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Functional analysis of mutant mitochondrial DNA polymerase proteins involved in human disease

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Abstract

DNA polymerase γ (pol γ) is the only DNA polymerase within the mitochondrion, and is thus essential for replication and repair of mtDNA. *POLG*, the gene encoding the catalytic subunit of pol γ , is a major locus for a wide spectrum of mitochondrial diseases with more than 100 known disease mutations. Thus, we need to understand how and why pol γ defects lead to disease. By using an extensive array of methods, we are developing a clearer understanding of how defects in pol γ contribute to disease. Furthermore, crucial knowledge concerning the role of pol γ in mtDNA replication and repair can be acquired. Here we present the protocols to characterize mutant DNA pol γ proteins, namely, assays for processive DNA synthesis, exonuclease activity, DNA binding, subunit interaction, and protein stability.

Keywords

DNA polymerase γ ; mitochondrial DNA polymerase; DNA replication; DNA repair; mitochondrial disease; enzyme assays; *POLG*; *POLG2*

1. Introduction

Mitochondrial diseases affect 1/2000 to 1/5000 people in the general population (1). A wide spectrum of mitochondrial disease affects both children and adults. Mutations in nuclear genes encoding mitochondrial DNA (mtDNA) replication components, such as *POLG* and *POLG2*, have been associated with disease (2). DNA polymerase γ (pol γ) is a heterotrimeric enzyme consisting of a 140 kDa catalytic subunit (encoded by *POLG*) with high fidelity DNA polymerase and proofreading activities, and a homodimeric 55 kDa accessory subunit (encoded by *POLG2*) for tight DNA binding and processive DNA synthesis (3,4). Pol γ is the only DNA polymerase within the mitochondrion, thus it is essential for replication and repair of mtDNA. In particular, *POLG* is a major locus for mitochondrial disease (see Fig. 1), with more than 100 mutations associated with the fatal early childhood Alpers syndrome, midlife-onset ataxia neuropathy syndromes, late onset progressive external ophthalmoplegia (PEO), male infertility, and susceptibility to nucleoside reverse transcriptase inhibitor drugs commonly used to treat HIV (see <http://tools.niehs.nih.gov/polg/> for references). Furthermore, mutations within the accessory subunit gene (*POLG2*) are known to cause PEO (5).

We, and others have characterized a number of disease mutations, in order to determine the nature of the biochemical defects arising in these mutant proteins (6,7,8,9,10,11,12,13). For

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example, the most common amino acid substitution in *POLG*, A467T, was shown to have a severe catalytic defect, as both polymerase and exonuclease activities were greatly reduced compared to wild-type (WT) enzyme (11). Interestingly, this mutation also disrupts physical association between the catalytic and accessory subunits (11). As more mutant pol γ variants are analyzed the structural basis for the biochemical defects in each enzyme is becoming clear (12). Likewise, the involvement of single nucleotide polymorphisms in disease has been found to be important. In the case of pol γ with both W748S and E1143G in the same allele, the E1143G single nucleotide polymorphism contributes both beneficial and detrimental effects to the protein (12). Since more patients with mitochondrial disease are found with *POLG* mutations every year, it is imperative that we understand the mechanisms by which these mutations cause mtDNA instability and ultimately mitochondrial disease, and the contributions of each mutation towards pathology.

By utilizing an extensive array of methods, we are developing a clearer understanding of how defects in pol γ contribute to disease. Furthermore, crucial knowledge concerning the role of pol γ in mtDNA replication and repair can be acquired. Here we present the protocols we use in the laboratory to characterize mutant DNA pol γ proteins, namely, assays to quantify polymerase activity, processivity, exonuclease activity, DNA binding, interaction between subunits, and protein stability.

2. Materials

2.1. Site-directed mutagenesis to introduce DNA polymerase γ mutations

Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

2.2. Processivity and primer extension assays

1. Enzyme dilution buffer: 50 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 50 μ g/mL acetylated bovine serum albumin (BSA), 0.1% NP-40, 0.1 M NaCl.
2. T4 polynucleotide kinase.
3. Phosphor storage screen.
4. Typhoon 9400 phosphorimager (GE Healthcare, Piscataway, New Jersey).
5. NIH Image or ImageJ software (<http://rsb.info.nih.gov/nih-image/>).

2.3. Exonuclease assay

1. Gel-purified 40mer oligonucleotide (5' -TCA CTG ATA TAC GTC CGA CTC TGA TGT ACA TGG TCA TGG T- 3').
2. Formamide stop solution: 95% deionized formamide, 0.01 M EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol.
3. 12% polyacrylamide gel: 19:1 acrylamide, 6.7 M urea, 0.084% ammonium sulfate, 0.02% TEMED in 1X Tris Borate EDTA buffer.
4. Phosphor storage screen.
5. Typhoon 9400 phosphorimager (GE Healthcare, Piscataway, New Jersey).
6. NIH Image or ImageJ software (<http://rsb.info.nih.gov/nih-image/>).

2.4. Determination of DNA binding by electrophoretic mobility shift assay

1. 38mer oligonucleotide (5'-TTA TCG CAC CTA CGT TCA ATA TTA CAG GCG AAC ATA CT-3').
2. Complementary 34mer oligonucleotide (5'-GTA TGT TCG CCT GTA ATA TTG AAC GTA GGT GCG A-3').
3. Binding buffer: 10 mM HEPES-OH (pH 8.0), 0.2 mg/mL acetylated BSA, 2 mM dithiothreitol.
4. Gels: 6% native polyacrylamide (60:1 w/w acrylamide/bis-acrylamide), 20 mM HEPES-OH (pH 8.0), 0.1 mM EDTA, 0.06% ammonium persulfate, 0.1% TEMED.
5. Phosphor storage screen.
6. Typhoon 9400 phosphorimager (GE Healthcare, Piscataway, New Jersey).
7. NIH Image or ImageJ software (<http://rsb.info.nih.gov/nih-image/>).

2.5. Immunoprecipitation assay to determine physical association of both subunits

1. Rabbit polyclonal antibodies (DPg) against recombinant pol γ (6).
2. Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Piscataway, NJ).
3. NuPAGE 4X LDS Sample Buffer (Invitrogen, Carlsbad, CA).
4. NuPAGE Novex 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA).
5. NuPAGE MOPS SDS Running Buffer (Invitrogen, Carlsbad, CA).
6. NuPAGE Antioxidant solution (Invitrogen, Carlsbad, CA).
7. Immobilon-P PVDF membrane (Millipore, Billerica, MA).
8. NuPAGE 20X Transfer Buffer (Invitrogen, Carlsbad, CA).
9. PBSN-BSA buffer: 0.05 M potassium phosphate buffer, pH7.5, 0.15 M NaCl, 0.1% NP-40, and 0.1 mg/mL bovine serum albumin (BSA).
10. Buffer TNT: 0.1% Triton X-100, 50 mM Tris-HCl, pH7.5, 0.5 M NaCl.
11. Buffer TN: 50 mM Tris-HCl, pH7.5, 0.5 M NaCl.
12. Anti-penta-His monoclonal antibody (Qiagen, Valencia, CA).
13. PBSN buffer: 0.05 M KPO₄, pH7.5, 0.15 M NaCl, 0.1% NP-40.
14. Goat anti-mouse IgG alkaline phosphatase conjugated secondary antibody (Bio-Rad, Hercules, CA).
15. Western Blue stabilized substrate for alkaline phosphatase reagent (Promega, Madison, WI).
16. 3% (w/v) trichloroacetic acid (TCA): Make a 100% (w/v) stock solution by dissolving 500 g crystalline TCA in 227 mL water to make 500 mL total volume. From this stock solution, dilute to make a 3% solution.

2.6. Circular dichroism measurements to determine protein stability

1. Jasco 810 Spectropolarimeter equipped with a Peltier thermal controller and 1 cm cell.

2. Dilution buffer: 10 mM potassium phosphate buffer (pH 7.5), 5% glycerol, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol and 200 mM NaCl.
3. Graphing software with sigmoidal curve fitting (for example, KaleidaGraph from Synergy, Reading, PA).

3. Methods

3.1 Site-directed mutagenesis to introduce DNA polymerase γ mutations

We use Stratagene's Quikchange site-directed mutagenesis kit to introduce the desired mutations into the pol γ construct.

1. The pQVSL11.4 baculoviral transfer vector encodes *POLG* cDNA without its mitochondrial targeting sequence (14). This baculovirus transfer vector is the template used to introduce the mutation of interest, using site-directed mutagenesis. For example, codon 1143 was converted to the E1143G derivative with the QuikChange site-directed mutagenesis kit and the mutagenic primers 5'-CCT GGT GCG GGG GGA GGA CCG CTA CC' and 5'-GGT AGC GGT CCT CCC CCC GCA CCA GG-3'.
2. After Quikchange, DNA is treated with the enzyme DpnI to digest and remove methylated template, and then XL1 Blue supercompetent cells are transformed according to the Quikchange protocol.
3. Introduced mutations are confirmed by sequencing the resultant baculovirus transfer vector.

3.2. Expression and purification of the catalytic subunit of DNA polymerase γ

1. Select recombinant baculovirus expressing the mutant pol γ catalytic subunit and amplify.
2. Transfect Sf9 insect cells in 30 confluent T₁₇₅ tissue culture flasks.
3. Process and purify protein as described previously (6,8,15).
4. MonoQ fractions containing each p140 derivative are flash-frozen in small aliquots in liquid nitrogen and stored at -80°C (see Notes 1 and 2; Fig. 2).

3.3. Expression and purification of the accessory subunit of DNA polymerase γ

Express the His6 affinity-tagged p55 accessory subunit in *E. coli* and purify to homogeneity as described previously (16) (see Fig. 2).

3.4. DNA processivity assay

3.4.1. Primer extension assay—The processivity of WT and mutant forms of pol γ is estimated by primer extension assay on an end-labeled, singly-primed M13 DNA substrate as described (6), without the pre-incubation step.

3.4.1.1 Phosphorylation

1. In a chilled 1.5 mL polypropylene microfuge tube, add 10X T4 polynucleotide kinase buffer (to produce a final concentration of 1X), 20 pmol of the gel-purified 38mer, 25 pmol [γ -³²P]ATP, 12 U T4 polynucleotide kinase and dH₂O to 25 μ L.
2. Incubate reactions at 37°C for 60 min.
3. Heat at 80°C for 5 min to heat-kill the kinase.

4. Cool tube on ice.

3.4.1.2 Hybridization

1. Hybridize 5 pmol ^{32}P -labeled 35mer with 5.25 pmol M13mp18 DNA. Add TE buffer to 100 μL .
2. Vortex gently. Heat tube in a 400 mL beaker containing 95°C H_2O for 5 min.
3. Cool slowly to room temperature by leaving the beaker at room temperature.
4. Store at -20°C until needed.

3.4.1.3 Extension

- 1 Dilute polymerase in enzyme dilution buffer (*see Subheading 2.2*) to desired concentration. Assemble reaction mixtures (10 μL) on ice with 0 or 100 mM NaCl, 25 μM of each dNTP, and 12 ng purified WT or mutant p140, with or without 9.6 ng of the p55 accessory subunit (gently mix p140 and p55 in a 1:2 molar ratio).
- 2 Reactions are incubated at 37°C for 30 min.
- 3 Quench reactions with 10 μL formamide stop solution.
- 4 Heat samples at $> 85^\circ\text{C}$ for 3 min and analyze the products by denaturing polyacrylamide gel electrophoresis.
- 6 Dry the gel and expose to a phosphor storage screen.
- 7 Image the radioactivity on a Typhoon 9400 phosphorimager.
- 8 Quantify bands using NIH Image software (*see Fig. 3*).

3.4.2. Processivity assay—The method is the same as above for the primer extension assay, except that heat-denatured calf thymus DNA harboring approximately 18 pmol of random 3'-ends is added to each of the reaction mixtures in **Subheading 3.4.1.3**. This acts as a polymerase trap, such that processivity can be determined for a single binding event.

3.5. Exonuclease assay

The exonuclease assay we use in our laboratory has been described previously (6,11). Briefly, we assess exonuclease activity by monitoring degradation of a 5'- ^{32}P -labeled 40mer in the absence of dNTPs.

1. Set up 1.5 mL polypropylene microfuge tubes on ice. Assemble reaction mixtures (10 μL) to contain 25 mM HEPES-KOH, pH 7.6, 1 mM 2-mercaptoethanol, 5 mM MgCl_2 , 0.05 mg/mL heat-treated BSA, 10 mM NaCl, 5 pmol of the labeled oligonucleotide, and 0.4 – 3.5 nM purified WT or mutant p140.
2. Incubate reactions at 37°C for 15 min.
3. Terminate reactions by placing tubes on ice and adding 10 μL formamide stop solution.
4. Heat samples at $> 85^\circ\text{C}$ for 3 min and analyze 2.5 μL of the products by denaturing 12% polyacrylamide gel electrophoresis as described in **Subheading 3.4.1.3** (*see Fig. 4*).

3.6. Determination of DNA binding by electrophoretic mobility shift assay

The $K_d(\text{DNA})$ value for each form of p140 was determined by electrophoretic mobility shift assay (17).

3.6.1. Substrate preparation - Phosphorylation—As described in Subheading 3.4.1.1.

3.6.2. Hybridization

1. To the chilled 1.5 mL polypropylene tube containing labeled 38mer, add 26 pmol unlabeled 34mer and water to a total volume of 40 μ L. The labeled 38mer oligonucleotide primer is hybridized with a 1.3-fold molar excess of complementary 34mer to generate a primer-template substrate possessing a three base recessed 3'-end.
2. Mix gently, and hybridize by placing tube in a 400 mL beaker containing 95°C water. Let the water slowly cool to room temperature (about 1 hr). Once the water is approximately 30°C the substrate is ready for use.
3. Assemble binding reactions (20 μ L) on ice: binding buffer (*see Subheading 2.4*), 1 pmol of 32 P-primer-template, and 0, 0.5, 1, 1.5, 2 or 4 pmol of WT or mutant pol γ protein.
4. Bind at room temperature for 10 min.
5. Add 5 μ L 50 mM HEPES-OH pH8.0, 50% glycerol to each reaction.
6. Resolve bound and unbound primer-template molecules by polyacrylamide gel electrophoresis through prechilled 6% native polyacrylamide gels at 4°C.
7. After 3 hr at 120 V, dry the gel and expose to a phosphor storage screen.
8. Image the radioactivity on a Typhoon 9400 phosphorimager.
9. Quantify bands using NIH Image software (*see Fig. 5*).

3.7. Immunoprecipitation assay to determine physical association of the subunits

To determine subunit-subunit interaction, an immunoprecipitation experiment using anti-pol γ antibodies bound to Sepharose can be performed (5,11).

3.7.1. Construction of DPg-Sepharose

1. Resuspend 1.5 mL slurry of Protein G Sepharose 4 Fast Flow beads in 10 mL Buffer PBSN (*see Subheading 2.5*).
2. Gently spin down beads at 27 *g* for 5 min, remove ethanol/supernatant.
3. Resuspend pellet in 15 mL Buffer PBSN.
4. Add 1.5 mL DPg anti-serum, mix end-over-end at 4°C for 6 hr.
5. Recover beads by spinning down at 27 *g* for 5 min.
6. Wash beads with 3 $\times$ 10 mL Buffer PBSN, store at 4°C, do not freeze.

3.7.2. Immunoprecipitation experiment

1. Equilibrate the beads in PBSN-BSA (1 volume beads to 1 volume PBSN-BSA).
2. Aliquot 30 μ L DPg-Sepharose beads per 1.5 mL polypropylene microfuge tube, place tube on ice.
3. Spin 2,700 *g* 4°C for 2 min.
4. Remove supernatant, add 400 μ L PBSN-BSA to each tube and mix.

5. To separate tubes containing prepared DPg-Sepharose beads and PBSN-BSA add: 3 μ g WT pol γ only; 3 μ g WT p55 accessory subunit only; 3 μ g mutant pol γ or p55 accessory subunit only; 3 μ g WT pol γ and 3 μ g WT p55; 3 μ g mutant pol γ protein and 3 μ g WT p55 (or 3 μ g mutant p55 accessory subunit with 3 μ g WT pol γ protein).
6. Rotate tubes end-over-end for 45 min at 4°C.
7. Collect beads by microcentrifugation at 2,700 *g* for 2 min at 4°C.
8. Remove supernatant carefully by pipeting.
9. Wash beads twice with 1 mL cold PBSN-BSA, then once with PBSN.
10. Resuspend beads in 25 μ L 2X LDS loading buffer (4X LDS loading buffer made 2X with PBSN).
11. Heat samples for 10 min at 70°C prior to analysis on a 4–12% polyacrylamide gel using NuPAGE MOPS running buffer and antioxidant solution (*see Note 3*).
12. After electrophoresis, electroblot the proteins onto an Immobilon-P PVDF membrane overnight at 80 mA using NuPAGE transfer buffer with 15% methanol and antioxidant solution.
13. Wash the membrane in Buffer TNT (*see Subheading 2.5*) for 15 min.
14. Block membrane with 5% dried milk in Buffer TN (*see Subheading 2.5*) at room temperature.
15. Incubate the blot with 0.2 μ g/mL anti-penta-His monoclonal antibody in Buffer TN containing 0.1 mg/mL BSA for 2 hr.
16. Wash the blot 3 times with Buffer TN for 10 min (*see Note 4*).
17. Incubate the blot in a 1/3,000 dilution of goat anti-mouse IgG alkaline phosphatase conjugated secondary antibody for 1 hr.
18. Wash blot thoroughly using three 10 min washes in Buffer TNT followed by three 10 min washes in Buffer TN.
19. Develop the blot with Western Blue alkaline phosphatase reagent.
20. Rinse in H₂O, then place the blot in 3% TCA for 15 min.
21. Wash in water for 2 min, air dry and scan blot (*see Fig. 6*).

3.8. Circular dichroism measurements to determine protein stability

CD studies were performed according to the method of DeRose *et al.* (18) with the following modifications (12). With circular dichroism spectroscopy, the relative amounts of alpha helix, beta sheet and random coil in a particular protein, as well as the intrinsic stability of the protein of interest can be determined.

1. Turn on spectropolarimeter and computer. Allow N₂ to equilibrate for 15 min. Allow water bath to cool down to 4°C.
2. Thaw pol γ protein aliquots on ice.
3. In a cooled 1 cm cell, add 1 mL pol γ protein at a concentration of 10 μ g/mL (diluted with cold dilution buffer; *see Notes 5 and 6*) and a stir bar.
4. Measure in quadruplicate the CD spectra from 260 nm down to 190 nm at 4°C (Fig. 7A).

5. Determine the thermal stabilities of α -helices within the p140 proteins by measuring the ellipticity at 220 nm as the temperature is increased from 28°C to 60°C by 1°C per minute, with stirring. Ellipticity should be sampled every 1°C.
6. Using a graphing program with sigmoidal curve fitting, determine the equation of each thermal denaturation curve. The melting point (T_m) will be the point of inflection of the thermal denaturation curve, while the value for the enthalpy change upon unfolding (ΔH_m) is the slope of the curve for each p140 protein (*see* Fig. 7B).

4. Notes

1. Make up all reactions in an ice bucket. Pol γ has a functional half-life at 42°C of less than 1 minute without the accessory subunit or DNA (11).
2. Refreeze as soon as possible in liquid nitrogen and store at -80°C. Enzyme preparations begin to lose DNA polymerase activity after approximately 3 freeze-thaw cycles. The enzyme buffer contains glycerol, salt and β -mercaptoethanol, which are important for stability.
3. This combination of loading buffer and polyacrylamide gel helped to minimize background due to cross-reactivity with rabbit immunoglobulin heavy chains.
4. In the Western blot protocol, it is important to thoroughly wash the blot in order to further reduce cross-reactivity and background.
5. Pol γ prefers to be stored in a high concentration of both glycerol and NaCl. However, these ingredients are in conflict with producing optimal CD spectra, as a higher background may result at shorter wavelengths (around 190 nm). Thus, a compromise is needed, as the conditions are not ideal for determining the amount of β -sheet. As pol γ appears to be highly α -helical, we have determined the thermal stability of the α -helices of pol γ at 220 nm.
6. Renaturation of pol γ is not possible, thus denatured protein samples must be discarded after melting curves are obtained.

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Mutations in DNA polymerase γ , *POLG*

Fig. 1.
Known disease mutations in *POLG*.

Fig. 2. DNA polymerase γ subunit composition. Lane 1: purified recombinant pol γ catalytic subunit (p140); lane 2: purified recombinant pol γ accessory subunit (p55).

NaCl	-	-	+	-	+
p140	-	+	+	+	+
p55	-	-	-	+	+

Fig. 3.
Primer extension assay. Reactions were assembled with the indicated components.

Fig. 4. Exonucleolytic degradation of DNA by WT and A467T pol γ . Lane 1: no enzyme control; lane 2: WT pol γ ; lane 3: A467T pol γ .

Fig. 5.
DNA binding of WT pol γ catalytic subunit as assessed by electrophoretic mobility shift assay.

Fig. 6. Immunoprecipitation assay. The G451E mutant p55 subunit fails to co-immunoprecipitate with p140. (Reproduced from ref. (5), University of Chicago Press.)

Fig. 7. Circular dichroism spectroscopy. **(A)** Circular dichroism spectrum of WT pol γ catalytic subunit; **(B)** thermal denaturation curve of WT pol γ at 220 nm.