Study on protein-protein interaction between scRad51 and Tpa1

Mohammed Tanveer Ahmed

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Department of Biotechnology

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Tanveerahmed

MOHAMMED TANVEER AHMED

(BO14MTECH11004)

Approval Sheet

This thesis entitled 'Study on protein-protein interaction between scRad51 and Tpa1' by Mohammed Tanveer Ahmed is approved for the degree of Master of Technology from IIT Hyderabad.

N. K. Raghavendra

Dr. N. K. Raghavendra

Assistant Professor

Department of Biotechnology, IIT Hyderabad

Internal Examiner

G. Satyanarayan

Dr. G. Satyanarayan

Associate Professor

Department of Chemistry, IIT Hyderabad

External Examiner

Anindya Roy

Dr. Anindya Roy

Associate Professor

Department of Biotechnology, IIT Hyderabad

Adviser

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Abstract

In this study, the interactions between the yeast proteins Tpa1 (involved in direct demethylation repair) and scRad51 (involved in recombination repair) are analysed. The interactions between the bacterial homologs, AlkB and RecA were shown in previous research done in this lab. For this study, the proteins were purified using nickel-affinity columns and the interactions were analysed through FPLC. Even though the interactions between bacterial proteins were shown, the same could not be proved for these proteins.

Contents

Declaration i

Approval Sheet ii

Acknowledgements iii

Abstract iv

Chapter 1: Introduction 1

Chapter 2: Materials and Methods 8

Chapter 3: Result and Discussion 19

Chapter 4: Figures 21

References 26

Chapter 1

Introduction

1.1 DNA damaging alkylating agents are cytotoxic:

The genome of every cell is exposed to environmental and/or endogenously generated DNA damaging alkylating agents. Even cancer chemotherapy uses some of the alkylating agents (1). Methylating agents, such as methylmethane sulfonate (MMS), methylate double-stranded DNA bases and generate 7-methylguanine (7meG) and 3-methyladenine (3meA) (2). These block DNA synthesis and hence are considered to be lethal lesions. MMS also methylates single-stranded DNA and generates 1-methyladenine (1meA) and 3-methylcytosine (3meC) (3). These sites participate in base pairing, so they are protected within double-stranded DNA, but they are momentarily exposed during certain processes, such as transcription or recombination. Therefore, MMS causes genotoxicity mainly by generating these major lesions, viz., 3meA, 1meA and 3meC (3, 4).

1.2 *E. coli* AlkB repairs methylation damage:

The *E. coli* AlkB protein eliminates damaged DNA by mechanism of oxidative dealkylation. Comparative structural analysis of the AlkB with no ligand and AlkB with ssDNA having 1-meG (PDB IDs: 3KHB and 3KHC, respectively) unveils many

fascinating mechanistic details (5). The protein utilizes both hydrophobic interactions and hydrogen bonding, and spreads over five nucleotides to maintain the protein-DNA complex. The phosphodiester backbone of ssDNA binds within the DNA binding groove (electropositive) created by Thr 51, Tyr 55, Ser 129, and Lys 127 positions. This helps in the flipping of alkyl-lesions into the substrate binding center of the AlkB protein where the metal binding residues (His 131, Asp 133, and His 187) and substrate binding residues come close to each other. The base substrate binds in the active site patch formed by non-polar stacking interaction of Trp 69 and His 131 (5). These interactions help to stabilize the flipped conformation of 1meG in substrate binding center. It guides to the proper positioning of the methyl group of the 1-meG adjacent to the bound metal ion for oxidative repair. The common interactions between the amino acid residues of AlkB protein and the phosphodiester backbone or methylated base of the nucleotide does not distinguish between ribonucleotide and deoxyribonucleotide substrates, which explains the capability of AlkB to function on both RNA and DNA (5).

A crystal structure of *E.* coli AlkB complex containing a trinucleotide and 1meA revealed several details regarding the catalytic site. The nucleotide backbone is bound in a cooperative hydrogen bonding (H-bonding) network in which the two phosphates, that are 5' to the alkylated adenine, interact with Thr 51, Tyr 76 and Arg 161. The

first two residues are located in flexible segments of the nucleotide-recognition lid, while Arg 161 comes from a flexible surface loop comprising residues 159–164 in the dioxygenase core. The centre of the H-bonding network is Tyr 76, which forms a bidentate H-bond bridging the two phosphates. Arg 161 forms salt bridge to the 5'phosphate of the alkylated adenine, which explains the importance of this phosphate for substrate affinity. However, there are no other salt bridges to the trinucleotide, and the substrate binding pocket does not carry significant net charge. The alkylated 1-methyladenine base is bound in a deep, predominantly hydrophobic cavity, where it is sandwiched between invariant residues Trp 69 from the nucleotide-recognition lid and His 131, an iron cation-ligating residue in the dioxygenase core (6, 7).

1.3 Structure of *Saccharomyces cerevisiae* Tpa1 DNA Repair Protein:

Several homologs of AlkB have been discovered in several organisms ranging from bacteria to humans, except in *Saccharomyces cerevisiae*. Upon scanning the genome of budding yeast, an uncharacterized ORF was found that shared significant sequence homology with AlkB. It was named Termination and Polyadenylation protein (Tpa1) as the protein was part of a ribonucleoprotein complex along with eRF1, eRF3 and Poly-A binding protein (8).

Tpa1 is a 644aa long protein with a mass of about 74kDa. A study showed that it belonged to a family of 2-oxoglutarate and iron-dependent dioxygenases, of which even

AlkB is a member (9). Structural analysis of Tpa1 showed that it consists of two domains: an N-terminal domain and a C-terminal domain (10). Only the N-terminal domain of Tpa1 was shown to contain the binding sites for iron and 2-oxoglutarate (10). Hence, it was deduced that the Tpa1 N-terminal domain (NTD) could be structurally similar to AlkB. The structure of the N-terminal domain of Tpa1 protein could be superimposed upon the AlkB structure. Another study showed that Tpa1 indeed is involved in the repair of MMS-induced DNA methylations, in a similar manner to AlkB (11).

1.4 AlkB interacts with RecA:

The *E. coli* RecA protein is a 352 amino acid polypeptide and essential for recombination. The RecA structure shows a large core domain, and two smaller domains at the N- and C-termini (12-14). In the active RecA filament, ATP is bound at the subunit-subunit interface (15). RecA protein binds to the single-stranded DNA with one RecA monomer for every three bases of DNA and forms nucleoprotein filament accompanied by ATP hydrolysis. These RecA filaments promote alignment of the ssDNA with a homologous duplex DNA, strand exchange and the subsequent branch migration (16). Beside nucleoprotein filament formation, RecA also has coprotease activity, that stimulates the autocatalytic cleavage of the LexA repressor. LexA is the repressor of many DNA damage-inducible genes, including *recA* and

cleavage of LexA repressor promote induction of many *lexA* regulated genes. This response to DNA damage is known as SOS response (17). RecA also directly facilitates replicative bypass of DNA lesions by associating with DNA polymerase-V (pol-V) during SOS response (18). It has been reported that AlkB prefers damaged ssDNA over undamaged ssDNA as a substrate (6) and AlkB identifies alkylated base lesions by scanning the genome (7).

To understand the mechanism of recruitment of AlkB to the damaged DNA, AlkB was purified and a targeted proteomic analysis of proteins co-purified with AlkB protein was performed using mass spectrometry (Unpublished observation). It was found that AlkB physically interacts with the N-terminal domain of recombination repair factor RecA. RecA protein is present in every organism and is necessary for genetic recombination and recombinatorial DNA repair (19, 20). The functional significance of RecA-AlkB interaction is not yet clear.

1.5 *Saccharomyces cerevisiae* scRad51 protein:

The *Escherichia coli* RecA protein is a very well-characterized protein. The RecA family consists of proteins which function in similar ways. Rad51 is a part of that family, which also includes archaeal RadA and eukaryotic Dmc1 (23). The *S. cerevisiae* Rad51 is a 400aa long protein with a mass of approx. 43kDa (24). It is a part of a recombination complex that includes Rad51, Rad52 and others (25). It is involved in

the repair of DNA strands through recombination, both during mitosis and meiosis, unlike the Dmc1 protein that is involved only during meiosis (26). It is also known to be regulated during the cell cycle, peaking during the G1-S transition (27). The protein shares 30% sequence identity with *E. coli* RecA protein, yet has a significant structural similarity. It binds to the DNA molecules in a very similar way as the RecA protein. The helical filaments formed by the Rad51 appear similar to the filaments formed by the RecA proteins (28).

1.6 N-terminal domain of RecA is similar to that of Rad51:

The RecA NTD contains only a helix (residues 1–23) and a β-loop (residues 24–33). By contrast, the NTDs of archaeal and eukaryotic RecA family proteins are composed of two helix-hairpin-helix (HhH) motifs. It has been reported that the α-helix of the RecA NTD (residues 1–23) shows significant amino acid sequence conservation with the second HhH motif of archaeal and eukaryotic RecA family proteins. First, Gly15 and Lys23 of RecA are conserved in all RecA family members. The equivalent respective residues in human Rad51 are Gly65 and Lys73. Second, Lys8 of E. coli RecA also conserved in eukaryotic Rad51 and Dmc1 proteins. The positively charged side chains of Lys8, Lys19, and Lys23 all point outward to the outermost surface of the active RecA-ssDNA presynaptic nucleoprotein filament. Intriguingly, these three lysine

residues are conserved among most, if not all, prokaryotic RecA proteins. Third, Glu18 of RecA is also conserved in several other RecA family proteins.

Objectives:

As we have strong evidence that proves that purified AlkB and RecA forms stable complex, where RecA enhances AlkB-catalyzed repair of methyl ssDNA adducts, we wanted to examine whether similar interaction also exists between the eukaryotic homologs of AlkB and RecA, which are Tpa1 and scRad51 for *S. cerevisiae* respectively. Hence, the objectives of this project are:

1. Cloning, expression and purification of His-scRad51
2. Interaction study of Tpa1 and scRad51 *in-vitro*.

Chapter 2

Materials and Methods

2.1 General Techniques

2.1.1 Agarose gel electrophoresis:

Horizontal agarose gel (0.6-1.2%) electrophoresis was routinely performed for separation of DNA fragments of various lengths (between 100bp-10kb). Appropriate amount of agarose (SeaKem®LE, LONZA, Cat. no. 50005) was dissolved in 1X TAE buffer (40mM Tris-acetate; 1mM EDTA pH 8.0) by heating in microwave oven. After cooling, ethidium bromide of concentration 1µg/ml was added into the gel solution and then poured into a mould and a comb was inserted to generate wells. After 30-45 minutes, when the gel got solidified completely, gel was mounted into electrophoresis chamber containing 1X TAE (40mM Tris-acetate; 1mM EDTA pH 8.0) and comb was removed gently. DNA samples and size marker were mixed with appropriate volume of 6X DNA loading dye (6x stock: 0.25% bromophenol blue; 0.25 xylene cyanol FF; 30% v/v glycerol (Thermo scientific, Cat. No. R0611). Electrophoresis were generally carried out at 60-120 volt till the xylene cyanol dye migrated to distance of half of the gel. DNA bands were visualized in gel documentation system from SynGene (Model no: Chemi XR5, S.No DR4V2/2355). DNA ladder of 1kb (Cat no: SM0311 Thermo scientific) was used for Tpa1 and scRad51, and linearized plasmid DNA for fragment size determination.

2.1.2 Preparation of competent *E. coli* cells:

Competent *E. coli* cells of DH5α and BL21(DE3)pLysS were prepared as described by Alexander by using MnCl2 and CaCl2. 20µl of *E. coli* strains (DH5α and BL21(DE3)pLysS) were taken from frozen glycerol stock and inoculated in 3ml of LB medium containing no antibiotics and incubated for overnight in a shaking

incubator at 200 rpm. 1ml of this overnight grown culture was inoculated in a pre-warmed 100 ml of LB medium (1% tryptone, 0.5% yeast extract) prepared in 250ml Erlenmeyer flask. Inoculated culture was grown for about 4h at 30°C at 200 rpm in shaking incubator and the culture growth was monitored by measuring OD600 spectrophotometer every 20 minutes. When culture had reached OD600 of ~0.35 it was taken out and incubated on ice for 1hr. Cells were harvested in two sets by centrifugation at 4000 rpm for 15 min at 4°C. The supernatant was discarded and the cell pellets were resuspended in 12ml of acid salt buffer-A (ASB-A, sodium acetate- 40mM, CaCl2- 100mM, MnCl2- 70mM, pH 5.5) and incubated on ice for 1 hour. Then solution from both the falcon tube were combined in one falcon tube and the ASB treated cells were then pelleted by centrifugation carried out at 3500 rpm for 15 min at 4°C and resuspended in 2ml of ASB-B (Sodium acetate- 40mM, CaCl2- 100mM, MnCl2- 70mM, 15% glycerol, pH 5.5) and were stored in Eppendorf tubes (aliquots of 50ml) in deep freezer at -86 °C for future use.

2.1.3 Purification of DNA from agarose gels (gel extraction):

DNA was purified from agarose gels using GeneJET gel extraction kit (Fermentas Life Sciences, cat. No. K0691) following the instructions recommended by the manufacturer.

2.1.4 Restriction digests and Ligation:

All restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. Ligation reactions were performed using T4-DNA ligase (New England Biolabs), used as recommended by the manufacturer. Whole ligation reactions were used for transformation into *E. coli* (DH5α).

2.1.5 Plasmid DNA Isolation from mini bacterial culture:

A single colony was picked out from the plate containing colonies of DH5α transformed cells and inoculated in 2ml of LB medium containing kanamycin. The culture was incubated in a shaking incubator at 37°C, 200 rpm for overnight. Entire culture was used for isolation of plasmid DNA using Plasmid Mini-Prep kit (Fermentas Life Sciences, cat. No. K0502) following the instructions recommended by the manufacturer.

2.1.6 Transformation of competent *E. coli* cells:

(I) *Transformation of competent DH5α cells*: DH5α competent *E. coli* cells were prepared as described above. 5µl of a ligation mixture was added to 25µl of competent DH5α cells and incubated on ice for 30 minutes. After that heat shock was given at 42°C for 30 seconds, cells were again briefly incubated on ice for 5 minutes and transformed competent cells were plated on LB- agar plates containing 100µg/ml kanamycin. Plates were incubated at 37°C overnight.

(II) *Transformation of competent BL21(DE3)pLysS cells*: BL21(DE3)pLysS competent *E. coli* cells were prepared as described above. 1µl of a plasmid DNA was added to 20µl of competent cells and the mixture was incubated on ice for 5 minutes. After a 42°C heat shock for 30 seconds, the cells were briefly incubated on ice for 5 minutes. After this incubation, entire transformation mixture was plated on LB-agar plates containing 100µg/ml kanamycin. Plates were incubated at 37°C overnight for growth of cells.

2.1.7 SDS-PAGE:

Recombinant proteins were analyzed through SDS-PAGE. SDS-PAGE was carried out using discontinuous buffer system as described by Laemmli using Bio-Rad gel electrophoresis apparatus (Cat. no: 165-8001). Throughout this study 1.5mm thick 10% and 12% polyacrylamide (30:0.8 acrylamide to bisacrylamide ratio) gel containing 0.1% SDS was used for electrophoretic separation of proteins. The

protein samples were mixed with half volume of 3X sample buffer (2.4ml 1M Tris-HCl, pH 6.5; 3ml 20% SDS, 3ml 100% Glycerol, 1.6ml β-mercaptoethanol, 0.006g Bromophenol blue) and heated at 100°C for 10 minutes and were loaded into the wells of precast polyacrylamide gel. Electrophoresis was performed at a constant voltage range of 100-140V by diluting 10x buffer (Tris base 30.3g, Glycine 144g, SDS 10g in 1000ml). After the electrophoresis, the gel was stained with coomassie brilliant blue (0.4% w/v Coomassie blue R250, 30% v/v Methanol, 10 % Acetic acid). Spectra multicolor broad range protein ladder was used to confirm the size of protein. Composition of 10% and 12% SDS-PAGE gel is given below:

SDS-PAGE Gel Composition

Resolving Gel			Stacking Gel	
Components	10%	12%	Components	4%
30% Acrylamide bisacrylamide	1.65 ml	2.0ml	30% Acrylamide bisacrylamide	0.65 ml
Tris-HCl, pH 8.8, 1.5M	1.25 ml	1.25 ml	Tris-HCl, pH 6.8, 0.5M	1.25 ml
H_2O	2.05 ml	1.70 ml	H_2O	3.05 ml
SDS (10%)	50.0µl	50.0µl	SDS (10%)	50µl
APS	45.0µl	45.0µl	APS	25 µl
TEMED	20.0µl	20.0µl	TEMED	10.0µl

2.1.8 Media and Solutions:

De-ionized water was used for all buffers solutions and media.

Media	Composition
LB media	1%Tryptone w/v, 0.5% w/v Yeast Extract
TB media	1.2% Tryptone w/v, 2.4% Yeast Extract, 0.4% v/v glycerol

2.1.9 Other solutions:

i) Isopropyl β-D-1-thiogalactopyranoside (1M): IPTG 4.7g, H_2O 20ml.

ii) 20% SDS: SDS (20g), H_2O (80ml). Make final volume 100 ml with stirring.

iii) 20% Ammonium per Sulfate: APS (0.2g), H_2O (0.8 ml).

2.1.10 General buffers used in the study:

Buffers	Composition
TE (Tris-EDTA) 50X	1mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0. A 10X stock solution was routinely used to prepare 1X TE.
SDS-PAGE Resolving gel buffer	1.5 M Tris-HCl, (for Resolving Gel) ,pH 8.8
SDS -PAGE Stacking gel buffer	0.5 M Tris-HCl (for stacking gel), pH 6.5
10X SDS-PAGE gel running buffer	Tris base 30.3g, Glycine 144g, SDS 10g (add last) , Make final volume to 1000ml.
1X Gradient gel running buffer	25mM Tris Base, 192mM Glycine, 0.1 % (w/v) SDS, methanol 20%.
3X SDS-PAGE loading dye (10ml)	1M Tris-Cl, pH 6.5 (2.4 ml), 20% SDS (3 ml), Glycerol (100%) (3 ml), β-mercaptoethanol (1.6 ml), Bromophenol blue (0.006g).
SDS-PAGE staining solution.	Coomassie blue R250-0.4% w/v, Methanol-30% v/v, Acetic acid 10%v/v
SDS-PAGE de-staining solution	Methanol- 30 % v/v, Acetic acid 10 %v/v.

2.2.1 Cloning of His-scRad51:

2.2.1.1 PCR amplification of scRad51:

The genome of *Saccharomyces cerevisiae* was isolated, purified and used as template to amplify the scRad51 gene. The PCR was carried out using Phusion High-Fidelity DNA Polymerase (Cat. No: M0530S). The gene sequence was retrieved from NCBI and forward and reverse primers were designed accordingly. The primers scRad51-BamHI-Sen (5' AATTCCCGGGATCCATGTCTCAAGTTCAAGAAC 3') and scRad51-XhoI-Anti (5' AGATCTCGAGCTACTCGTCTTCTTCTCTGGG 3') were synthesized by Imperial Life Sciences, Haryana, India. Full-length scRad51 gene was amplified using the specified primers. The PCR reaction had dNTP-200 µM, primers-1.0 µM each and enzyme 1 unit. The PCR condition for amplification was as follows: Initial denaturation: 98° C for 30 seconds: followed by 98° C for 30 seconds: 57° C for 30 seconds: 72° C for 1 min: final extension 72° C for 10 min: and 4° C on hold. PCR was carried out for 32 cycles of amplification. The product was checked in 0.8 % agarose gel, run in 1X TE-buffer, pH 8.0 (10 mM Tris, 1 mM EDTA) at 120 V for 15 min. Standard 1 kb DNA ladder (NEB) was used to compare the size of the amplicon. After the run, the gel was viewed under Syngene Gel Doc system.

2.2.1.2 Restriction digestion of PCR product:

The PCR product was cleaned-up using Gene-Jet PCR clean-up kit (Cat.no: #K0701). Reaction conditions for the restriction digestion were as follows: Cleaned-up PCR DNA-34 µl, 10X NEB buffer 4- 4.0 µl, BamHI-1.0 U, XhoI-1.0 U, incubated at 37° C for 4 h. The entire digested product was analyzed by 0.8 % agarose gel at 70 V for 30 min. After completion of the run, the digested PCR product was seen under transilluminator and the gel was cut with a sterile scalpel. The digested

product was extracted from the gel using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The product was eluted in molecular grade water. The eluted product was analyzed in 0.8 % agarose gel run at 120 V for 15 min.

2.2.1.3 Restriction digestion of vector:

For cloning of scRad51, pET28a (xxxxxxxx), a vector with N-terminal His-tag was used. Vector digestion was carried out with plasmid DNA-17.0 µl, 10X NEB buffer 4- 2.0 µl, BamHI-0.5 U, XhoI-0.5 U and incubated at 37° C for 4 h. The digested product was separated in 0.8 % agarose gel, at 70 V for 30 min. To visualize the linearized plasmid DNA, the gel was exposed under transilluminator and the band was cut using a scalpel. The DNA was extracted from the gel plug using GeneJET Gel extraction kit (Thermo Scientific: #K0691). The eluted product was analyzed in a 0.8 % agarose gel.

2.2.1.4 Ligation of BamHI, XhoI digested scRad51 and pET28a:

Ligation of BamHI-, XhoI-digested scRad51 and pET28a was carried out using T4-DNA ligase (NEB: M0202). The composition of the ligation was as follows: BamHI, XhoI cut scRad51 (insert) 7.0 µl, BamHI, XhoI cut pET28a (vector) - 1.0 µl in a 10.0 µl reaction. The ligation reaction was incubated at 16° C for 15 h.

2.2.1.5 Transformation of ligation and colony screening:

The ligation mixture after incubation was transformed into DH5α competent cell. 5 µl of the ligation mix was added to 25 µl DH5α competent cells and incubated on ice for 30 min. The incubation was followed by heat shock at 42° C for 30 sec. After incubation on ice for 5 min, entire transformation mix was plated on LB-agar plate (Tryptone-1 %: Yeast extract- 0.5 %: agar-1.5 %) containing Kanamycin (final conc. 100 µg/ml). The plates were incubated at 37° C for 12 h. Isolated single colonies obtained after the transformation were inoculated into 2 ml LB-Kan broth and incubated at 37° C / 200 rpm / 12 h. Alkaline lysis method was followed to isolate

plasmid from the grown culture. Cells were pelleted at 8000 rpm / 5 min / 4° C. Pellet was resuspended in 250 µl P1-buffer (50 mM Tris, pH 8.0: 10 mM EDTA) by vortex. Then 250 µl P2-buffer (0.2 M NaOH: 1 % SDS) was added and gently mixed; finally, 350 µl of P3-buffer (3 M Potassium acetate, pH 5.5) was added and gently mixed. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min at 4° C. Supernatant was taken to new tube and equal volume of Isopropanol was added to the supernatant and incubated at -20° C for 40 min. Plasmid DNA was precipitated by centrifugation at 14000 rpm for 10 min at 4° C. Plasmid DNA pellet was washed with 250 µl of 80 % ethanol and centrifuged at 14000 rpm for 5 min at 4° C. Pellet was air dried and resuspended in 50 µl of molecular grade water. The plasmid DNA thus obtained was analyzed on 0.8 % agarose gel, run at 120 V for 20 min. Later the positive clones were regrown and isolated using GeneJET plasmid purification kit (Thermo Scientific: Cat. #K0502).

2.2.1.6 Confirmation of the clones:

Plasmids containing insert were initially selected based on the slower mobility in the agarose gel analysis. These clones were further confirmed for the presence of insert and orientation of the insert by restriction analysis. Two restriction sites were chosen, one from the vector backbone (BamHI) and one from the gene of interest (NdeI). In a ligated vector the position of BamHI and NdeI are 945 and 1640 nucleotide respectively and the presence of correct insert would result in release of an insert of size 695 nucleotide. The reaction conditions were as follows: Plasmid DNA: 4.0 µl, BamHI and NdeI- 0.4 U, in a total volume of 10 µl. The reaction was incubated at 37° C for 5 h and 10 µl of the reaction was then analyzed in 0.8 % agarose gel run at 120 V / 20 min.

2.2.2 Expression of recombinant scRad51:

2.2.2.1 Transformation of pET28a-scRad51 into BL21(DE3)pLysS

The positive clones, after restriction analysis, were selected for transformation into bacterial expression system, BL21 (pLysS). 1.0 µl of plasmid DNA was added to 25 µl of BL21(DE3)pLysS competent cells and the transformation mix was incubated on ice for 5 min. Heat shock was given at 42° C for 30 sec. After incubation on ice for 5 min., entire transformation mix was plated on LB-Kan agar plates. The plates were then incubated at 37° C for 13 h.

2.2.2.2 Small-scale expression analysis of recombinant His-scRad51

After the transformation, 5-10 colonies were inoculated into 5 ml of LB-Kan broth. Cells were allowed to grow till log phase (approximately 3 h) at 37° C, 200 rpm. 2 ml of the culture was aliquoted as uninduced sample and the remaining 3 ml was induced with 1 mM Isopropyl β-D-thiogalactoside (IPTG, Sigma I6758). Induction was carried out at 30° C for 4 h. After the induction, 1 ml of uninduced and induced cells were pelleted at 8000 rpm for 5 min. Pellet was then resuspended in 200 µl of Protein Extraction Buffer (Tris, pH 8.0 - 50 mM; NaCl - 250 mM; Triton X – 0.005 %) followed by incubation on ice for 5 min. 40 µl of resuspended cells were mixed with 20 µl of 3X SDS-loading dye (Tris, pH 6.5 – 250 mM; SDS – 6 %; Glycerol – 30 %; β-mercapto ethanol – 2.28 M; Bromophenol blue – 0.06 %). The sample thus prepared was incubated in dry bath at 100° C for 10 min. 15 µl of the uninduced and 10 µl of the induced samples were loaded into 10 % SDS-PAGE, run at 140 V for 70 min. Prestained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps.

2.2.3 Purification of recombinant scRad51

2.2.3.1 Large scale culture of recombinant scRad51

The positive clone(s) for expression was inoculated into 100 ml LB-Kan broth and grown overnight at 37° C / 200 rpm. The preinoculum was then diluted into 1 L Terrific Broth (Tryptone – 1.2 %; Yeast extract – 2.4 %; Glycerol – 0.4 %). The culture was grown till mid-log phase at 37° C / 200 rpm / 3 h using incubator shaker (JSR, Model No. JJSI-100C). The culture was then induced with 1 M IPTG (Sigma, Cat. No. I6758) (final concentration 1 mM). Induction was carried out at 30° C / 200 rpm / 4 h. Cells were then pelleted at 8000 rpm / 15 min / 4° C using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Pellet was stored at -80° C refrigerator (Thermo Fischer Scientific, Model No. FORMA 900 Series).

2.2.3.2 Purification of recombinant scRad51 protein by Ni-NTA affinity

The cell pellet was resuspended in 20 ml of extraction buffer (Tris, pH 8.0 – 50 mM; NaCl 300 mM; Triton X100 0.1 %; Imidazole – 1 mM) by vortex. Total extract was then subjected to sonication on ice for 15 min with pulse 10 sec on and 10 sec off, at 25 % amplitude using a sonicator (Sonics Vibra Cell, Model No. VCX 130, Frequency- 20 kHz). Cell debris were pelleted at 14000 rpm / 20 min / 4° C using centrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Clear supernatant was taken for Ni-NTA (Qiagen, Cat. No. 30210) bead binding. Before addition of the beads to supernatant, beads were equilibrated with the extraction buffer. Binding was carried out by gentle rotation at 10 rpm / 4° C for 4 h. After binding unbound fraction was removed by centrifugation at 1000 g / 4° C / 5 min using refrigerated microcentrifuge (Thermo Scientific, Model No. SORVALL Legend XTR). Protein bound beads were then washed 3 times with wash buffer (Tris, pH 8.0 – 50mM; NaCl 350 mM; Triton X100 0.1 %; Imidazole – 5 mM).

Washing step was repeated 3 times with 15 ml wash buffer. The bound protein was eluted using elution buffer (Tris – 10 mM; NaCl 100 mM; Imidazole – 250 mM). Protein was collected in 3 different fractions as elute – 1, 2 and 3. The protein elutes along with soluble, insoluble, unbound and wash fractions were loaded and checked in 10 % SDS-PAGE at 140 V / 50 min. Prestained protein ladder (Fermentas, Cat No.: PMT2522) was used as the molecular weight standard. SDS-PAGE gel was stained with staining solution (Coomassie brilliant blue R250 Sigma, CatB7920 – 0.4 %; Methanol – 30 %; Acetic acid – 10 %) for 20 min. Excess stain was removed by destaining solution (30 % Methanol, 10 % Acetic acid) in several steps. After destaining, the protein elutes, which has protein, were pooled together for dialysis against dialysis buffer (Tris 10 mM, NaCl 100 mM, Glycerol – 5 %) using dialysis membrane of molecular weight cut-off 3500 Da (Spectrum labs, Cat. No.: 132590 Spectra Por). The dialysis buffer was changed three times. After dialysis protein was aliquoted as 50 µl and 100 µl and stored at -80̊ C.

2.3 Analysis of scRad51-Tpa1 interaction by size exclusion chromatography

Samples of purified recombinant proteins were applied to Superose-12 (GE Healthcare) gel filtration column and analyzed using an AKTA Prime FPLC system (GE Healthcare). For analyzing the formation of scRad51-Tpa1 complex, 0.5 mg of Tpa1 was mixed with 0.5 mg of scRad51 in 0.5 ml buffer containing 25mM NaCl and 20mM HEPES, pH 7.0 or 20mM Tris-HCl, pH 8.0. The samples were analyzed with a flow rate of 0.3 ml/min and 0.5 ml fractions were collected.

Chapter 3

Results

3.1 Cloning, Expression and purification of scRad51:

Full length hRad51 was PCR amplified and examined by 0.8% agarose gel electrophoresis. The expected PCR amplification of size 1020 bp was obtained (Figure 1A). The full length scRad51 PCR product and plasmid pET28 were sequentially digested by BamHI and XhoI and ligated. The clone was selected by slower mobility compared to negative control (pET28) (Figure 1B). Sequencing was performed to finally confirm the clone.

For recombinant protein expression, *Escherichia coli* BL21(DE3) cells were transformed with pET28-scRad51. These cells were grown and induced for protein expression by IPTG. Expression of the recombinant protein was confirmed by SDS-PAGE analysis in 12% gel and staining with coomassie-brilliant blue (Figure 2A). Since the proteins had the C-terminal his tag, they were purified by Ni-NTA affinity chromatography (Figure 2B).

3.2 Purification of Tpa1:

Tpa1 was cloned in pET28 vector by Ms Monisha Mohan (PhD student in Dr Anindya Roy's lab) (Figure 3A). The purity of the protein was checked by SDS-PAGE (Figure 3B).

3.3 scRad51 does not interact with Tpa1

To examine if scRad51 forms stable complex with Tpa1 or they interact transiently, we analyzed their interaction by size exclusion chromatography (SEC). First, recombinant scRad51 and Tpa1 purified from *E.coli* were applied separately to a Superose-12 SEC column equilibrated with 25 mM NaCl and 20 mM Tris-HCl, pH 8.0. Eluted fractions were applied to SDS-PAGE to detect and identify the protein contents. SEC analysis of scRad51 (0.5 mg) showed that scRad51 protein was eluted predominantly near the void volume of the column (8

ml) (Fig 4B). This is probably due to existence of scRad51 as various oligomers in equilibrium. In the absence of DNA, scRad51 protein can self-assemble into a variety of multimeric forms, including rings, rods and highly aggregated structures. Tpa1 protein was found in a distinct peak at an elution volume of 12ml, which corresponds to a molecular mass of approximately 75 kDa (Fig 1A). This indicates that Tpa1 purified from *E. coli* exists as a monomer. To elucidate characteristics of the Tpa1-scRad51 protein complex, equal amount (0.5 mg) of the proteins were mixed and assessed by SEC. The highest concentrations of scRad51 and Tpa1 were eluted at 8ml and 13ml, respectively, indicating the individual proteins remained separate and there was no evidence of an interacting peak (Fig 1C).

Chapter 4

Figures

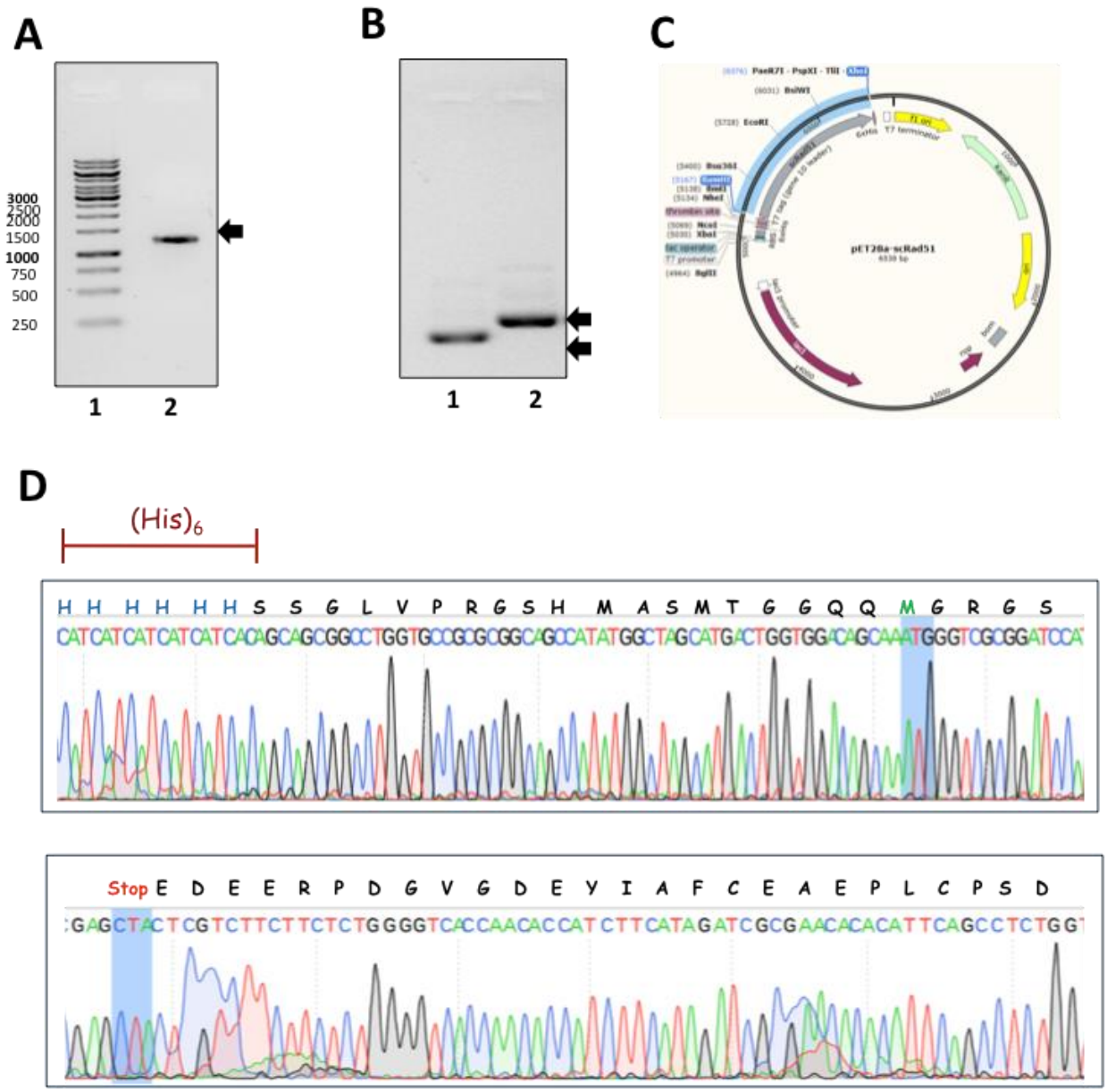

Figure 1. Cloning of scRad51. A. The PCR amplification of scRad51 gene was confirmed by running the sample on Agarose Gel. A band between 1000 and 1500 bp marks of ladder suggested the amplification of the gene as the length of the gene is 1203bp. B. Comparison of mobility of pET28a-scRad51 and pET28a. Slower mobility (lane 2) suggest the presence of insert. C. Plasmid map of pET28a-scRad51 generated using SnapGene software. Unique restriction sites are indicated in bold. D. Sequencing and confirmation of scRad51 clone.

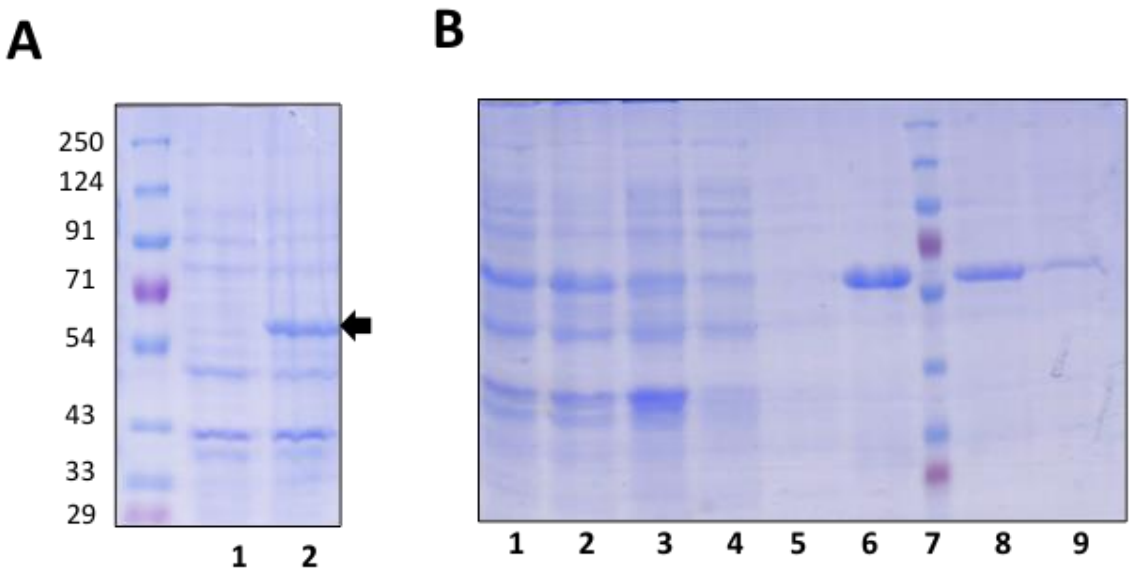

Figure 2. Expression and purification of scRad51. A. pET28a-scRad51 was transformed into BL21(DE3)pLysS and was analyzed for expression of His-scRad51 by induction, using 1 mM IPTG. The expression level of the induced cell extract was compared with the uninduced cell extract, analyzed in 10% SDS-PAGE. B. Recombinant His-SSB was purified from 1 L culture by one-step Ni-NTA-affinity chromatography. Bacterial lysate (lane 1) was loaded along with Soluble (lane 2), insoluble (lane 3), unbound (lane 4) and wash fraction (lane 5). The purified His-scRad51 (lane 6, 8 and 9) was collected as different fractions (elute 1, 2 and 3). All the fractions were loaded in 10% SDS-PAGE along with Pre-stained Protein Marker (lane 7) and the bands were visualized by Coomassie staining.

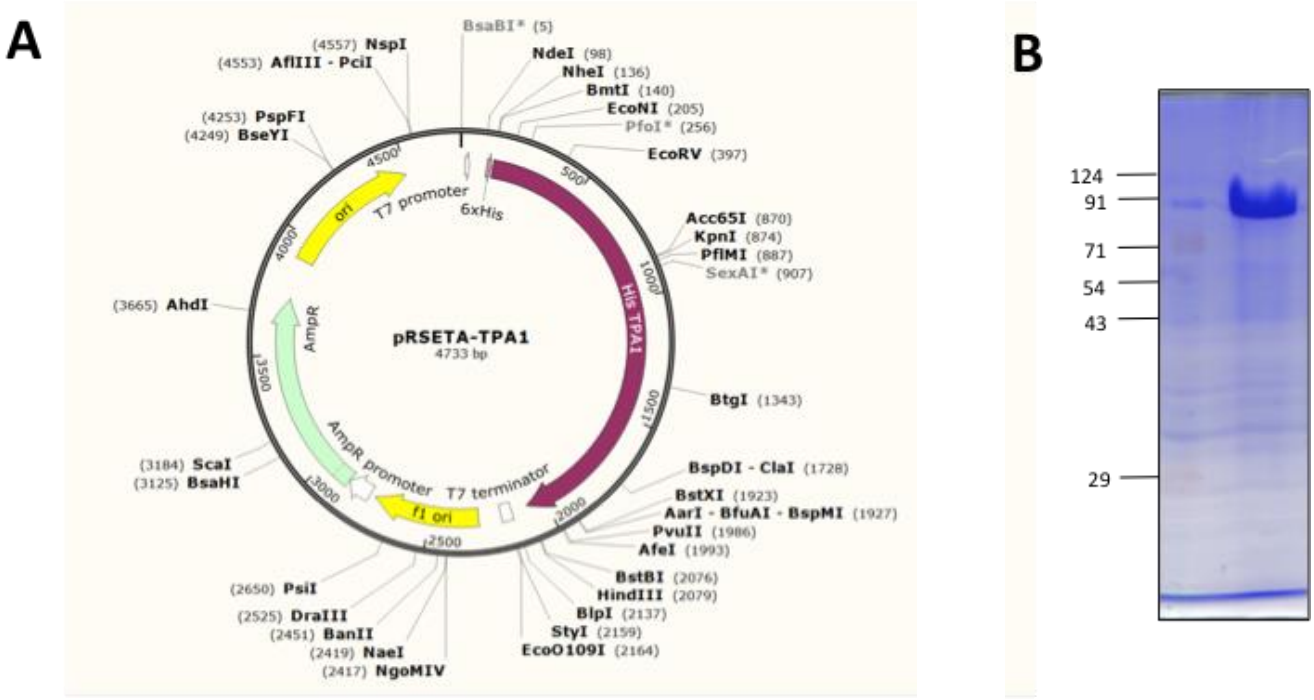

Figure 3. Cloning and expression of Tpa1. A. Plasmid map of pRSET-Tpa1 generated using SnapGene software. Unique restriction sites are indicated in bold. B. SDS-PAGE analysis of purified Tpa1.

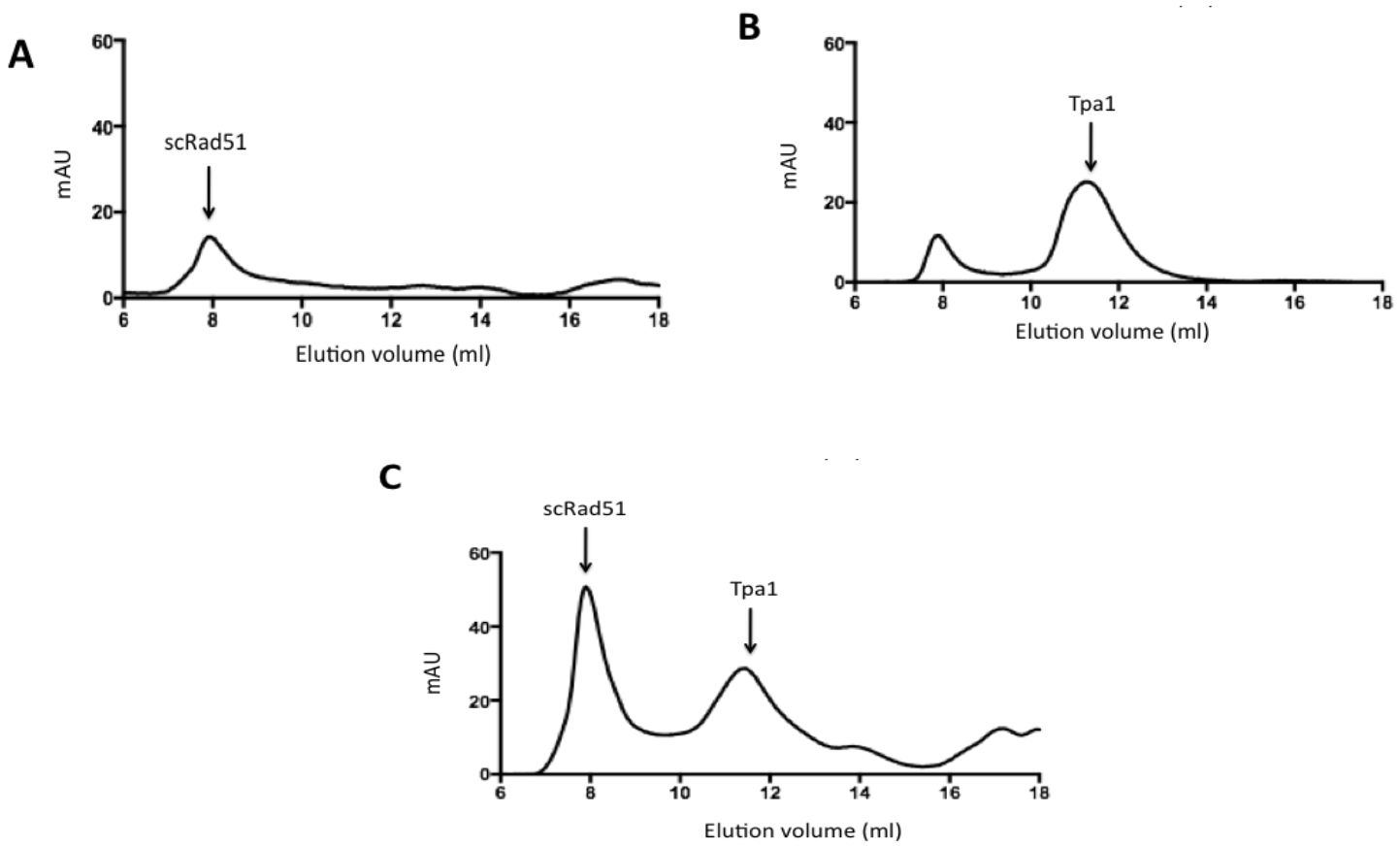

Figure 4: FPLC analysis of scRad51 and Tpa1. A. Elution profile when only scRad51 is injected. **B.** Elution profile when only Tpa1 is injected. **C.** Elution profile when both scRad51 and Tpa1 are injected.

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