

## Expression and Purification of Tumor Suppressor Protein TIP30 for Structural Studies by NMR Spectroscopy

Joseph Blas

### ABSTRACT

TIP30 (Tat-Interacting Protein, 30 kD) is a putative metastasis suppressor that promotes apoptosis and inhibits angiogenesis. To provide structural insight into the function of TIP30, structural studies by NMR spectroscopy were pursued. A TIP30 segment (101-166) was expressed and purified by His-tagged affinity chromatography followed by FPLC purification. Successful <sup>15</sup>N-labeling of this protein fragment by two-dimensional heteronuclear single-quantum proton-nitrogen correlated spectroscopy was demonstrated. The fragment exhibited no single favored conformation under the studied conditions.

Keywords: *FPLC, HSQC, IPTG, NMR, TIP30, Tumorigenesis.*

### INTRODUCTION

TIP30 (Tat-Interacting Protein, 30kD) is a putative metastasis suppressor that promotes apoptosis, inhibits angiogenesis of hyperproliferative cells, and suppresses the growth of present tumor cells (Ito, M et al. 2003; King, FW, E Shtivelman. 2004; Shtivelman, E. 1997). TIP30 was originally implicated as a transcription cofactor that enhances Tat-interactive transcription of Human Immunodeficiency Virus-1 (HIV-1) (Xiao, H et al. 1998). Amino acid sequence homology data shows that TIP30 exhibits similar sequences to members of the short-chained dehydrogenases/reductases (SDR) enzyme family, suggesting that it may exhibit a similar elementary structure (Baker, ME, L Yan, and MR Pear. 2000) (Figure 1). TIP30 has shown intrinsic serine-threonine kinase activity suggested to play a role in enhancing transcription of HIV-1 and inducing apoptosis in oncocytes (Xiao, H, et al. 2000). TIP30 has also been demonstrated to interact with an estrogen receptor, ER- $\alpha$ , suggesting a mechanism for tumorigenesis promoted by its deficiency (Jiang, C, et al. 2004). Functional studies have been well-developed; however, there is no current published solution-state structure for TIP30 (Baker, ME, L Yan, and MR Pear. 2000). Preliminary data on protein expression, <sup>15</sup>N isotope labeling, and purification of a TIP30 segment, corresponding to amino acid residues 101-166 are presented and analyzed. To provide structural insight into the function of TIP30, NMR spectroscopy was utilized.

### MATERIALS AND METHODS

#### *Plasmid Constructs and reagents*

To construct the pRSET-TIP30<sub>106-166</sub> plasmid, PCR of TIP30<sub>106-166</sub> DNA was performed with 1 $\mu$ l template, 0.5 $\mu$ l each of the primers (both 3' and 5'), 1 $\mu$ l dNTP, 1 $\mu$ l vent polymerase, 5 $\mu$ l 10x Buffer, and 41 $\mu$ l MQ

H<sub>2</sub>O (total 50 $\mu$ l/PCR tube). Following PCR, the products were placed on a 1.0% agarose gel and electrophoresed at 100V to isolate the 200bp of DNA. The gel piece containing the desired DNA was excised and purified, then digested with NdeI, BamHI restriction enzymes. The pRSET vector was also digested with NdeI and BamHI. The digestion product was placed on a 1.0% agarose gel at 100V and the desired segment of DNA (2.9kb) was excised and purified.

Ligation of the vector and insert was done with 7 $\mu$ l of insert DNA, 1 $\mu$ l vector, 1 $\mu$ l T4 ligase buffer, and 1 $\mu$ l T4 ligase (total of 10 $\mu$ l). The mixture was allowed to sit at room temperature overnight. The following day, the ligated plasmids were transformed to BL21 competent *E. coli* cells. The transformation was done with 100 $\mu$ l competent cells to 1 $\mu$ l of ligation mixture. The mixture was placed on ice for 30 minutes and then immediately placed at 37°C for two minutes. 0.5mL of Luria-Bertani (LB) media without antibiotics was then added. The mixture was placed at 37°C and shaken for one hour and fifteen minutes. Two plates for each plasmid transformant were made for 100 and 400 $\mu$ l, respectively. The plates were placed agar-side up in a 37°C incubator and incubated overnight. Ten to 12 single colonies for each plasmid were picked and streaked.

Mini-prep was done the following day after colony samples were grown with Kanamycin in 37°C incubation overnight with shaking. Following mini-prep, a 5 $\mu$ l sample of DNA from each mini-prep procedure was digested with NdeI and BamHI so that the plasmid could be cleaved at two sites. After electrophoresis at 100V, two visible bands of DNA indicated that the plasmid contained the desired insert in the correct direction.

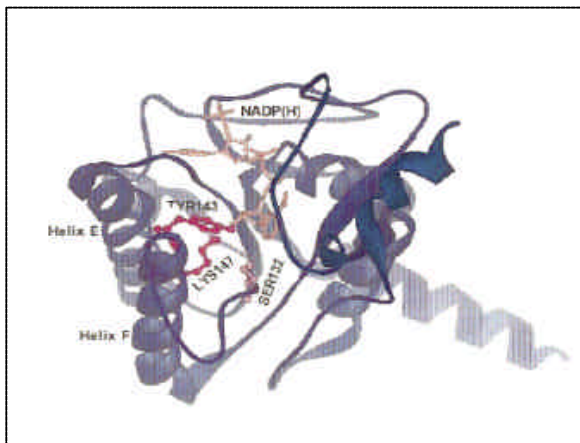


Figure 1. Three-Dimensional Model of SDR Enzyme Family

### Expression and $^{15}\text{N}$ Labeling

A small inoculation culture of 50mL of Luria-Bertani media was made with the TIP30-containing *E. coli* bacteria and left to grow overnight. The following day, a 1 liter amount of sterilized Luria-Bertani media with Ampicillin was inoculated with the overnight culture and allowed to grow to logarithmic phase ( $\text{Abs}_{600} = 0.5$ ). Protein expression was induced with the addition of 0.4mM IPTG. Bacteria were left to grow for another four hours, and then centrifuged at 5,000 x g for 10 minutes. Cells were re-suspended in 30mL of 25mM Tris/200mM NaCl buffer at pH 8.0. The solution was then french-pressed five times at 10,000psi. The cells were centrifuged at 20,000rpm for 40 minutes. Protein was located in the pellet after confirmation using SDS-PAGE at 35mV for one to two hours (14.4kDa). To achieve the  $^{15}\text{N}$  labeled product, the inoculation broth was made using LB media. One liter of minimal labeling media with  $^{15}\text{N}$ - $\text{NH}_4\text{Cl}$  was then inoculated with the overnight culture. Subsequent procedure modeled that of the non-labeled cells.

### Purification and NMR spectroscopy

After centrifugation to pellet the protein, it was re-suspended using 8M urea. The solution was mixed with 5mL of Qiagen Ni-NTA His-tagged protein affinity chromatography resin and left to shake in a cold room. The solution was centrifuged at 900rpm for 5 minutes. The resin was washed three times with 25mM Tris/200mM NaCl and 10mM imidazole and left to incubate 5 minutes at room temperature while shaking. The solution was centrifuged again and resin was re-suspended in Tris/NaCl buffer. The solution was loaded to an affinity column. The solution was washed with increasing concentrations of imidazole (25mM-100mM) and fractionated. FPLC purification was done with an Amersham Biosciences (GE Healthcare) FPLC with a SuperDex 75 10/300 gel filtration column. Tris buffer containing 8M urea

was used to elute protein and fractionation was determined by UV absorbance at 280nm (Figure 2). The peptide was concentrated with a Pall Corporation Macrosep centrifugal device. Buffer exchange to 50mM Tris/100mM NaCl at pH 7.0 was also done with Macrosep. A second buffer exchange was done to 50mM phosphate buffer at pH 7.0. NMR spectra were obtained on a Varian INOVA 600MHz spectrometer equipped with a cryoprobe.

## RESULTS

An  $^{15}\text{N}$ -HSQC spectrum was obtained from the isolated and purified TIP30 fragment (Figure 3). The HSQC spectrum was obtained in a 50mM phosphate buffer at pH 7.0. Additional NMR spectra of the TIP30 fragment were not successfully obtained.

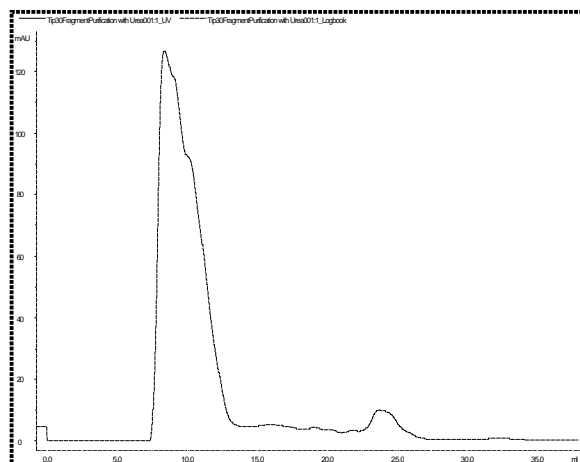


Figure 2. Elution Profile of TIP30 Fragment

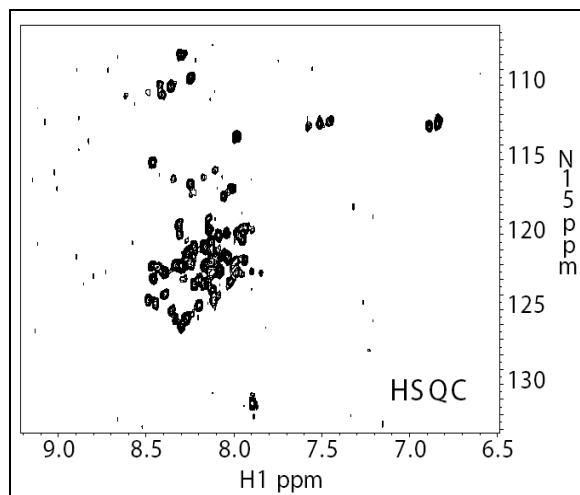


Figure 3. HSQC of TIP30 Fragment

## DISCUSSION

We have demonstrated that a fragment of TIP30 is able to be isotope-labeled, purified, and analyzed by

NMR spectroscopy. The <sup>15</sup>N-HSQC NMR spectrum shows a narrow dispersion of the amide region, suggesting that this fragment does not adopt a particular conformation under these conditions. This can be explained because the TIP30 fragment analyzed does not contain the active site. The methodologies developed to acquire a spectrum are novel approaches to establishing a definitive conformation of the TIP30 protein. Alternative optimization techniques and procedures will be investigated and developed. This experiment has contributed a rudimentary set of steps to help obtain the entire structure of the TIP30 tumor suppressor protein at close to physiological conditions. Future work on TIP30 includes further protein purification for additional structural studies using the procedures developed in this study.

### ACKNOWLEDGEMENTS

Joseph Blas would like to thank the Eppley Institute for Cancer and Allied Diseases for the opportunity to do summer research. Joseph Blas also thanks Dr. Guangshun Wang and his laboratory for their guidance. He would like to acknowledge the financial support from both the CURE grant and Dr. Wang's startup fund. Joseph Blas thanks Paul Keifer for maintaining the NMR instrument and advising me about NMR theory and practice. He thanks Dr. Jonathan Frye for his recommendation and guidance. Joseph Blas also would like to acknowledge Dr. Tim Hubin, his advisor and mentor, for advice and guidance. Finally, Joseph Blas acknowledges Dr. Karrie Rathbone and Dr. Alan Van Asselt for their advice on this project and their intelligent conversations.

### LITERATURE CITED

- Baker, ME, L Yan, and MR Pear. 2000. Three-dimensional model of human TIP30, a coactivator for HIV-1 Tat-activated transcription, and CC3, a protein associated with metastasis suppression. *Cellular and Molecular Life Sciences*. 57(5):851-858.
- Ito, M, C Jiang, K Krumm, X Zhang, J Pecha, J Zhao, Y Guo, RG Roeder, and H Xiao. 2003. TIP30 Deficiency Increases Susceptibility to Tumorigenesis. *Cancer Research* 63:8763-8767.
- Jiang, C, M Ito, V Piening, K Bruck, RG Roeder, and H Xiao. 2004. TIP30 Interacts with an Estrogen Receptor  $\alpha$ -interacting Coactivator CIA and Regulates c-myc Transcription. *Journal of Biological Chemistry*. 279(26):27781-27789.
- King, FW and E Shtivelman. 2004. Inhibition of Nuclear Import by the Proapoptotic Protein CC3. *Molecular and Cellular Biology*. 24(16):7091-7101.
- Shtivelman, E. 1997. A link between metastasis and resistance to apoptosis of variant small cell lung carcinoma. *Oncogene* 14:2167-2173.
- Xiao, H, V Palhan, Y Yang, and RG Roeder. 2000. TIP30 has an intrinsic kinase activity required for up-regulation of a subset of apoptotic genes. *EMBO Journal*. 19:956-963.
- Xiao, H, Y Tao, J Greenblatt, and RG Roeder. 1998. A cofactor, TIP30, specifically enhances HIV-1 Tat-activated transcription. *Proceedings of the National Academy of Science of the United States of America*. 95(5):2146-2151.