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Expression, Purification and NMR studies of SH3YL1 SH3 domain

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Abstract : SH3YL1, a novel protein containing one Src homology 3 domain at the carboxyl terminus was first detected in mouse anagen skin cDNA. This protein had a significant homology with YHRO 16c/Ysc 84, the yeast Src homology 3 domain-containing protein. The sequence identity was remarkable at the carboxyl and amino-terminal Src homology 3 domain, suggesting that the novel protein is a mouse homolog of the yeast protein and thus was termed as SH3YL1. SH3YL1 is composed of two domains, a DUF500 at N-termini and a SH3 domain at C-termini. In our study we cloned the SH3 domain in bacterial expression system in *Escherichia coli* using pET32a vector with TEV protease cleavage site and purified as a monomer using affinity chromatography. The N-terminal poly-Histidine tag was cleaved with TEV protease and target protein was used for backbone studies. Our study showed that SH3 domain primarily consists of β-sheet which is in consistence with previous result performed on the truncated SH3 domain of SH3YL1.

Keywords : Src Homology 3 domain, DUF500 domain, NMR spectroscopy, TALOS analysis, Cloning, Purification.

INTRODUCTION

SH3YL1 consists of a DUF500 and a SH3 (Src homology 3) domain at N- and C- termini respectively. SH3YL1 was first discovered in the mouse cDNA of mouse anagen skin[1]. The whole protein comprises of 323 amino acids and has a size of around 35.5 KDa. NOX1, an NADPH

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oxidase expressed predominantly in colon epithelium, which shows a high degree of similarity to the phagocyte NADPH oxidase helps protect the cell by producing superoxide [2].

Within the NOX1 complex the interaction between the SH3 domain of cytosolic NOXA1 with PRR domain of NOXO1 is crucial for NOX1 acivation[3-6]. The SH3 domain typically binds to the proline-rich region[7-9]. However, the interaction between the NOXA1 and NOXO1 is terminated upon the involvement of SH3 domain of SH3YL1. We hope to find out the molecular mechanism of this behavior using structural analysis and get an insight as to how it works. Furthermore, sequence comparison between the SH3YL1_SH3 domain and NOXA1_SH3 domain showed high degree of similarity, which could be the cause for SH3 domain of SH3YL1 interacting with NOXO1 and in turn displacing the SH3 domain of NOXA1 causing the inhibition of NOXA1-NOXO1 interaction.

In this report, we present the cloning, expression, purification and backbone assignment[10,11] of SH3 domain of SH3YL1. Additionally we also performed the TALOS analysis[12] for its secondary structure prediction which will be beneficial in the future studies in understanding the overall inhibition mechanism of this SH3YL1 SH3 domain in NOXA1-NOXO1 interaction.

EXPERIMENTAL

Cloning and expression of SH3 domain of SH3YL1.

The full length SH3YL1 was obtained from EWHA Women's University. SH3 (Src Homology 3 domain) was cloned in the expression vector, pET32a containing a TRX and poly-Histidine tag at N-terminal. PCR (Polymerase Chain Reaction) was used to amplify the SH3 domain from full SH3YL1 with BamHI/XhoI restriction site. TEV recognition site (ENLYFQG) was included in the sense primer to later aid in the cleavage of the target SH3 domain from the recombinant fusion partner. Sense primer was 5' – CAG GGA TCC GAA AAC CTG TAT TTT CAG GGC AAC TCT GGC TCT CAA AGC-3' and anti-sense primer 5'- CCG CTC GAC TCA TTA ATT CAT GGT TAC GTA-3'. After cloning the correct DNA sequence was confirmed at COSMO Co. Plasmid containing SH3 domain was overexpressed in *Escherichia coli* strain BL21 (DE3) and positive colonies were selected in Lauria-Bertani (LB) plate with 0.1mg/ml Ampicillin. The expression of protein was done in Lauria-Bertani broth using 1mM IPTG at 25 ^{o}C in 160 rpm after induction till the $O.D._{600}$ value was over 1.5. Protein expression was confirmed using the SDS_PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis).

Isotope ($^{13}C/^{15}N$) labeling of SH3 domain.

For hetero-nuclear NMR, we prepared isotope ($^{13}C/^{15}N$) labeled form of SH3 domain. Initially cells were cultured in $^{12}C/^{14}N$ M9 minimal medium at 37 ^{o}C and 220 rpm until the $O.D._{600}$ of 0.65. The cells were then washed with PBS buffer to remove all the leftover M9 medium and cell down was done at 4500 rmp at room temperature, cell pellet obtained were then suspended in $^{13}C/^{15}N$

{$^{13}C/^{15}N$, 99 %, Cambridge Isotope Laboratories, Inc.} M9 media and $O.D._{600}$ was maintained at 0.5 - 0.6. Induction was done by 1mM IPTG (isopropyl-β-D-thiogalactopyranoside) and cultured at 25 ^{o}C at 160 rpm for 18 hours till $O.D._{600}$>1.5 which was harvested at 6000 rpm at 4 ^{o}C for 30 mins.

Purification of SH3 domain

The cell containing fusion SH3 protein (TRX-$His_{(6)}$-TEV-Target protein), were sonicated in lysis buffer (25 mM NaPi, 300 mM Nacl, 5 mM β-mercaptoethanol, pH 7.5). Protease inhibition cocktail (Roche) 50 µl was added to inhibit protease activity. The lysate was then centrifuged at 14000 rpm at 4 ^{o}C for 30 minutes. The supernatant was loaded in Ni-NTA (affinity) open column and imidazole was removed from elution by dialysis. Presence of target protein was confirmed by SDS-PAGE which showed only on band of fusion protein in the elution fraction.

Cleavage of Tag and size exclusion chromatography

Fusion protein was incubated with 0.5 X TEV protease (Purified in laboratory) at 25 ^{o}C for 8 hours. Second column was performed in Ni_NTA open column to obtain cleaved target protein in flow through and first washing fraction.

NMR spectroscopy

The target protein was dissolved in four different buffer conditions(Buffer 1: 10 mM HEPES, 200 mM NaCl, 2 mM DTT, 0.01 % NaN_3 and pH 7.4; Buffer 2: 10 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01 % NaN_3 and pH 6.5; Buffer 3: 10 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01 % NaN_3 and pH 8.0 and Buffer 4: 10 mM HEPES, 200 mM NaCl, 2 mM DTT, 0.01 % NaN_3 and pH 6.5). Buffer 2 was found to be the best buffer condition for the Target protein. The protein concentration was about 1.3 mM. All the NMR experiments were performed in Bruker DRX 500MHz equipped with Cryoprobe^{TM}. Backbone assignment was performed by using standard backbone experiments namely (^{1}H-^{15}N HSQC; HNCACB; HNCA and CBCACONH) at 25 ^{o}C.

RESULTS AND DISCUSSION

Domain construct of SH3YL1 SH3 domain

We designed the construct of SH3 domain from 256 - 323 amino acid. SH3 domain (256 - 323) was cloned in the *E. coli o*ver-expression vector, pET32a, and was expressed as TRX_$His_{(6)}$_TEV protease recognition sequence_target protein (Fig. 1A). Sequence alignment for SH3 domain from SH3YL1 already studied (SH3YL1_SH3_P); SH3YL1 and NOXA1 were executed using CLUSTALW, EBI (Fig. 1B). All three sequences showed high homology with each other suggesting they share similar structure and may be function as well.

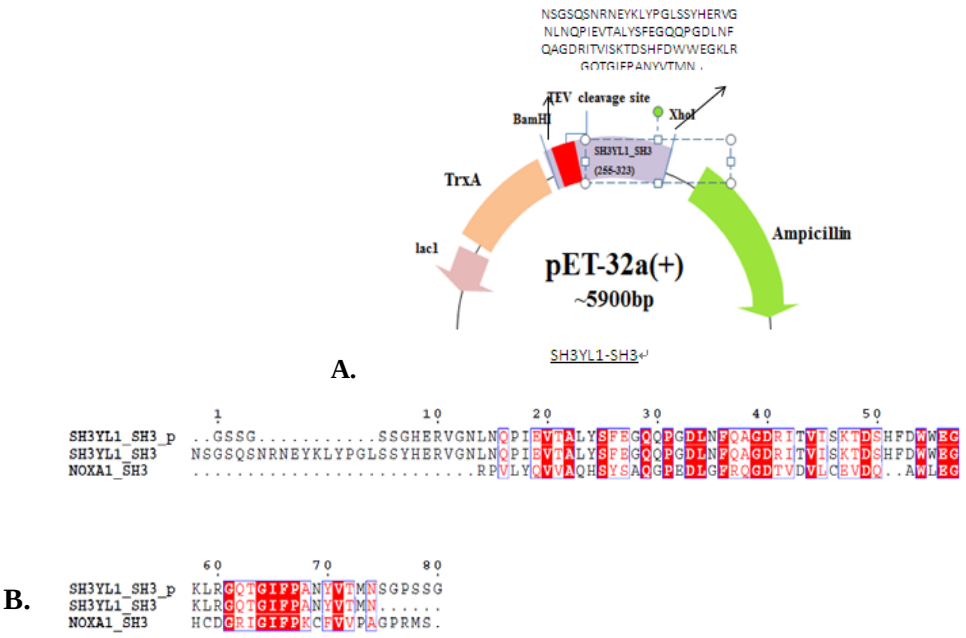

Figure. 1. Vector map of SH3YL1_SH3 domain cloning and sequence alignment. (A) Construct was designed with SH3 domain (256 - 323). (B) Amino acid sequence alignment for SH3 domain from SH3YL1_SH3_P (truncated and studied); SH3YL1_SH3 domain and NOXA1_SH3 domain. All three sequences showed high sequence homology suggesting similar structure.

Purification of SH3YL1_SH3 domain

E. coli cells cloned with SH3YL1_SH3 domain were cultured in LB broth. At $O.D._{600}$ of 0.6, protein induction was done by 1 mM IPTG and cultured at 25 ^{o}C for adequate induction and harvested after 18 hours. The harvested cells were suspended in (25 mM NaPi, 300 mM NaCl, 5 mM β-mercaptoethanol, pH 7.5) and sonicated to rupture the cells. Ni-NTA open column was used to

purify the fusion (TRX-HIS_6-TEV recognition sequence-Target protein) and later cleaved by TEV protease (0.5 X for 8 hours at 25 ^oC) to obtain target SH3 domain (Fig. 2 A and 2 B).

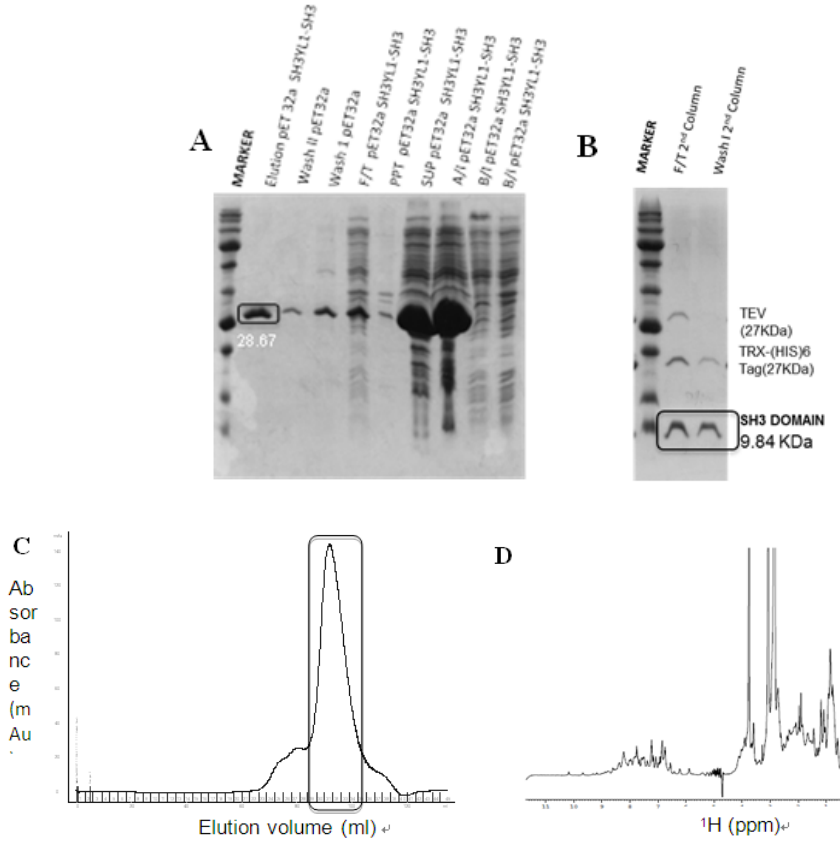

Figure. 2. Expression, Purification, Gel filtration and ^1H NMR spectroscopy of phosphatase domain.(A) SDS-PAGE of purified SH3YL1_SH3 domain from Ni_NTA open column work.(B) Second Ni_NTA column work after TEV cleavage of Fusion TRX_$(HIS)_6$_SH3 protein; 28.67 KDa. (C) Gel filtration using analytical column to confirm the monomeric form of SH3 domain. (D) ^1H NMR spectrum recorded in 10 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% NaN_3, pH 6.5 at 25^oC.

Size exclusion chromatography of SH3YL1_SH3

To further purify the SH3 domain and to know the actual size of the target protein, we performed the gel filtration experiment (Fig. 2C) using HiLoad^{TM} 16/60 Superdex^{TM} 75 and confirmed the target protein existed in monomeric state shown by a peak in the graph from 84 ml to 100 ml.

Structural studies of SH3YL1_SH3 domain

In order to perform the backbone assignment we first performed ^{1}H NMR spectroscopy which showed a stable protein sample condition (Fig. 2D). We next performed ^{1}H-^{15}N HSQC experiments with different buffer conditions(Fig. 3A and 3B) and found that Buffer 2 (10 mM HEPES; 100 mM NaCl; 2 mM DTT; 0.01 % NaN_3 and pH 6.5) was the optimum buffer condition for NMR studies. The spectrum in Buffer 2 was clear with most of the peaks seen in it, so we choose this buffer condition for backbone assignments. Backbone Assignment was completed using the ^{1}H-^{15}N HSQC, HNCACB, CBCACONH and HNCA NMR spectrum. For the Backbone assignment SPARKY program was used and all the backbone residues were assigned completely (Fig. 3C). The secondary structure was predicted from TALOS analysis along with PSI-PRED program (Fig. 4 A, B and C). Finally homology modeling of SH3YL1_SH3 with truncated SH3YL1_SH3 domain (PDB 2D8H,

Chain A) showed that the structure of SH3 domain is dominated by β-sheet, five in this case of SH3YL1_SH3 (Fig.5)

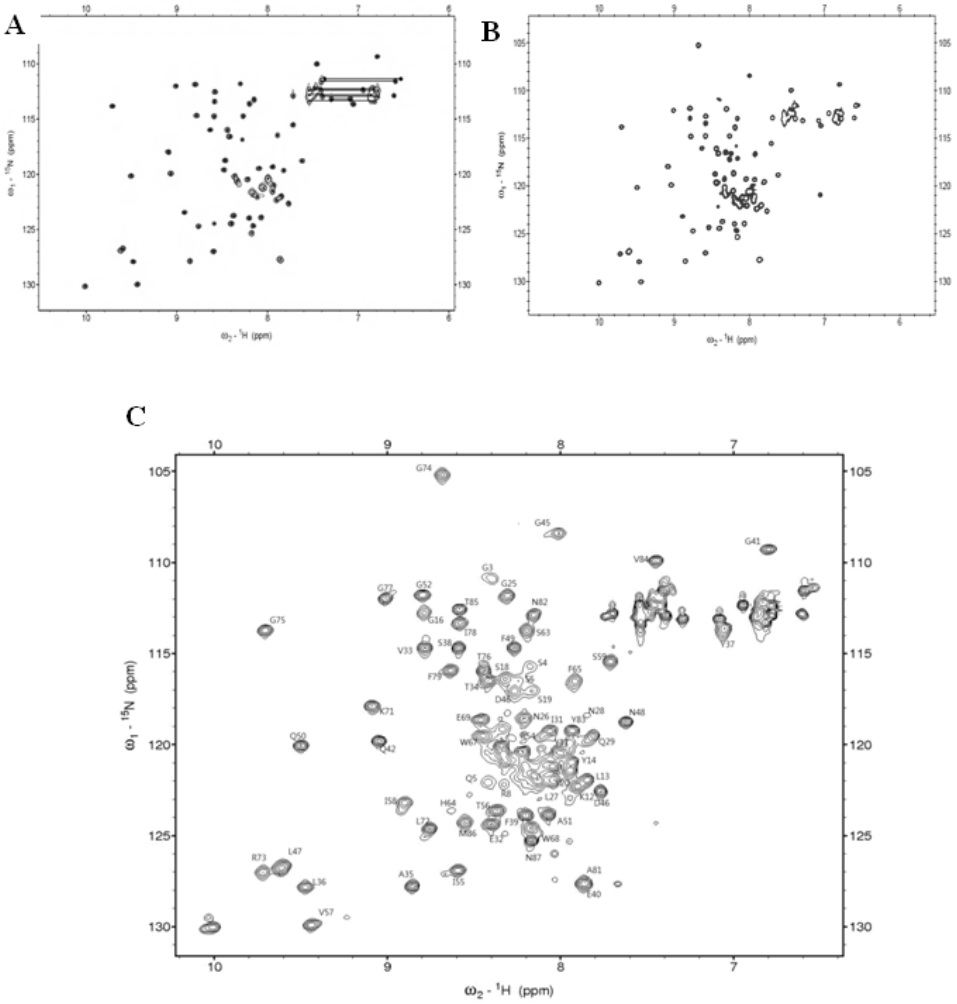

Figure. 3. ^{1}H-^{15}N 2D-HSQC spectrum of SH3YL1_SH3 domain. (A)The spectrum was recorded on 1.3 mM solution with 10 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01 %NaN_3, pH 7.4 at 25^{o}C with lower number of peaks.(B) Optimized Buffer condition with 10 mM HEPES,100 mM NaCl, 2 mM

DTT, 0.01 %NaN_3, pH 6.5 recorded at 25^oC showing improved spectrum with additional peaks in it.(C) ^{1}H-^{15}N 2D-HSQC in optimized buffer condition, spectrum labeled with assigned amino acid residues.

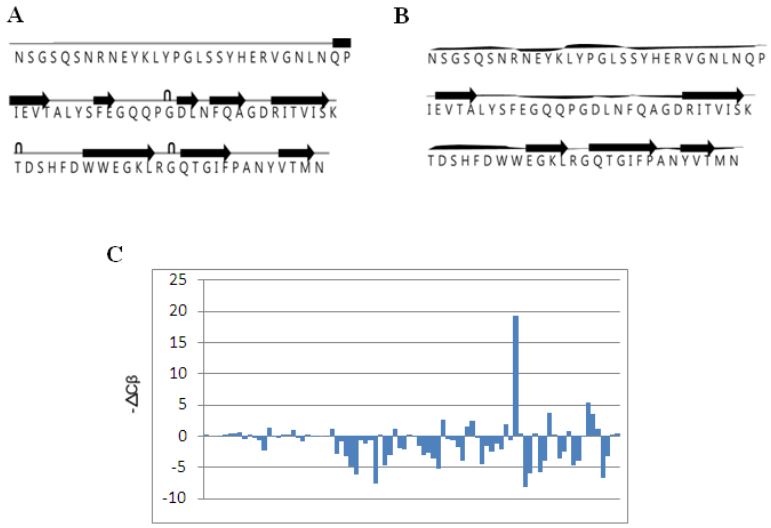

Figure. 4. Secondary structure prediction of SH3YL1_SH3 domain. (A) TALOS analysis of SH3 domain showing secondary structure dominated by β-strand. (B) PSI-PRED secondary structure prediction of SH3 domain. (C) Delta values (ΔCα-ΔCβ) of backbone carbon to random coil chemical shifts were plotted for secondary structure prediction.

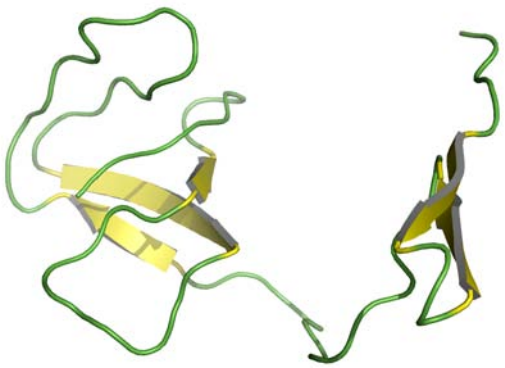

Figure. 5. Homology modeling of SH3YL1_SH3 using SWISS_MODEL, ExPASy, (PDB 2D8H, Chain A) truncated SH3YL1_SH3 structure was taken as template for homology modeling. The SH3 structure was dominated by β-strand, five in this case of SH3 domain.

CONCLUSION

SH3 domain of SH3YL1 has been successfully cloned into E. coli expression vector, with TEV cleavage site. The SH3 domain was purified using Ni_NTA open column and gel filtration chromatography as a monomer. Furthermore study of its secondary structure showed that SH3 domain structure is dominated by β-strand, 5 β-strands in this case. This study will help in further understanding as to how the SH3 domain of SH3YL1 inhibits the NOXA1-NOXO1 interaction in NOX1 complex.

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