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Purification and Comparative Assay of Human Mitochondrial Single-Stranded DNA-Binding Protein

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Abstract

The mitochondrial single-stranded DNA-binding protein (mtSSB) coordinates the function of replisome components at the mitochondrial replication fork. In recent years, it has been demonstrated that mtSSB stimulates the activities of DNA polymerase γ (Pol γ) and mitochondrial DNA (mtDNA) helicase in a concentration-dependent manner. Here we present a new approach to purify the human mtSSB and our standard assays to evaluate its biochemical properties, including a Gel Mobility Shift Assay (GMSA) to assess single-stranded DNA (ssDNA) binding activity, and an assay to assess SSB stimulation of Pol γ activity.

Keywords

Mitochondrial DNA replication; Mitochondrial single-stranded DNA-binding protein; DNA polymerase γ ; Human

1 Introduction

In vitro studies suggest that three proteins, Pol γ , the mitochondrial replicative DNA helicase (also known as Twinkle), and mtSSB are the key components of the mitochondrial DNA replisome [1, 2]. They are responsible for mtDNA synthesis and proofreading, unwinding of double-stranded DNA (dsDNA), and protection of ssDNA, respectively. In addition, mtSSB has been shown to coordinate the functions and stimulate the activities of Pol γ and mtDNA helicase [3–8]. mtSSBs are 13–16 kDa proteins that function as homotetramers [3, 9]. *In vivo*, depletion of mtSSB protein causes a reduction of mtDNA copy number in cultured cells [10]. Moreover, absence of mtSSB in *D. melanogaster* is lethal at the third larval stage of development [11]. Unlike Pol γ and mtDNA helicase, no mtSSB mutations have yet been associated with human diseases, which may imply that dysfunction of mtSSB is lethal for humans at an early stage of development.

Our group has developed and published protocols to overexpress efficiently mtSSB variants in *E. coli* cell cultures, and to purify the target protein to near-homogeneity, which has enabled the study of various recombinant mtSSB variants of human and fruit fly origin [3–5, 7, 9, 12]. Here, we present an updated purification scheme to obtain near-homogenous recombinant human mtSSB in two key steps: affinity chromatography using Blue Sepharose and either a cation exchange chromatography using phosphocellulose, and/or sedimentation

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in a glycerol gradient (Fig. 1). Some mtSSB variants bind tightly to the Blue Sepharose column and cannot be eluted using NaCl. A sodium thiocyanate (NaSCN) elution is used instead, which takes advantage of the ability of SSB proteins to retain their functional conformation after denaturation and subsequent renaturation [13–16]. Inclusion of the phosphocellulose chromatography step serves both to purify further the protein, and to ensure the removal of a dsDNA-unwinding contaminant [9].

We present a GMSA as a method to assess the ssDNA-binding activity of mtSSB [7]. In this assay, we employ a 48-mer ssDNA oligonucleotide that is close to the binding-site size previously determined for human mtSSB (Fig. 2) [17, 18]. Given that mtSSB is a key component of the mitochondrial replisome, it is important to understand its properties during replication, and in particular, how it interacts with other key proteins of the replisome. To this end, we have developed an assay to evaluate the capacity of mtSSB to stimulate the DNA polymerase activity of Pol γ *in vitro* (Fig. 3) [5]. Similarly, we describe an assay to evaluate the stimulation of mtDNA helicase activity.

2 Materials

2.1 Human mtSSB Purification

1. pET11a encoding human mtSSB without the mitochondrial presequence (available from this lab).
2. *E. coli* BL21 (λ DE3) pLysS.
3. Bacterial media (L broth).
4. 100 mg/mL Ampicillin (Filter sterilize and store at -20°C).
5. 100 mM IPTG, stored at -20°C .
6. 1 M Tris-HCl, pH 7.5, pH 8.0.
7. 2 M Tris-HCl, pH 6.8, pH 8.8.
8. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
9. 5 M Sodium chloride (NaCl).
10. 3 M Sodium thiocyanate (NaSCN).
11. 1 M Potassium phosphate ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH 7.6.
12. 80 % Sucrose, ultrapure.
13. 100 % Glycerol, anhydrous.
14. 0.2 M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Aliquots are stored at -20°C .
15. 1 M Sodium metabisulfite, 1.0 M stock solutions at pH 7.5, stored at -20°C .
16. 1 mg/mL Leupeptin prepared in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, stored at -20°C .
17. 1 M Dithiothreitol (DTT), stored at -20°C .

18. Sodium cholate.
19. 5 % Tween 20.
20. 30 % Polyacrylamide (29:1; acrylamide:bisacrylamide), stored at 4 °C.
21. 10 % Sodium dodecyl sulfate (SDS).
22. 4× Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8, 0.4 % SDS.
23. 4× Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8, 0.4 % SDS.
24. 5× SDS-PAGE running buffer: 0.125 M Tris base, 0.95 M glycine, 0.5 % SDS.
25. 5× SDS-PAGE loading buffer: 50 % glycerol, 2 M Tris base, 0.25 M DTT, 5 % SDS, 0.1 % bromophenol blue, stored at –20 °C.
26. 10 % Ammonium persulfate (APS).
27. *N,N,N',N'*-tetramethylethylene-diamine (TEMED).
28. Blue Sepharose CL-6B (Amersham Pharmacia Biotech).
29. Phosphocellulose P-11 (Whatman) prepared as per manufacturer's directions.
30. Tris-sucrose buffer: 50 mM Tris–HCl, pH 7.5, 10 % sucrose.
31. 5× Lysis buffer: 1 M NaCl, 10 mM EDTA, 10 % sodium cholate.
32. Dilution buffer: 30 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA.
33. Blue Sepharose equilibration buffer: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 0.2 M NaCl.
34. Blue Sepharose wash buffer: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 0.25 M NaSCN.
35. Blue Sepharose elution buffer 1: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 0.4 M NaSCN.
36. Blue Sepharose elution buffer 2: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 1.2 M NaSCN.
37. Blue Sepharose elution buffer 3: 35 mM Tris–HCl, pH 7.5, 10 % glycerol, 2 mM EDTA, 1.5 M NaSCN.
38. Dialysis buffer: 60 mM KPO₄, pH 7.6, 10 % glycerol, 2 mM EDTA.
39. Phosphocellulose equilibration buffer: 60 mM KPO₄, pH 7.6, 10 % glycerol, 2 mM EDTA.
40. Phosphocellulose wash buffer: 60 mM KPO₄, pH 7.6, 10 % glycerol, 2 mM EDTA.
41. Phosphocellulose elution buffer 1: 60 mM KPO₄, pH 7.6, 10 % glycerol, 2 mM EDTA.
42. Phosphocellulose elution buffer 2: 150 mM KPO₄, pH 7.6, 10 % glycerol, 2 mM EDTA.

43. Phosphocellulose elution buffer 3: 350 mM KPO₄, pH 7.6, 10 % glycerol, 2 mM EDTA.
44. Centricon-30 spin concentrator.

2.2 GMSA of Human mtSSB

1. 48-mer oligonucleotide: 5'-GGACTATTTATTAAATATATTTAAGAACTAATTCCAGCTGAGCGCCG-3'.
2. T4 polynucleotide kinase.
3. 10× PNK buffer: 700 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 50 mM DTT.
4. [γ -³²P] ATP.
5. DE-81 Chromatography paper cut into 1 × 1 cm squares.
6. 0.3 M Ammonium formate, pH 7.5.
7. 95 % Ethanol.
8. Micro Bio-Spin P-30 Tris Chromatography column.
9. Human mtSSB ~1 mg/mL in 35 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 20 % glycerol (*see* Note 1).
10. 1 M Tris-HCl, pH 7.5, pH 8.0.
11. 1 M Magnesium chloride (MgCl₂).
12. 5× SSB buffer: 100 mM Tris-HCl, pH 7.5, 20 mM MgCl₂.
13. 30 % Polyacrylamide (59:1; acrylamide:bisacrylamide), stored at 4 °C.
14. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
15. 5× TBE buffer: 450 mM Tris-HCl-borate, 10 mM EDTA.
16. 0.8 M Sodium chloride (NaCl).
17. 1 M Dithiothreitol (DTT).
18. 10× sample loading buffer: 50 % glycerol, 0.25 % bromophenol blue.
19. Phosphor Screen.
20. Storm 820 Scanner.

2.3 Assay of Stimulation of Pol γ Activity by Human mtSSB

1. Pol γ α 200 nM in 30 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 5 mM β -Mercaptoethanol, 20 % glycerol.

¹Following the Blue Sepharose chromatography step, if the presence of a dsDNA-unwinding contaminant is not a concern (e.g., as is the case for GMSA and the assay for stimulation of DNA polymerase activity), the protein can be purified further using glycerol gradient sedimentation [9].

2. Pol $\gamma\beta$ 275 nM in 30 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM β -Mercaptoethanol, 20 % glycerol.
3. mtSSB 20 μ M in 35 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 20 % glycerol (*see* Note 1).
4. Singly-primed M13 DNA (6407 nt), 125 nM in 10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 30 mM Na citrate, 8 mM EDTA.
5. Bovine serum albumin (BSA).
6. 1 M Tris-HCl, pH 8.5.
7. 1 M Magnesium chloride (MgCl_2).
8. 1 M Dithiothreitol (DTT).
9. 2 M Potassium chloride (KCl).
10. 100 % Glycerol, anhydrous.
11. Dilution buffer: 30 mM Tris-HCl, pH 7.5, 8 % glycerol.
12. 5 $\times$ γ N buffer: 250 mM Tris-HCl, pH 8.5, 20 mM MgCl_2 , 2 mg/mL BSA.
13. 50 $\times$ dNTPs mix: 1 mM dATP, 1 mM dTTP, 1 mM dGTP, 0.5 mM dCTP.
14. α - ^{32}P dCTP 10 $\mu\text{Ci}/\mu\text{L}$.
15. 100 % Trichloroacetic acid (TCA).
16. 100 % Sodium pyrophosphate (NaPPi).
17. Stop solution (10 % TCA, 0,1 M NaPPi).
18. 12.1 M Hydrochloric acid (HCl).
19. Acid wash solution: 1 M HCl, 0,1 M NaPPi .
20. 95 % Ethanol.
21. Glass-fiber filter paper.
22. Glass vials, 20 mL.

3 Methods

3.1 Human mtSSB Purification

3.1.1 Bacterial Cell Growth and Protein Overproduction (See Note 2)

1. Use *E. coli* BL21 (λ DE3) pLysS containing pET11a-human mtSSB (complete cDNA without the mitochondrial presequence) to inoculate 2 L of L broth containing 100 $\mu\text{g}/\text{mL}$ ampicillin at $A_{595} = 0.06$, and grow with aeration at 37 $^{\circ}\text{C}$.
2. After an $A_{595} = 0.6$ is reached:

²Using the pET11 system, we express 50–100 mg of mtSSB protein per liter of cell culture.

- a. Dispense a 1 mL aliquot of uninduced cells in a microcentrifuge tube. Centrifuge cells to pellet, remove supernatant and resuspend pellet in 200 μ L of 1 $\times$ SDS loading buffer. Use 10 μ L of the aliquot as a control for the SDS-PAGE analysis.
 - b. Induce expression of target protein with the addition of IPTG to a final concentration of 0.2 mM and continue to incubate for 3 h at 37 °C.
3. Cell harvest:
 - a. Save a 1 mL aliquot of the induced cells as in **step 2a**. Use 5 μ L for the SDS-PAGE analysis of protein overexpression. Use Coomassie Blue to stain the gel (*see* Note 3).
 - b. Harvest cells by spinning at 3600 $\times$ g for 5 min at 4 °C in Sorvall GSA bottles. Discard the supernatant.
 - c. Resuspend resulting pellets in 1/10 volume of the original culture with Tris-sucrose buffer. Use one-half of the total resuspension volume to resuspend pellets, then transfer to a pre-chilled beaker. With the remaining one-half of the volume wash the GSA bottles and combine in the beaker.
 - d. Divide the washed cells into Sorvall SS-34 tubes, and centrifuge at 3000 $\times$ g for 5 min at 4 °C. Remove the supernatant and freeze the resulting pellets in liquid nitrogen. Store at –80 °C.

3.1.2 Cell Lysis and Preparation of Soluble Fraction

1. Thaw cell pellets on ice for 30 min.
2. Resuspend cell pellet in 1/25 volume of Tris-sucrose buffer as described above.
3. Add 5 $\times$ lysis buffer to a 1 $\times$ final concentration and incubate on ice for 30 min with gentle inversions every 5 min.
4. Use liquid nitrogen to freeze cell suspension, thaw partially in ice water, then transfer to ice until completely thawed.
5. Centrifuge cells at 17,500 $\times$ g for 30 min at 4 °C in Sorvall SS-34 tubes.
6. Collect supernatant (soluble fraction, Fraction I) by pipetting into a fresh, pre-chilled tube.
7. Use dilution buffer to adjust the sample to 200 mM NaCl equivalent.

3.1.3 Blue Sepharose Chromatography

1. Pack a Blue Sepharose column (5–7 mg of total protein/mL of resin) and equilibrate with 10 column volumes (CV) of equilibration buffer at a flow rate of 1 CV/h.

³The SDS-PAGE gel must be stained with Coomassie Blue. A silver-stained gel is not suited for detection of human mtSSB, especially when analyzing the level of induction in the bacterial cell extract.

2. Load the salt-adjusted sample onto the column at a flow rate of 1 CV/h.
3. Wash column with 1 CV of wash buffer at a flow rate of 2 CV/h, collecting 1 CV fractions.
4. Elute column with 8 CV of the elution buffers containing 0.4–1.2 M NaSCN in a linear gradient at 2 CV/h. Collect 1/6 CV fractions.
5. Perform a final elution step with 2 CV of elution buffer containing 1.5 M NaSCN. Collect 1/4 CV fractions (*see* Note 4).
6. Analyze fractions by SDS-PAGE on 17 % minigels followed by Coomassie Blue staining, and pool fractions accordingly (Fraction II).
7. Dialyze Fraction II against buffer containing 60 mM KPO₄, pH 7.6, 10 % glycerol, 2 mM EDTA, until the fraction is at an ionic equivalent of 60 mM KPO₄ (Fraction IIb).

3.1.4 Phosphocellulose Chromatography (See Note 1)

1. Pack a phosphocellulose column (0.5 mg of total protein/mL of resin) and equilibrate with 10 column volumes (CV) of equilibration buffer at a flow rate of 1 CV/h.
2. Load Fraction IIb onto the column at a flow rate of 0.8 CV/h.
3. Wash column with 2.5 CV of wash buffer at a flow rate of 2 CV/h, collecting 1/2 CV fractions.
4. Elute column with 5 CV of the elution buffers containing 60–150 mM KPO₄ in a linear gradient at 2 CV/h. Collect 1/6 CV fractions.
5. Perform a final elution step with 2 CV of elution buffer containing 350 mM KPO₄. Collect 1/4 CV fractions (*see* Note 5).
6. Analyze fractions by SDS-PAGE on 17 % minigels followed by Coomassie Blue staining, and pool fractions accordingly (Fraction III).
7. Use a Centricon 30 concentrator (pre-treated with 5 % Tween 20 overnight at room temperature) to spin concentrate the sample to ~1 mg/mL, (Fraction IIIb) (*see* Note 6).
8. Aliquot Fraction IIIb into microcentrifuge tubes, freeze in liquid nitrogen and store at –80 °C.

⁴The wild-type *Hs* mtSSB can be eluted from the Blue Sepharose column with NaCl by increasing the ionic strength of the elution buffers [9].

⁵*Hs*mtSSB proteins elute at ~80 mM KPO₄.

⁶After overnight treatment with 5 % Tween 20, rinse thoroughly with Milli-Q water.

3.2 Electrophoretic Mobility Shift Assay of Human mtSSB

3.2.1 5'-End Labeling of 48-mer Oligodeoxyribonucleotide

1. The 50 μL kinase reaction contains $1\times$ PNK buffer, $[\gamma\text{-}^{32}\text{P}]$ ATP ($0.44\text{ }\mu\text{M}$, 4500 Ci/mmol), 340 pmol (as nt) of oligonucleotide, and 20 units of T4 polynucleotide kinase.
2. Incubate the reaction for 30 min at $37\text{ }^{\circ}\text{C}$.
3. Spot $0.25\text{ }\mu\text{L}$ of the reaction onto 5 squares of DE-81 chromatography paper ($1\times 1\text{ cm}$). Wash 3 of the squares in 0.3 M ammonium formate, twice for 5 min, then once in 95 % ethanol. Dry all 5 squares under a heat lamp for 5 min. Count radioactivity on each square in scintillation fluid, and calculate incorporation (*see* Note 7).
4. Purify labeled oligonucleotide using a Micro Bio-Spin P-30 Tris chromatography column.

3.2.2 Gel Mobility Shift Assay

1. Prepare a master mix such that each reaction contains $1\times$ SSB buffer, 1 mM DTT, 36 fmol of ^{32}P -48-mer substrate, and 50 mM NaCl. Prepare the reaction mixture in a microcentrifuge tube, heat the mix to $100\text{ }^{\circ}\text{C}$ for 5 min, vortex and centrifuge briefly.
2. Dispense the mix into microcentrifuge tube(s) on ice. Adjust with water for a final volume of $20\text{ }\mu\text{L}$ after considering the addition of desired amount of the mtSSB.
3. Add the desired amount of mtSSB (for example: 0, 1, 2, 4, 6, 10, and 15 nM as tetramer) to the microcentrifuge tube(s) and incubate at $20\text{ }^{\circ}\text{C}$ for 10 min.
4. Add $2\text{ }\mu\text{L}$ of $10\times$ sample buffer to the reaction(s).
5. Load sample(s) onto a pre-run (200 V for 2 h) 6 % native polyacrylamide gel and electrophorese for 3 h at 200 V in $1\times$ TBE (*see* Note 8).
6. Dry gel under vacuum with heat, and expose to a Phosphor Screen from 4 h to overnight.
7. Scan Phosphor Screen using the Storm 820 scanner. Determine the volume of each band and subtract background using computer integration analysis (*see* Note 9).

3.3 Assay of Stimulation of Pol γ Activity by Human mtSSB

1. Adjust a water bath to $37\text{ }^{\circ}\text{C}$.

⁷Incorporation is calculated as: $[(\text{average cpm of three washed papers})/(\text{average cpm/pmol of unwashed papers})]/\text{pmol ends in the reaction}$.

⁸Use a fan for cooling, dye-front migration should be approximately 10 cm.

⁹The amounts of shifted and free oligonucleotide are calculated as: $\% \text{ ssDNA bound} = [V_S/(V_S + V_F)] \times 100$; V_S represents the volume of the shifted and V_F represents the volume of unshifted oligonucleotide in the lane of interest.

2. Prepare a master reaction mix such that each 25 μ L reaction contains 1 $\times$ γ N buffer, 10 mM DTT, 1 $\times$ dNTPs, 2.4 nM M13 template, 2 μ Ci α -³²P dCTP, and 15 mM KCl.
3. Dispense the mix into pre-chilled microcentrifuge tube(s) on ice. Adjust with water for a final volume of 25 μ L after considering the addition of desired amounts of the mtSSB and Pol γ .
4. Add the desired amount of mtSSB as 5 μ L aliquots (for example, the final concentration of: 0.25, 0.40, and 0.65 μ M as tetramer) to the microcentrifuge tubes. Include a reaction with no mtSSB as a control.
5. Prepare dilutions of Pol $\gamma\alpha$ and β at 7.2 and 40 nM, respectively. Mix equal volumes of the protein dilutions and dispense 10 μ L into the tubes containing the master mix and mtSSB (*see* Note 10).
6. Incubate the tubes for 30 min at 37 °C, then transfer to ice.
7. Stop the reactions with 0.5 mL of stop solution and incubate on ice for 5 min.
8. Filter samples through glass-fiber filters. Wash the reaction tube twice with acid wash solution, then wash the filtration funnel three times with acid wash solution and once with 95 % ethanol.
9. Dry the filters under a heat lamp for 5 min, then count in scintillation fluid.
10. Spot 1 μ L of mix directly onto two glass fiber filters, dry, and count in scintillation fluid without filtration to calculate the specific radioactivity of the mix and determine nucleotide incorporation (*see* Note 11).

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¹⁰We use an ~sixfold molar excess of the Pol $\gamma\beta$ dimer over Pol $\gamma\alpha$ to ensure complete reconstitution of Pol γ .

¹¹The DNA polymerase activity of Pol γ is measured as pmol of nucleotides incorporated into the nascent DNA strand. The molar amount of incorporated nucleotide is calculated as follows:

- a. Incorporated nucleotide = average cpm of washed filters / [(average cpm/ μ L of unwashed filters) / (pmol/ μ L of nucleotides spotted)]
- b. To obtain fold of stimulation, calculate the ratio of nucleotide incorporation in the samples containing mtSSB as compared to those without mtSSB.

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Fig. 1.

Purification of *HsmtSSB*. Protein fractions were denatured and electrophoresed in a 15 % SDS-polyacrylamide gel, and the proteins were detected by staining with Coomassie Blue. *Lane 1*: molecular weight standard; *lane 2*: soluble cell extract (Fraction I, 2.3 μg); *lane 3*: Blue Sepharose pool (Fraction II, 4.7 μg); *lane 4*: concentrated Fraction II (Fraction IIb, 8.6 μg); *lane 5*: glycerol gradient pool (Fraction III, 4.2 μg)

Fig. 2. GMSA of *HsmtSSB*. Concentrations of 1, 2, 4, 6, 10, and 15 nM (as tetramer) of *HsmtSSB* were incubated with a radiolabeled 48-mer oligonucleotide in buffer containing 50 mM NaCl. “-” denotes no added mtSSB, U and B denote the unbound and mtSSB-bound fractions, respectively

Fig. 3.

Stimulation of *Hs* DNA polymerase γ by mtSSB. The stimulation assay was performed as described under Subheading 3, using 60 fmol of singly primed M13 DNA, 35 fmol of *Hs* Pol γ A, 218 fmol of *Hs* Pol γ B and increasing amounts of *Hs*mtSSB: 0, 6.4, 10.7, 16, and 32 pmol. The data were normalized to the nucleotide incorporated by *Hs* Pol γ in the absence of mtSSB