forms a hexameric ring owing to the fact that the primase and the helicase activities reside on the same peptide (17). Recent studies suggest that E. coli DnaG primase also forms a higher order trimeric structure when interacting with the DnaB helicase (18). In the T4 system, several analytical methods previously failed to detect the oligomerization of gp61 in the absence of DNA and gp41 (7, 19). In the presence of DNA and/or gp41, a study using gel mobility shift analysis suggested a monomeric primase as the active component of the primosome (19), although transient or unstable protein-protein interactions may not be detected by such an approach. In contrast, a more recent investigation that employed isothermal titration calorimetry (ITC) and chemical cross-linking techniques revealed the existence of oligomeric states of the gp61 primase (7).

In this work, we confirm the oligomerization of gp61 upon binding to DNA by monitoring the change in fluorescence anisotropy. Moreover, a recent electron microscopy study revealed a symmetric, hexameric structure for the primase in the presence of ssDNA (10). The measured 6:1 stoichiometry of gp61/DNA binding further supports the formation of a hexameric T4 primase. The priming kinetics for various, mixed populations of oligomers derived from wt and an inactive mutant gp61 demonstrate that the multimeric gp61 is the functional form and that subunit interactions within the gp61 oligomer affect its priming activity. Our results also indicate that oligomer formation is an intrinsic property of gp61 upon interacting with DNA and takes place even in the absence of the gp41 helicase.

MATERIALS AND METHODS

[\( \alpha^{32}\mathrm{P} \)]CTP was purchased from New England Nuclear. Unlabeled ribonucleotides were purchased from Roche Biochemicals. Oregon Green 488 carboxylic acid, succinimidyl ester, S-isomer (OG) was obtained from Molecular Probes (Eugene, OR). Bacteriophage T4 proteins gp41, gp61 and gp61 (E234Q) were purified as previously described (20, 7). Pfu Turbo DNA polymerase was from Stratagene and all restriction enzymes were from New England Biolabs. All other chemicals were of analytical grade or better.

The DNA substrate

Two oligomeric single-stranded DNA substrates were used in this study. Oligo A (67mer) has the sequence of 5’ GAT GGG TAG AGA TGA GGT TAT TAG TGG TAG TGT GTA GTG AGT GTG ATT CGT AGT GGT CAT GGA TGG G 3’ and was used in all priming reactions. Oligo B (20mer) has the sequence of 5’ TGG GTG ATG TTG ATG TCT AG 3’ and was used in the fluorescence anisotropy and chemical crosslinking experiments. The priming sites in both oligos are underlined. Before use, the DNA oligos were purified on denaturing polyacrylamide Hoefer gels. The desired bands were cut out and recovered by a crush-and-soak method in pH 7.5 TE buffer (10 mM Tris-HCl, 1 mM EDTA), purified on Sep-Pak columns, dried by speed-vac and redissolved in deionized water. The concentration of DNA oligos was determined by measuring the absorbance at 260 nm. The molar absorption coefficient was calculated as the sum of those of individual nucleotides: \( \varepsilon = 15400 \) (dA), 9100 (dC), 13700 (dG), and 9600 M\(^{-1}\)cm\(^{-1}\) (dT) (21). The purity of all oligos was analyzed on a 16% denaturing acrylamide sequencing gel and is greater than 95%.

The procedure for labeling oligo B with fluorophores is given below. Oligo B was synthesized with a 5’ amino-modifier C2-dT (Glenn Research, Sterling, VA) at its 5’ end using standard \( \beta \)-cyanoethyl phosphoramidite chemistry and purified as described above. The oligo was then labeled with a 50-fold excess of Oregon Green 488 carboxylic acid, succinimidyl ester, S-isomer (OG) in a pH 8.5, 100 mM sodium tetraborate buffer for 4 h at room temperature protected against light. The reaction mixture was then precipitated with ethanol and microcentrifuged for at least 30 minutes. The resulting pellet was rinsed with 70% ethanol,
dried, redissolved in water, and purified by anion-exchange HPLC (Solvent A: pH 7.5, 20 mM Tris-HCl/1 mM EDTA; solvent B: pH 7.5, 20 mM Tris-HCl/1 mM EDTA/1 M NaCl; Gradient: 90% A to 70% A over 5 min, then to 45% A over 10 min, then to 25% A over 15 min and to 0% A over 5 min). The labeled oligo was collected and subject to reverse phase HPLC (Solvent C: acetonitrile; solvent D: 100 mM triethylamine acetate, pH 6.8; Gradient: 0% C for 5 min, to 30% C over 10 min, then to 90% C over 1 min and maintained at 90% C for 13 min). The desired labeled oligo was collected and dried.

The standard priming reactions

Priming reactions were carried out in a complex buffer containing 25 mM Tris-acetate (pH 7.5), 125 mM KOAc and 10 mM Mg(OAc)2. The standard priming reactions in the presence of gp41 helicase contained 2 μM oligo A, 3.4 μM gp41, 4 mM ATP, 400 μM each of CTP, GTP, and UTP and various ratios of wt gp61 to mutant gp61 (E234Q) (as indicated in figure legends) in a typical reaction volume of 20 μl. The [α-32P]CTP was used for labeling primer. In the absence of gp41 helicase, DNA (20 μM) and higher concentrations of wt gp61 and gp61 mutants (see figure legends) were used in the standard priming reactions. In all reactions, DNA and the proteins were first mixed and incubated at 37 °C for 5 min before the addition of rNTPs to initiate the reaction. Reaction aliquots were withdrawn at various times and quenched with an equal volume of 500 mM EDTA, pH 8.0. Priming products were analyzed by 20% denaturing acrylamide sequencing gel electrophoresis. Autoradiography was accomplished with a Molecular Dynamics Storm 800 Phosphoimager system (Amersham Biosciences, Piscataway, NJ). The amount of primer synthesized was quantified using Quantity One Quantitation Software (BioRad, Hercules, CA) and comparing it to a standard obtained by spotting a known amount of radioactive sample directly onto the DE81 filter paper.

Steady-state fluorescence anisotropy measurements

Steady state fluorescence anisotropy experiments were performed using an ISA Fluoromax-2 spectrofluorometer equipped with a polarizer (Edison, NJ). The OG label was irradiated at 492 nm and the emission was measured at 523 nm. Slit widths were set at a 2 nm band pass for the excitation and a 16 nm band pass for the emission. The integration time was 0.3 sec. The fluorescence anisotropy (A) measures the level of polarization of light emitted when a fluorophore is irradiated with polarized light: A = (I∥ - G*I⊥) / (I∥ + G*I⊥), where I∥ and I⊥ are intensities of the emitted light parallel to and perpendicular to the polarization plane of excitation and G is the correction for instrument response to polarized light at a particular emission wavelength (22). Titration experiments were performed by the addition of a constant volume of primase solution into a cuvette containing OG-labeled oligo B (100 nM) in the assay buffer (pH 7.5, 20 mM Tris-OAc, 50 mM KOAc, 6 mM Mg(OAc)2, and 1mM DTT). A typical sample volume was 1.4 ml and an average of 10 to 13 readings over a 2 to 4 minutes span was used to determine the final reading at each concentration of primase.

Assuming that primase has independent but identical binding sites, the dissociation constant and the molar ratio of primase to DNA substrate at saturation were determined by fitting the binding isotherm to equation (1) using a non-linear least square curve fit program, Origin 5. (MicroCal Software, Inc. Northampton, MA):

\[ A = A_f + (A_b - A_f) \frac{(L_i + K_d + nR_i) [([L_i - K_d - nR_i])^2 - 4L_i nR_i]^2]}{2L_i} \]

\[ L_i: \text{ the total added concentration of ligand} \]
\[ R_i: \text{ the total concentration of receptor} \]
\[ A_f: \text{ the anisotropy for the free ligand} \]
\[ A_b: \text{ the anisotropy for the fully bound ligand} \]
\[ A: \text{ observed anisotropy value} \]
\[ K_d: \text{ the dissociation constant between lignd and receptor} \]
\[ n: \text{ the binding ratio between ligand and receptor} \]

\[ \text{equation (1)} \]

Limited subtilisin proteolysis of gp61 primase and N-terminal sequencing of the proteolytic fragment

A typical subtilisin digestion was carried out in a 40 μl solution containing 10 mM Tris acetate (pH 7.8), 25 mM KOAc, 5 mM Mg(OAc)2, 2 mM DTT, 14 μM gp61 and 1.4 μM subtilisin
(Sigma). Before the addition of subtilisin, a 10 μl aliquot was removed, quenched with 2 μl of 5 mM phenylmethylsulfonyl fluoride (PMSF) and mixed with 4 μl of SDS gel loading buffer to serve as the zero time point of digestion. After the addition of subtilisin, 10 μl aliquots were removed at specified time points, quenched with 2 μl 5 mM PMSF and mixed with 4 μl of SDS-PAGE gel loading buffer. The samples were analyzed by SDS-PAGE using 12% polyacrylamide gels. A major proteolytic fragment with an apparent molecular weight of 28 kDa that built up during the proteolytic treatment was subjected to N-terminal sequencing (Micromolecular Core Facility, the Huck Institute of Life Sciences, The Pennsylvania State University). The N-terminal sequence of KEKGK was unambiguously determined for the gp61 C-terminal proteolytic fragment.

Cloning, expression and purification of the truncated gp61 C-terminal domain protein

The limited proteolysis of gp61 revealed a major cleavage site before Lys95. The C-terminal portion of gp61 (amino acid 94 – 342, gp61C) was cloned from wild type gp61 DNA by using the primers 61CterF and 61CterR with the sequences of 5’- GTA TAT CTT TGA ACA TAT GAA AGA AAA AGG TAA AAG and 5’- CGA TTC GCT CTT CCG CAA CCC TTA GCA TAT TTA G, respectively. These primers contain NdeI and SapI restriction sites used for cloning the target gene into the pTYB1 vector (New England Biolabs) to make gp61C intein-fusion protein construct. The PCR product and the pTYB1 plasmid were digested separately with SapI for 4 h at 37 °C. Then NdeI was added to the PCR product digest, and both NdeI and shrimp alkaline phosphatase were added to the pTYB1 plasmid digest. The digestion was allowed to continue for 10 h. The resulting insert and digested plasmid were purified by 0.8% agarose gel and then ligated using T4 DNA ligase (Promega) according to the manufacturer’s directions. The ligation mixture was used to transform E. coli DH5α cells and the plasmid was purified using a Qiagen miniprep kit. The presence and the sequence of the insert were verified by DNA sequencing (Pennsylvania State University Nucleic Acid Facility). The cloned gene encodes a protein with an N-terminal methionine and the 248 residues that correspond to amino acid sequence 95 to 342 of the wt T4 primase C-terminal portion and is present as an intein fusion protein.

The intin-fusion protein was purified using a chitin affinity column as described previously (7). The protein concentration was determined by using a calculated extinction coefficient of ε₂₈₀nm = 48960 M⁻¹ cm⁻¹. A typical preparation yielded approximately 20 mg of purified protein at >98% purity.

The primase in vitro activity complementation assay

Standard priming reactions were carried out in the presence of 19 μM oligo A, 2 μM gp41 and various amount of gp61(E234Q) (0 to 3.9 μM) and gp61C-terminal domain protein (0 to 4.8 μM). Reactions were allowed to proceed for 20 min and were quenched in pH 8.0, 500 mM EDTA solution. The priming products were analyzed by 20% denaturing acrylamide sequencing gel electrophoresis as described above.

Crosslinking of gp61 and gp61(E234Q)

Wt gp61 and the gp61(E234Q) mutant were dialyzed against a labeling buffer (25 mM HEPES, pH 6.8, 150 mM NaCl, and 10% glycerol) and each separately labeled with a 5-fold excess of biotinamidocaproate N-hydroxysuccinimide for 4 h at 4 °C. The labeled protein was then dialyzed against the crosslinking buffer (25 mM Tris-HCl, pH 7.3, 150 mM NaCl, and 10% glycerol) to remove the excess of biotin, frozen in aliquots, and stored at -70 °C. The labeling processes were performed in less than 30 h and the labeled wild type primase retained activity comparable to unlabeled primase in the priming assay.

A typical crosslinking experiment was initiated by adding 1 μl of 20 μM Bis-maleimidylhexane (BMH, Pierce) in N, N-dimethyformamide to a mixture of wt gp61 and gp61(E234Q) and 0.1 μM oligo B in the crosslinking buffer (final volume 40 μl). The total concentration of wt and mutant gp61 was kept at 0.6 μM while ratios between the two were varied as required. Only one protein in the mixture carried a biotin tag, for instance, the biotinylated...
wt gp61 was mixed with the unlabeled mutant gp61 or vice versa. The reaction was allowed to proceed at room temperature for 10 min and was then quenched by the addition of 5 μl of SDS-PAGE loading buffer (60 mM Tris, pH 6.8, 2% SDS, 0.1% bromphenol blue, 25% glycerol, and 108 mM β-mercaptoethanol). The samples were separated and analyzed by 6% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Micron) which was blocked at room temperature for 1 h with 3% bovine serum albumin in pH 7.6, 20 mM Tris-HCl buffer containing 140 mM NaCl and 0.1% Tween 20 (TBST). The membrane was incubated with 1:8000 dilution of neutravidin horseradish peroxidase conjugate (Pierce) in 3% bovine serum albumin in TBST at room temperature for 1 h after the blocking step, then washed with TBST (4 x 10 min each) and developed with a luminol/hydrogen peroxide mixture (Pierce). Chemiluminescence was detected by BioMax film (Kodak) with typical exposure times of 20 - 60 s.

RESULTS AND DISCUSSION

**Fluorescence Anisotropy measurements of the gp61/DNA interaction**

Fluorescence anisotropy experiments were carried out to investigate the possible oligomerization state of the primase and to measure the binding affinity of the primase/ssDNA interaction. The 5'-OG labeled oligo B with one priming site in the middle of the sequence served as substrate, the same DNA substrate lacking a fluorescent label is active in the priming assay (data not shown). Changes in the fluorescence anisotropy of the labeled 20mer oligo B were measured and plotted as a function of gp61 concentration. The resulting data were fit to a binding isotherm (Fig. 1) that required a 6 ± 1 stoichiometry for gp61 to DNA assuming that each primase subunit acts independently. The $K_d$ value calculated for the gp61/DNA complex is 66 nM, in agreement with the $K_d$ of 50 to 100 nM measured in a previous study (7).

The observed stoichiometry most likely reflects the binding of the primase monomer to its priming site and concomitant formation of a hexameric ring structure, rather than simply coating the DNA in a “side-by-side” filament like fashion. The helicase gp41 (54 kDa) and the helicase loader gp59 (26 kDa) have been shown to form oligomeric structures when bound to DNA with a binding footprint of 12 to 20 nucleotides for gp41 (23) and 8 to 10 nucleotides for gp59 (24). Based on a recent EM study, the depth of gp61 ring structure is close to 3/4 of that of gp41 (10). Therefore, a binding site with an estimated size of 9 to 15 nucleotides was expected for gp61. A 20mer ssDNA substrate with a priming site at the middle of the sequence should eliminate the possibility of binding of two gp61 molecules in a side-by-side fashion on the DNA substrate. Furthermore, in a previous ITC experiment, 3 - 4 primase molecules were found to bind ssDNA oligos of two different sizes (24mer and 45mer) that contained only one priming site in the middle of the sequences (7), consistent with gp61 clustering around the priming site. The observed 6 to 1 stoichiometry, however, is different from the 3 – 4 primases bound per ssDNA oligo obtained earlier. We attribute this in part to the different sensitivities of the experimental approaches with the ITC protocol that requires higher levels of proteins, possibly compromised by aggregation (7).

This DNA binding stoichiometry was generated in the absence of the helicase. Therefore, unlike the T7 primase, gp61 is capable of forming oligomers even in the absence of its helicase. Given the strong stimulatory effect of the helicase on gp61 priming activity, we would expect that oligomer formation by the primase will be facilitated by gp41.

**Priming at various primase concentrations**

We next carried out kinetic experiments to determine whether the multimeric form of the primase is the functional form. Oligo A was used in all priming kinetic assays. We first measured the priming activity at different gp61 concentrations in the presence of saturating amount of DNA and gp41. In all reactions, the concentrations of DNA (2 μM) and gp41 (3.4 μM) were much higher than their $K_d$ values for primase binding (~100 nM , ref. 7) and were in large excess over those of the primase (30 to 480 nM). The $k_{cat}$ values thus can be measured directly from the observed rate of priming. If the primase acts only as monomer, the rate of priming should be...
proportional to the primase concentrations and no change in $k_{cat}$ ($V_{priming}/[primase]$) should be observed at below saturation primase concentrations. However, if there is a monomer/oligomer equilibrium where the turnover numbers for the two species are different, varying the primase concentration would induce a shift in this equilibrium and hence a change in the apparent $k_{cat}$ value. Specifically, if the multimeric form is more active, an increase in $k_{cat}$ will be observed with increasing gp61 concentrations. Likewise, a decrease in $k_{cat}$ would indicate that the monomeric form is the more active form (25, 26).

As shown in Fig 2, the apparent $k_{cat}$ for priming increases approximately by four hundred fold from 0.0036 to 1.375 min$^{-1}$ upon raising the primase concentration from 30 to 480 nM. This increase in $k_{cat}$ not only reflects the oligomerization of gp61, but also indicates that the oligomeric form of the primase is the major functional form.

**Priming at constant [wt primase] and increasing [E234Q primase] levels**

Mutant enzymes have been used in a number of cases to detect possible protein oligomerization involving transient protein/protein interactions (25, 27, 28, 29). In this work, a mutant primase (E234Q) was employed to study its effect on the priming activity of the wild type primase in order to provide additional evidence for the oligomerization of the primase. Sequence analysis suggests that residue E234 of gp61 aligns with residue E265 of the *E. coli* primase which is involved in metal ion binding that is crucial for catalysis (30). This mutant enzyme has been shown previously to lose the nucleotidyl transfer activity but retain normal binding activity towards DNA (16). Furthermore, both E234Q gp61 and wt gp61 have similar effects on the ATPase and unwinding activity of gp41 helicase (data not shown), suggesting that the interaction between gp41 and the mutant gp61 is not altered significantly.

Priming reactions were carried out as described under “Materials and Methods” to measure the inhibitory effect of gp61(E234Q) on the priming activity of wt gp61 (200 nM). A large excess of oligo A (2 μM) was present in all experiments to exclude the possibility that the mutant primase may inhibit the wt gp61 activity by competing for a limited number of priming sites. For the same reason, the gp41 helicase (3.4 μM, when present, was also in large excess over the primases. The amount of E234Q primase was chosen so as not to affect the limiting primase condition. In order to provide an equal opportunity for oligomer formation and DNA binding between these two enzyme forms, both wt and mutant primases were first mixed together for 10 min before the addition of the DNA substrate. Ribonucleotides were then added to initiate the reactions. In the presence of ample priming sites, if the primase only acts as a monomer, the addition of the mutant primase should have little inhibitory effect on the priming activity of the wt primase. Therefore, any inhibition induced by the mutant primase should come from a direct interaction between two primases through oligomer formation.

We first studied the effect of added mutant primase on the priming activity of 200 nM wt primase in the presence of 3.4 μM gp41 helicase. The result is shown in Fig 3A. The priming activity dropped sharply by ~ 3 fold with the addition of only 80 nM of the mutant primase. This activity further decreased by an additional 1.5 fold upon the addition of 160 nM mutant gp61. Next, we measured the inhibitory effect of the mutant primase in the absence of the gp41 helicase. Since the primase has a very weak activity in the absence of the helicase, nearly 10-fold more of the DNA substrate (20 μl) and wt gp61 (1 μl) were included in reactions lacking the helicase. A longer reaction time (five to ten min) was also needed for the primase to synthesize a measurable amount of primers in the absence of gp41. The results indicate a similar inhibition pattern (Fig. 3B). The priming activity decreases by 4.2 and 5.8 fold with the addition of 100 and 200 nM of the mutant primase, respectively.

In a control experiment, BSA was added instead of gp61(E234Q) and no inhibition of the priming activity was observed (data not shown). Taken together, these results strongly indicate the formation of hetero-oligomers between the wt and mutant primases both in the presence and absence of the gp41 helicase and suggest that the incorporation of the mutant enzyme into the primase oligomer severely affects its priming activity.
Priming at a constant total primase concentration with varying \([wt \text{gp61}] / [\text{gp61(E234Q)}]\) ratios

We next performed priming reactions at a constant total primase concentration (\([wt \text{primase}] + \text{[E234Q primase]}\)) with varying ratios of these two enzymes. By doing so, we intended to maintain a constant monomer/hexamer distribution of the primase in order to evaluate the effect on priming of increasing the mutant subunit level within the primase oligomer. For the reason stated above, both the DNA and the helicase (if present) were in large excess over the primase. Priming activity was measured at different \(wt/E234Q\) ratios and the results are given in Fig. 4. Two inferences can be made from the data curve. First, the decrease in priming activity does not follow a linear relationship with decreasing wt gp61 concentrations. Second, there is an initial sharp drop in activity when only a small percentage (10 to 20%) of the mutant primase is present, consistent with the observations from the previous experiment.

A curve that best overlays the data points can be drawn (Fig. 4A, solid line) when the following assumptions are made. 1) Based on the observed 6 to 1 stoichiometry of primase/DNA binding and the 400-fold increase in \(k_{\text{cat}}\) at higher gp61 concentrations, we neglect the activity contributed by the monomeric species or multimeric species other than the hexamer. 2) An average rate constant (\(k_3\)) is assigned to all the hetero-hexameric species that are composed of both wt and mutant primases. This assumption is based on the observation that the major decrease in activity already takes place upon the addition of 50 nM gp61(E234Q) (12.5% of the total primase concentration). At this concentration, the primase hexamers containing one gp61(E234Q) subunit constitute about 70% of all hetero-hexameric species based on the binomial distribution (see below). A further increase in gp61(E234Q) concentration only induced a small decrease in activity. Therefore it appears that the major change in activity is induced by the incorporation of one mutant gp61 subunit. We assign \(k_1\) as the rate constant for the wt homohexamer. The mutant homohexamer is inactive.

Addition of the mutant gp61 allows the formation of hetero-oligomers between the wt and mutant enzymes. The relative abundance of each species should follow a binomial distribution if similar noncooperative binding properties obtain for two forms of gp61 primase. The equation 2 was used to calculate the amount of each hexameric species present in the reaction mixture at any given concentrations of the two primases where \(A\) and \(B\) represent the wt and E234Q primase, respectively; \(A_n\) and \(B_n\) represent the homohexamer of the wt and E234Q primase, respectively; and \(A_nB_m\) to \(A_mB_n\) (where \(n = 1\) to \(5\)) represent all hetero-hexamer species formed with both wt and E234Q primases.

\[
(xA + yB)^6 = x^6A_6 + 6x^5yA_5B + 15x^4y^2A_4B_2 + 20x^3y^3A_3B_3 + 15x^2y^4A_2B_4 + 6xy^5AB_5 + y^6B_6
\]

\[
x = \frac{[A]}{[A]+[B]}; \quad y = \frac{[B]}{[A]+[B]}
\]

A linear decrease in the priming activity at increasing mutant gp61 concentrations should be observed as shown in the theoretical curve in Fig. 4A (dashed line) if one of the following two scenarios exists: 1) there is no oligomerization of gp61 or; 2) gp61 oligomerizes but the activity of each subunit is independent of adjacent subunits (namely, addition of each mutant subunit decreases the priming activity of the hexameric species by one-sixth). The current data clearly deviate from the theoretical curve and thus negate both scenarios. With the assumptions stated above, the experimental data in the main panel of fig. 4A was fitted to equation 3.

\[
A = C\left[k_1x^6 + k_2(1-x^6-y^6)\right]
\]

\(A\) stands for the priming activity and \(C\) is the hexameric primase concentration. \(k_1, k_2, x\) and \(y\) are as defined above. A \(k_1/ k_2\) ratio of approximately 5 to 1 is generated from the fitting. The value of \(C\) does not change this ratio but does affect the absolute values of \(k_1\) and \(k_2\). If we assume that all primase monomers under these conditions form hexamers, then the fitted \(k_1\) and \(k_2\) values are 2.6 ± 0.2 and 0.47 ± 0.09 min\(^{-1}\), respectively. This result indicates that the incorporation of an inactive subunit can affect the activity of the entire hexameric primase.
In order to provide further insights into the nature of the interactions between the mutant and wt primase subunits that caused the significant drop in the priming activity, we performed a series of chemical crosslinking reactions containing a mixture of wt: E234Q primase with various mixing ratios. With wt primase alone, higher order oligomeric states (n = 4, 5, and 6) were observed (Fig. 4B, lane 1), whereas with mutant primase alone, little pentamer and hexamer and much lower levels of tetramer were observed (lane 6). When the labeled mutant gp61 was mixed with the unlabeled wt gp61, all forms of higher order oligomers were observed, which probably consist of hetero-oligomers formed with both mutant and wt gp61 subunits, but with lower quantities compared to those with wt alone. Since residue E234 is likely involved in metal ion binding, its mutation may induce a different primase conformation due to its inability to bind Mg$^{2+}$. Such a conformational change has been observed in the E. coli primase (31). Mixing of mutant and wt gp61 therefore likely results in altered oligomeric structures compared to those formed by wt alone. These irregular oligomers may lower crosslinking efficiencies between crosslinking sites on adjacent subunits, which probably contributes to the decreasing amount of the high order crosslinking products upon addition of the mutant gp61. The conformational state of the mutant gp61 does not appear to affect the binding activities of the primase towards DNA and gp41. However, such a structural perturbation could potentially affect the activity of the wt subunits adjacent to the mutant one and thus lower the activity of the entire complex.

An alternative explanation for the decreased intensity of the high order crosslinking bands upon addition of the mutant gp61 is that the altered oligomeric structure results in a decrease in the number of higher order primase oligomers. Although this may change the distribution of various hexameric species, it does not affect our main conclusions drawn from the mutant inhibition study, namely, gp61 forms oligomers and the interactions between neighboring subunits affect the stability and/or activity of the entire complex. Below, we describe an in vitro complementation assay to provide further evidence for the existence of subunit interactions that affect the activity of the primase complex.

The same set of mutant inhibition reactions was also carried out in the absence of the helicase and similar results were obtained (inset, Fig. 4A). The values of $k_1$ and $k_2$ ($0.0060 \pm 0.0005$ min$^{-1}$ and $0.00066 \pm 0.00033$ min$^{-1}$ respectively) for fitting the primase activity in the absence of the helicase gave a ratio close to 10 suggesting that the effect of inclusion of a single mutant subunit in the hexameric primase causes even a greater loss of activity. However, this interpretation should be viewed cautiously since the low intrinsic priming activity of the primase generates a signal not much greater than background.

**In vitro priming activity complementation assay**

The crystal structure of the T7 primase has been solved recently showing two structural domains connected by a flexible linker region (32). The N-terminal zinc-binding domain (ZBD) is involved in DNA binding (33) and is also thought to play a role in priming site recognition (33, 34). The C-terminal polymerization domain contains the active site for priming as well as the region that interacts with the helicase (1). A sequence alignment using an enhanced threading program (3D-PSSM) (35) suggests that T4 and T7 primases share a very similar overall three dimensional structure. A linker region between the N-terminal ZBD domain and the C-terminal domain of gp61 was identified using the T7 primase structure as a template and was further confirmed by subtilisin digestion coupled with N-terminal sequencing as described in “Materials and Methods”. This information was used to direct the cloning of the N-terminal deletion mutant of gp61 (gp61C).

Deletion of the ZBD domain abolishes the priming activity since no primer was synthesized even at 50 μM gp61C in the presence of the helicase. Gp61(E234Q) lacks catalytic activity due to the mutation of the active site conserved glutamate residue in the C-terminal domain. When both gp61(E234Q) and gp61C were mixed together, however, priming was observed if gp41 was present (Fig. 5A). The rate of priming was comparable to that of the wt primase in the absence of gp41. The priming activity increased with increasing concentrations of either gp61(E234Q) or gp61C. No priming was observed if gp41 was omitted from the reaction mixture.
Similar in vitro complementation was also observed with several T7 primase mutants (36). The fact that the priming activity could be partially restored when both mutant primases, defective in either the C- or N-terminal domain, were mixed together, strongly suggests the formation of complex structures that allow the interaction between the intact ZBD domain of gp61(E234Q) and the active C-terminal polymerization domain of the neighboring gp61C subunit, leading to the restoration of the priming activity (Fig. 5B). This interaction may be facilitated by the flexible linker region as suggested in the T7 system (36).

We noted that the primers synthesized appear as two close but distinguishable bands on the gel (Fig. 5A). The lower band migrates in the same way as the primers formed by wt gp61 (lane 8). The upper band migrates slightly slower yet its mobility is distinctly faster than those of hexaribonucleotide primers. Pentaribonucleotide primers with different nucleotide compositions move slightly differently in the sequencing gel (37). This observation therefore indicates that the reconstituted complex between gp61(E234Q) and gp61C forms primers with different base compositions from a DNA substrate containing only one priming site. The ZBD domain has been proposed to play a role in priming site recognition in the T7 system (34). This decreased priming specificity likely results from a shifted orientation between the ZBD and C-terminal domains of the two neighboring subunits within the reconstituted primase complex, therefore suggesting that the gp61 ZBD domain is also involved in priming site recognition. The altered structure of the restored complex is probably also the cause for the lower priming activity observed in the complementation assay.

Although our data do not provide direct structural information about the reconstituted gp61 oligomer, we believe that the complex mimics a ring, rather than a “side-by-side” conformation on DNA. As discussed above, wt gp61 most likely forms a ring structure around the priming site that contains the major priming activity. In order for the two mutant proteins to complement each other, it is difficult to envision a “side-by-side” arrangement that can still assume a productive conformation. Furthermore, the helicase is absolutely required for the reconstitution of the priming activity. Since the C-terminal helicase-interacting domain is intact in both mutant primases, it is reasonable to suggest that the role of the helicase is to provide a hexameric platform to facilitate the oligomerization of two mutant primases through helicase/primase interactions (38) (Fig. 5B). The requirement of the helicase for the activity complementation suggests that the oligomerization of the primase and/or its DNA binding affinity is impaired, probably as a result of the deletion of the ZBD domain, and that the hexameric helicase facilitates the formation of the primase hexamer and enhances its affinity for DNA through both helicase/primase and helicase/DNA interactions.

CONCLUSION

Oligomerization is often observed for proteins that play specific functional or structural roles. For example, replicative helicases in many systems have been shown to form oligomers (39). A hexameric ring structure of the T4 gp41 helicase has been observed by EM (11) and was recently refined to higher resolution (10). This structure increases the processivity of gp41 during replication and also makes it possible for different helicase subunits to interact with multiple sites on DNA, a binding mode thought important in helicase unwinding (40). Recent data suggest that the T4 helicase loader (gp59) may also form a ring structure during primosome assembly (20). It thus appears that the oligomerization is a common structural motif for the T4 primosome during replication.

In many replication systems, the primase functionally interacts with the helicase to form the primosome complex. Such an interaction may induce correspondingly a ring structure in the primase. Oligomerization of the primase has been observed in both the T7 and E.coli systems (17, 18). In the T4 system, however, conflicting results as to the oligomeric state of the primase when it interacts with DNA or DNA/helicase have been reported. A single subunit of gp61 was found to associate with the hexameric helicase by gel shift analysis (19). ITC and crosslinking studies, on the other hand, provided evidence for the formation of higher order structures of gp61 (7). No such structures have been found in the absence of DNA and helicase (7, 19).
The current work provides further evidence for gp61 oligomerization and analyzes its functional role during the priming reaction. Fluorescence anisotropy established a 6:1 stoichiometry for a gp61:ssDNA complex, consistent with a recent observation of a hexameric ring structure of the primase by EM (10). Moreover, kinetic studies, including the \( k_{\text{cat}} \) dependency on primase concentration, the effect of substituting a kinetically inactive subunit within the hexameric structure, and the in vitro activity complementation assay, show that the oligomeric form of the primase is the major functional form during priming. These studies also implicate interactions between the primase subunits that affect the priming activity of the entire complex.

One potential function of primase oligomerization is to increase its catalytic efficiency. Compared to the DNA polymerases, the DNA primases are generally sluggish enzymes (1). The formation of a hexameric primase ring encircling DNA increases the probability for a primase subunit to orient correctly relative to its priming site on DNA. Besides, the subunit interactions among the primase oligomers increase the activity of the entire primase complex. In light of the innate tendency of the primase for oligomerization, the helicase may stimulate the priming activity by facilitating this process as well as by organizing the individual primase subunits within the hexameric structure.

The observation made from the current study about the functional oligomerization of T4 primase also has interesting implications for the repetitive Okazaki fragment synthesis on the lagging strand. We have shown that the primase dissociates and reassociates with the replisome (primosome remodeling) during lagging strand synthesis (16, 41). Two possible scenarios for the primosome remodeling process can be envisioned.

All six primase subunits may dissociate from the helicase ring at the same time. On the other hand, the dissociation of the primase, more likely, follows a stepwise process with one or several primase subunit(s) dissociating at a time. Reassembly of the primosome then would be more efficient given the presence of a nucleation site. The latter scenario also ensures that the required priming frequency is maintained during replication, namely, by the remaining primase subunits and ample priming sites on the lagging strand.

During lagging strand synthesis, the RNA primer synthesized by the primase must be transferred to the lagging polymerase in order to initiate a new Okazaki fragment synthesis. Although which of the various proteins in the replisome participate in the primer handoff has not been determined, the transfer is achieved most likely through a number of protein-protein and protein-DNA interactions executed in a coordinated and controlled cycle. In order for the macromolecular interactions to occur, the protein factors involved in this process need to be arranged in a specific orientation. A ring shaped hexameric primase complex may increase the probability for a primase subunit to assume the correct orientation relative to the next protein component during the primer handoff process.

The fact that gp61 can form a hexameric structure in the absence of the helicase differentiates the T4 from the T7 primases. No oligomerization was observed in the absence of the helicase domain of the T7 gp4 protein (42). These results indicate that although T4 and T7 primases share a certain degree of sequence homology and structural similarity, they differ in specific structural aspects that determine their ability to form oligomeric structures and most likely the pathway for primer handoff.

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Figure legends

Figure 1. **Fluorescence anisotropy study of the gp61/DNA interaction.** Primase solution (0 to 2152 nM) was titrated into a buffer containing 100 nM 5’-OG labeled 20mer oligo B with one priming site in the middle of the sequence. Fluorescence anisotropy measurements were plotted as a function of primase concentrations. Data were fitted to a $K_d$ of $66 \pm 20$ nM and a DNA/gp61 binding stoichiometry of $0.18 \pm 0.02$. The residual plot was also shown.

Figure 2. **Effect of primase concentrations on the apparent turnover number ($k_{cat}$) of the priming reactions.** The standard priming reactions were carried out in the presence of 2 μM 70mer oligo A and 3.4 μM gp41. Primase concentrations were varied from 30 to 480 nM and the initial priming rates were measured by monitoring the amount of [α-32P]CTP incorporation into the pentaribonucleotide primers. The RNA primers were separated from unincorporated CTP on a 20% denaturing acrylamide gel and the amount of primer formation was quantified as described in the “Materials and Methods”.

Figure 3. **The inhibitory effect of gp61(E234Q) on the priming activity of the wt primase in the presence or absence of gp41.** The priming reactions were carried out under saturating oligo A (2 μM and 20 μM in the presence or absence of gp41, respectively) and gp41 (3.4 μM when present) conditions with a constant and limiting amount of wt gp61 (200 nM and 1 μM in the presence or absence of gp41, respectively). Various amounts of E234Q gp61 (0, 78, 156 and 312.5 nM in the presence of gp41; 0, 100 and 200 nM in the absence of gp41) were included in the reactions and the initial priming rates were measured as described in Figure 2 legend. The priming rate in terms of CTP incorporation was plotted against mutant gp41 concentration. A. Reactions in the presence of gp41. B. Reactions in the absence of gp41.

Figure 4. A. **The inhibitory effect of gp61(E234Q) on the priming activity of the wt primase in the presence or absence of gp41 at a constant total primase concentration (wt + E234Q gp61) and various wt/E234Q ratios.** The priming reactions were carried out under saturating oligo A (2 μM and 20 μM in the presence or absence of gp41, respectively) and gp41 (3.4 μM when present) conditions with a constant and limiting amount of total primase concentration (400 nM and 1 μM as monomer in the presence or absence of gp41, respectively). The wt/E234Q gp61 ratio was varied as indicated in figure A. Figure A shows the priming reactions carried out in the presence of gp41. The initial priming rates (pmoles of primer per minutes, open triangle) were measured by monitoring the amount of [α-32P]CTP incorporation into the primers. The theoretical curve (dashed line) was generated assuming that the primase acted either as a monomer or as a hexamer but the activity of each subunit was independent of one another. The curve that overlays the data points (solid line) was generated based on the assumptions described in the text. The fitted $k_1$ and $k_2$ values are $2.6 \pm 0.2$ and $0.47 \pm 0.09$ min$^{-1}$, respectively, with the assumption that all primase monomers under these conditions form hexamers. Figure 4A inset shows the priming activities measured in the absence of gp41. An identical fitting procedure was used that provided $k_1$ and $k_2$ values of $0.0060 \pm 0.0005$ min$^{-1}$ and $0.00066 \pm 0.00033$ min$^{-1}$. B. **The crosslinking pattern of gp61 in the presence of increasing amount of gp61(E234Q).** A mixture of wt gp61 and mutant gp61(E234Q) in the presence of 0.1 μM of oligo B was crosslinked with BMH. The total concentration of wt and mutant gp61 was kept at 0.6 μM while the ratios between the two were varied. Only one protein in
the mixture carried a biotin label. Lane 1, 0.6 μM biotin-labeled wt gp61; lane 2, 0.4 μM labeled wt and 0.2 μM unlabeled mutant; lane 3, 0.4 μM unlabeled wt and 0.2 μM labeled mutant; lane 4, 0.2 μM labeled wt and 0.4 μM unlabeled mutant; lane 5, 0.2 μM unlabeled wt and 0.4 μM labeled mutant; lane 6, 0.6 μM labeled mutant; lane 7, MW marker. C. The zoom-in picture of hexameric species in B.

Figure 5. A. *In vitro* complementation of the priming activity between gp61(E234Q) and the N-terminal deletion mutant, gp61C. The standard priming reactions were carried out in the presence of 20 μM oligo A, 2 μM gp41, 0 to 3.9 μM gp61(E234Q), and 0 to 4.8 μM gp61C as indicated. The primers were separated and analyzed on a 20% denaturing acrylamide gel. The primer shown in lane 8 was the product of wt primase in a standard priming reaction using the same DNA substrate. B. Schematic illustration of the proposed mechanism for the *in vitro* complementation of the priming activity between gp61(E234Q) and gp61C. The hexameric helicase is shown as a segmented disc. The zinc-binding domain, the linker region and the C-terminal polymerization domain of the primase are shown by the small circle, the curved line, and the oval, respectively. The cross represents the E234Q mutation in gp61(E234Q). Only two primase subunits are shown here for clarity. The reconstituted primase complex restored the priming activity by the interaction between the zinc-binding domain of gp61(E234Q) and the C-terminal polymerization domain of gp61C mediated through the flexible linker region.
Figure 1.
Figure 3.
Figure 4A.
Figure 4B & C

B

Hexamer
Pentamer
Tetramer
Trimer

204 kDa
115 kDa
95 kDa

1 2 3 4 5 6 7

C

Hexamer

1 2 3 4 5 6
Figure 5.

| Lanes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-------|---|---|---|---|---|---|---|---|
| Gp61C (µM) | 4.8 | 4.8 | 4.8 | 0 | 1.6 | 3.2 | 4.8 |
| E234Q (µM) | 0 | 1.3 | 2.6 | 3.9 | 3.9 | 3.9 | 3.9 |

A

B

- No priming activity
- Restored priming activity
The oligomeric T4 primase is the functional form during replication
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